MICROENCAPSULATED FERULIC ACID ESTERASE ACTIVE *LACTOBACILLUS FERMENTUM* FOR THE REDUCTION OF INFLAMMATION AND CHOLESTEROL IN METABOLIC SYNDROME

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To my parents, family and friends
for being patient and supportive throughout this adventure

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PREFACE

In accordance with the McGill University Thesis Preparation guidelines, as an alternative to the traditional thesis format, I have elected to take the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more accepted or published papers. This collection of papers, of which I am the first author along with other co-authors, are presented in Chapters 3, 4, 5, 6, 7 and 8 and are each divided into sections consisting of an abstract, introduction, materials and methods, results and discussion. A common abstract, introduction, literature review, a final summary of results, conclusions and a bibliography are included in this thesis according to the guidelines.

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LIST OF ABBREVIATIONS

ABCG5/G8: ATP-binding cassette sub-family G members 5 and 8

ACE: Angiotensin-converting enzyme

APA: Alginate-polylysine-alginate

ATCC: American Type Culture Collection

AUC: Area under the curve

BMI: Body mass index

CAD: Coronary artery disease

CD14: Cluster of differentiation 14

CFU: Colony forming unit

CRP: C-reactive protein

CVD: Cardiovascular disease

DAD: Diode array detector

DMEM: Dulbecco's Modified Eagle Medium

EC: Esterified cholesterol

EFA: Ethyl ferulate

EGIR: European Group for Study of Insulin Resistance

ELISA: Enzyme-linked immunosorbent assay

FA: Ferulic acid

FAE: Ferulic acid esterase

FAO: Food and Agriculture Organization of the United Nations

FBS: Fetal bovine serum

FC: Free cholesterol

FDA: American Food and Drug Administration

FFA: Free fatty acid

GA: Glycosylated albumin

GALT: Gut-associated lymphoid tissue

GLM: General linear model

GLP-1: Glucagon-like peptide-1

GIT: Gastrointestinal tract

GRAS: Generally recognized as safe

Hb: Hemoglobin

HbA1C: Glycated hemoglobin

HDL-C: High-density lipoprotein cholesterol

HMG-CoA: Hydroxyl-3-methyl-glutaryl-CoA

HOMA-IR: Homeostasis Model Assessment of Insulin Resistance

HPLC: High-pressure liquid chromatography

IL: Interleukin

LAB: Lactic acid bacteria

LDL-C: Low-density lipoprotein cholesterol

LPS: Lipopolysaccharide

LXR: Liver X receptor

MALT: Mucosa-associated lymphoid tissue

MetS: Metabolic syndrome

MIP-2: Macrophage inflammatory protein 2

MRS: de Man, Rogosa and Sharpe

NCEP/ATP III: National Cholesterol Education Program's Adult Treatment Panel III

NCIMB: National Collection of Industrial, Food and Marine Bacteria

NEFA: Non-esterified fatty acids

NIH: National Institutes of Health

NPC1L1: Niemann-Pick C1-like

NSAID: Non-steroidal anti-inflammatory drugs

PAI-1: Plasminogen activator inhibitor-1

PBS: Phosphate buffered saline

PEG: Polyethylene glycol

PLL: Poly-L-lysine

PPAR-γ: Proliferator-activated receptor-γ

PS: Physiological solution

ROS: Reactive oxygen species

RSV: Respiratory syncytial virus

SEM: Standard error of the mean

SGF: Simulated gastric fluid

SIF: Simulated intestinal fluid

STZ: Streptozotocin

T2DM: Type 2 Diabetes Mellitus

TER: Transepithelial electrical resistance

TC: Total cholesterol

TG: Triglyceride

TLR-4: Toll-like receptor 4

TNF-α: Tumor necrosis factor alpha

VLDL-C: Very-low-density lipoprotein cholesterol

WHO: World Health Organization

ZDF: Zucker diabetic fatty

ABSTRACT

Metabolic syndrome (MetS) is an important public health concern of industrialized countries. MetS is a cluster of metabolic disturbances, including abdominal obesity, atherogenic dyslipidemia, elevated blood pressure, insulin resistance and the presence of a proinflammatory and prothrombotic state. Currently, there is no successful therapeutic intervention for the prevention and treatment of this disorder. Chronic systemic inflammation and hypercholesterolemia are two important therapeutic targets of MetS. The goal of this work is to develop a novel microencapsulated ferulic acid-producing probiotic *Lactobacillus* formulation as a MetS biotherapeutic. Specifically, a number of selected probiotic strains were screened for their ferulic acid esterase activity to select the best probiotic producer of ferulic acid from ethyl ferulate, a natural substrate. Ferulic acid is a molecule with important therapeutic properties relevant to the development of a MetS biotherapeutic. Due to the harsh conditions of the gastrointestinal tract, we propose microencapsulation as a carrier capable of protecting the viability and activity of the probiotic cells. Hence, the best ferulic acid-producing *L. fermentum* was microencapsulated using alginate-polylysine-alginate microcapsules. The probiotic viability and activity in the microcapsules was significantly higher than that of non-microencapsulated probiotic cells following exposure to gastrointestinal conditions in an *in vitro* model, demonstrating its suitability for oral delivery. The probiotic *L. fermentum* formulation was shown to possess anti-inflammatory properties, *in vitro*. Specifically, the probiotic reduced the secretion of macrophage pro-inflammatory cytokines and provided protection of the intestinal epithelial integrity in a co-culture model of the epithelium. As well, the probiotic formulation demonstrated important cholesterol-lowering activity, by

assimilating cholesterol and via inhibition of colon epithelium cholesterol uptake, determined *in vitro*. Results also demonstrate that *L. fermentum*, administered in animal models of MetS, significantly reduced a number of MetS-associated risk factors, including cholesterol, adiposity, insulin resistance and hyperglycemia. This novel work indicates the potential of a ferulic acid-producing microencapsulated probiotic formulation as a biotherapeutic for MetS treatment and prevention. Further studies, including clinical investigations, are required to demonstrate the full potential of this probiotic biotherapeutic for the management of MetS.

RÉSUMÉ

Le syndrome métabolique (SMet) est une préoccupation importante des pays industrialisés au niveau de la santé publique. SMet est un groupe de troubles métaboliques, qui comprend l'obésité abdominale, la dyslipidémie athérogènique, l'hypertension artérielle, la résistance à l'insuline et la présence d'un état pro-inflammatoire et pro-thrombotique. Actuellement, il n'existe aucune intervention thérapeutique efficace pour la prévention et le traitement de ce syndrome. L'inflammation systémique chronique et l'hypercholestérolémie sont deux cibles thérapeutiques importantes du SMet. Le but de cette recherche est de développer une nouvelle formulation biothérapeutique de microcapsules probiotiques produisant de l'acide férulique pour combattre le SMet. En particulier, des souches probiotiques ont été évaluées pour leur activité d'estérase d'acide férulique pour choisir le probiotique produisant le plus d'acide férulique. L'acide férulique est une molécule possédant des propriétés thérapeutiques importantes relatives au développement d'une biothérapeutique SMet. La souche de *L. fermentum* sélectionnée a été microencapsulée à l'intérieur de microcapsules d'alginate-polylysine-alginate. La viabilité des cellules probiotiques et leur activité dans les microcapsules était significativement supérieure à celle des cellules probiotiques non-microencapsulées une fois soumises à des conditions gastro-intestinales dans un modèle in vitro, ce qui démontre leur aptitude à l'administration orale. La formulation probiotique *L. fermentum* a démontré des propriétés anti-inflammatoires, in vitro. En particulier, le probiotique *L. fermentum* réduit la sécrétion de cytokines pro-inflammatoires par les macrophages et assure la protection de l'intégrité épithéliale intestinale dans un modèle *in vitro* de l'épithélium. La formulation probiotique a aussi

démontré une activité anti-cholestérol importante en assimilant le cholestérol et par l'inhibition de l'absorption du cholestérol par l'épithélium du côlon, déterminée in vitro. Les résultats démontrent également que *L. fermentum*, administré dans des modèles animaux de SMet, réduit de manière significative plusieurs facteurs associés au SMet, y compris le cholestérol, l'adiposité, l'insulinorésistance et l'hyperglycémie. Cette nouvelle recherche démontre le potentiel d'une formulation probiotique microencapsulée, produisant de l'acide férulique, comme un biothérapeutique pour le traitement et la prévention du SMet. D'autres études, y compris des études cliniques, sont nécessaires pour démontrer la pertinence de ce produit.

CHAPTER 1: INTRODUCTION, RESEARCH HYPOTHESIS, THESIS OBJECTIVES AND OUTLINE

1.1 General introduction

Metabolic syndrome (MetS), encompassing Type 2 Diabetes Mellitus (T2DM) and cardiovascular diseases (CVD), is a public health concern (Ford ES 2002; Zimmet et al. 2005). In the USA in 2005, the incidence of T2DM was estimated at 16.2 million individuals and is projected to rise to 48.3 million by 2050 (Narayan et al. 2006b). T2DM is characterized by insulin resistance, hyperglycemia, low-grade inflammation and hyperinsulinemia. CVD, responsible for 16.7 million deaths worldwide, is the leading cause of global mortality (Tarride et al. 2009). CVD factors include dyslipidemia, hypercholesterolemia, hypertension and obesity. Furthermore, in MetS, there is increased permeability of the gut epithelium, leading to the translocation of factors such as lipopolysaccharide (LPS), resulting in low-grade systemic inflammation. There is also an alteration in the gut microbiota composition, with an increase in the Gram-negative to -positive ratio, specifically an increase in *Bacteroides*/ *Clostridium* and a decrease in *Lactobacillus*/ *Bifidobacterium*. Current treatment methods for MetS primarily involve lifestyle modifications, but also pharmacological agents. However, the majority of patients are far from achieving the necessary goals for lipid, cholesterol and glucose levels (Lebovitz 2011).

Ferulic acid (FA) is a phenolic acid abundantly bound to foods consumed by humans. FA is a potent antioxidant able to neutralize free radicals, such as Reactive Oxygen Species (ROS) (Rice-Evans et al. 1996) implicated in DNA damage (Lombard et al. 2005a), cancer (Hu et al. 2011), accelerated cell aging (Ishii et al. 1998), obesity

(Sonta et al. 2004) and T2DM (Donath and Shoelson 2011; Sonta et al. 2004). FA has been shown to regulate blood glucose levels by modulating insulin secretion and pancreatic β-cell survival and by reducing inflammatory markers linked to antioxidant activity (Adisakwattana et al. 2008). Orally-delivered FA is quickly absorbed in the stomach, greatly reducing its residence time (Zhao et al. 2004). Interestingly, a number of gastrointestinal tract (GIT) bacterial strains have the enzyme feruloyl esterase (FAE) (Bhathena et al. 2007; Bhathena et al. 2008; Bhathena et al. 2009; Lai et al. 2009a; Tomaro-Duchesneau et al. 2012a; Tomaro-Duchesneau et al. 2012b), that hydrolyses and releases free FA from its bound state.

Some of these FAE active organisms include probiotic bacteria, microorganisms which have gained interest for the treatment of a number of disorders, including colon cancer and inflammatory bowel disease (Prakash et al. 2011b). Probiotic bacteria with FAE activity can be used to increase the FA concentrations in the intestinal lumen as well as to increase the ratio of "good" bacteria in the gut. Hence, FAE-active probiotic bacteria, administered orally, should prove beneficial in the treatment/prevention of MS by decreasing inflammation and cholesterol levels. To enhance the number of viable and active probiotic bacteria delivery to the lower GIT, microencapsulation can be used as a vehicle (Tomaro-Duchesneau et al. 2013b).

1.2 Research hypothesis

A novel microencapsulated FAE-active *Lactobacillus* formulation can be designed and used as a MetS biotherapeutic, by reducing chronic systemic inflammation and hypercholesterolemia.

1.3 Research objectives

The overall objective of this project is to develop a novel microencapsulated FAE-active *Lactobacillus* formulation for the prevention and treatment of MetS, with the following specific objectives:

1. To screen, select and characterize a FA-producing probiotic *Lactobacillus* bacterium based on the utilization of the natural substrate ethyl ferulate (EFA).
2. To characterize the microencapsulated *Lactobacillus* for its viable delivery to the targeted site using an *in vitro* simulated gastrointestinal model and exposure to acidic conditions as well as gastric and pancreatic enzymes.
3. To investigate and characterize the anti-inflammatory properties of the probiotic *in vitro* using macrophage cells and a co-culture model of the colon epithelium, measuring antioxidant production, pro-inflammatory cytokine secretion and colon epithelial integrity.
4. To investigate and characterize the probiotic effects on cholesterol metabolism *in vitro*, measuring probiotic cholesterol assimilation, probiotic adhesion to the colon epithelium and inhibition of cholesterol uptake by colon epithelial cells.
5. To investigate the final FAE-active probiotic *Lactobacillus* formulation *in vivo* for its effects on diet-induced MetS in a BioF1B hamster animal model.
6. To validate the probiotic *Lactobacillus* formulation's efficacy *in vivo* for its effects on genetically induced MetS using the Zucker Diabetic Fatty rat animal model.

1.4 Thesis outline

The presented thesis contains 11 chapters. A general introduction and the specific objectives of the thesis research are presented in Chapter 1. An exhaustive literature review

of the project is presented in Chapter 2. Six original research manuscripts that are published, accepted or submitted to peer-reviewed journals are presented from Chapters 3-8. These research articles are associated with each of the research objectives aforementioned. Chapter 9 provides a summary of the observations presented in the thesis, with Chapter 10 identifying the original contributions to knowledge and conclusions. Recommendations for future research are provided in Chapter 11, the final chapter of the thesis.

CHAPTER 2: LITERATURE REVIEW

2.1 Metabolic Syndrome

2.1.1 Metabolic syndrome epidemiology and prevalence

The National Cholesterol Education Program's Adult Treatment Panel III (NCEP/ATP III) defines MetS as a constellation of risk factors that increase a person's risk of developing cardiovascular disease (2001; Grundy et al. 2004). **Table 2.1** highlights the diagnostic guidelines for MetS. MetS is an emerging public health concern in a number of Western countries but especially in the United States where, as of 2006, 34% of the adult population was affected (Bethene Ervin 2009). It is a syndrome that increases with age and body mass index (BMI). Genetic factors demonstrate that a family history of hyperlipidemia, obesity, Type 2 Diabetes Mellitus (T2DM) and coronary artery disease increases the risk of MetS development. There are a number of disorders associated with MetS including polycystic ovary syndrome, fatty liver disease with steatosis, fibrosis and cirrhosis, acute pancreatitis, chronic kidney disease and sleep-disordered breathing, including obstructive sleep apnea. There are six risk factors associated with MetS in respect to cardiovascular disease and, according to the guidelines provided by NCEP/ATP III, individuals who have three or more of the risk factors, described below, are considered as having MetS.

2.1.2 Metabolic syndrome risk factors

MetS is not considered a disease but, rather, a cluster of metabolic disturbances, defined by Grundy *et al.*: abdominal obesity, atherogenic dyslipidemia, elevated blood pressure, insulin resistance, proinflammatory state and prothrombotic state, highlighted in **Figure 2.1** (Grundy et al. 2004).

Abdominal obesity: Abdominal obesity, also termed central obesity, is highly correlated with the risk of developing CVD. In humans, a waist circumference of more than 102 cm in men and more than 88 cm in women is indicative of central obesity (Yusuf et al. 2004). Central adiposity in animal models may be assessed by weighing of fat masses following euthanasia.

Atherogenic dyslipidemia: Austin *et al.* were the first to describe the term atherogenic dyslipidemia (Austin et al. 1990). Atherogenic dyslipidemia is a typical feature of MetS (Musunuru 2010), comprised of elevated low-density lipoprotein (LDL) blood levels, reduced high-density lipoprotein (HDL) blood levels and increased triglyceride concentrations. These markers can be routinely measured by serum/plasma collection in fasted humans and rodents.

Elevated blood pressure: Elevated blood pressure, also termed hypertension, is strongly correlated with obesity and is common in insulin-resistant patients. Blood pressure has two measurements, systolic (contracting heart) and diastolic (relaxing heart), taken by sphygmomanometer readings. A normal resting blood pressure has a systolic range of 100-140 mm Hg and a diastolic range of 60-90 mm Hg. A blood pressure that is above these ranges is considered characteristic of hypertension.

Insulin resistance: Insulin resistance, and hyperinsulinemia, is often considered as the key component of all of the MetS risk factors (Ferrannini et al. 1991). It is strongly correlated with the other metabolic risk factors and, more importantly, with CVD risk. Insulin, produced by the islets of Langerhans in the pancreas, signals the cells of the body to take up glucose from the blood. In a state of insulin resistance, the body's cells fail to

respond adequately to the insulin hormone, contributing to hyperglycemia and glucose intolerance, as characteristic of T2DM.

Proinflammatory state: A proinflammatory state, with elevated levels of proinflammatory cytokines and markers, is commonly present in patients with MetS. The role of inflammation in MetS is detailed later.

Prothrombotic state: MetS is also characterized by the presence of a prothrombotic state. The term prothrombotic is defined as an agent or condition that promotes the formation of thrombi in blood vessels, leading to the obstruction of blood circulation. A prothrombotic state is characterized by increased levels of plasma plasminogen activator inhibitor-1 (PAI-1) and fibrinogen, an acute-phase protein.

2.1.3 Metabolic syndrome current therapies and limitations

MetS diagnosis is guided by several guidelines, detailed in **Table 2.1**, with the NCEP/ATP III and World Health Organization (WHO) guidelines as the most used. Following diagnosis of MetS, the primary goal is to treat the underlying causes of the disorder (ex: obesity, physical inactivity), by intensive lifestyle modifications such as diet changes and increased physical activity. If the lifestyle modifications are insufficient, pharmacologic treatment is necessary for the treatment of the metabolic risk factors. Currently used pharmacologic treatments are presented in **Table 2.2**. Dyslipidemia therapies include statins, ezetimibe, fibrates, nicotinic acid and bile acid sequestrants. Hypertension therapies may include Angiotensin-converting enzyme (ACE) inhibitors, beta-blockers, thiazides, alpha-blockers and calcium-channel blockers. Insulin resistance is typically treated with metformin and thiazolidinediones. Many of the therapeutics related to the treatment and management of MetS have important side-effects which may

limit their use. For example, statins, which inhibit 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, a key liver enzyme involved in the production of cholesterol, have shown side effects related to myalgia, muscle cramps, liver enzyme derangements and other symptoms (Thompson PD 2003). In addition, ezetimibe, a therapeutic that inhibits the absorption of cholesterol in the intestine has presented side-effects related to diarrhea, myalgia, hepatits, pancreatitis, hepatic dysfunction and rhabdomyolysis (Stolk et al. 2006). Strikingly, beta-blockers, commonly used for the management of hypertension, increase a patient's risk of developing diabetes, limiting their use as a first-line treatment (Prisant 2004). Metformin, a widely used drug to combat insulin resistance, also has important side effects, including diarrhea, nausea and lactic acidosis (Stang et al. 1999). Importantly, there is currently no single available therapeutic that targets all the risk factors of MetS. With the limitations and shortcomings associated with the current MetS therapeutics, it is clear that there is a need for the development of a novel therapeutic. The role of inflammation in MetS holds great importance in the development of a successful therapeutic.

2.2 Metabolic Syndrome and inflammation

As aforementioned, research has demonstrated an important association between MetS and low-grade chronic systemic inflammation. This inflammatory state has also been termed "metaflammation" to emphasize the condition as metabolically triggered inflammation and "parainflammation" to define MetS as centered between a non-inflammatory and inflammatory state (Hotamisligil 2006; Medzhitov 2008). In MetS, adipose tissue has been shown to secrete adipocytokines, inflammatory molecules whose

levels are inversely correlated with insulin resistance, cardiovascular disease and obesity (Chandran et al. 2003). Several studies have demonstrated an association between insulin resistance and increased interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α), two markers of inflammation, as well as increased leukocyte counts (Vozarova et al. 2001; Weyer et al. 2002). Recent findings have demonstrated that elevated levels of inflammatory markers, such as C-reactive protein (CRP), are a risk factor for MetS and can be used to predict cardiovascular risk (Haffner 2006; Ridker et al. 2002). The results have even suggested that CRP levels may be a stronger predictor of cardiovascular events than low-density lipoprotein (LDL) cholesterol levels (Ridker et al. 2002). These inflammatory characteristics of MetS suggest that the low-grade chronic systemic inflammation may play a causative role in its pathogenesis. This causative role was investigated by Hotamisligil *et al.* who demonstrated that the neutralization of TNF-α in an obese rat model improved insulin sensitivity (Hotamisligil et al. 1993). The characterization of MetS as an inflammatory disorder has important implications for the development of therapeutics. For example, peroxisome proliferator-activated receptor-γ (PPAR-γ), such as rosiglitazone, pioglitazone and troglitazone, were shown to improve endothelial function in patients with insulin resistance (Watanabe et al. 2000), to prevent T2DM (Buchanan et al. 2002) and to inhibit the formation of early atherosclerotic lesions (Collins et al. 2001). In addition, salicylates, non-steroidal anti-inflammatory drugs (NSAIDs), were shown to reverse insulin resistance in obese and diabetic animal models (Hundal et al. 2002; Kim et al. 2001; Yuan et al. 2001). There have been further associations made linking inflammation, intestinal epithelial integrity and MetS (Brun et al. 2007), described later.

2.3 The role of the gut microbiota in metabolic syndrome

2.3.1 The gut microbiota

The gut microbiota contains a broad spectrum of microorganisms, totalling 10^{13} to 10^{14} bacterial cells, but has not been completely explored as of yet (Cani 2009). The microbiome, a term coined by Lederberg, is the totality of microbes, their genetic elements (genomes), and environmental interactions in a particular ecosystem (Lederberg and Mccray 2001). The importance of the gut microbiota is exemplified by the fact that the number of bacterial cells outnumbers human cells by a factor of ten (Turnbaugh et al. 2007). The human intestinal habitat contains 300 to 500 different species of bacteria, varying significantly in content between individuals (Guarner and Malagelada 2003). Most gut bacteria reside in the lower part of the digestive tract, in the large intestine, since the upper tract consists of high levels of acid, bile and pancreatic secretions which are toxic to most microorganisms, as shown in **Figure 2.2** (Guarner and Malagelada 2003). Even though some bacterial species of the gut are potential pathogens, the constant interaction between the host and its microbes usually remains beneficial to the health of the host (Salminen et al. 1998). It has been demonstrated that the gut bacterial population plays an important role in their host's metabolism and energy consumption, especially in the digestion and absorption of nutrients (Cani 2009; Tilg et al. 2009). The upper portion of the gastrointestinal tract (GIT), made up of the stomach and the duodenum, harbours very low numbers of microorganisms, with less than 1000 bacterial cells per gram of contents, with the predominant microorganisms present being Lactobacilli and Streptococci (Dicksved et al. 2009; Guarner et al. 2008). The relatively low number of microorganisms found in the upper digestive tract, although some are of

great importance in human disease, can be explained by the presence of high levels of acid, bile and pancreatic secretions, as aforementioned (Guarner et al. 2008; Mainville et al. 2005). One important organism found in the stomach, which can withstand these harsh conditions, is *Helicobacter pylori*, a microorganism responsible for ulcers and stomach cancer (Montecucco and Rappuoli 2001). There is also a phasic propulsive motor activity in the upper GIT which impedes any stable bacterial colonization (Guarner 2006). Lower in the digestive tract are found the jejunum and the ileum where there is a gradual increase in the bacterial numbers from 10^4 to approximately 10^7 cells per gram of contents by the time the distal ileum is reached (Guarner et al. 2008). Once in the large intestine, the tract is heavily populated by anaerobes with up to 10^{12} cells per gram of luminal contents (Guarner 2006).

Bacteria are classified into genera and species based on their individual phenotypic and genotypic characteristics, with a number of different genera found actively residing in the human GIT. The dominant anaerobic genera are *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Peptococcus*, *Peptostreptococcus* and *Ruminococcus* (Salminen et al. 1998; Willing et al. 2010). The main genera of facultative anaerobic bacteria are *Escherichia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus* and *Proteus* (Salminen et al. 1998; Willing et al. 2010). The proportion and numbers of these bacteria can vary, depending on a number of genetic and environmental factors, including disease state and one's food intake (Cani 2009; Culligan et al. 2009; Davis and Milner 2009; Willing et al. 2010). The main functions of the microbiota were mostly elucidated by investigations with animals bred under germ-free conditions, with

the functions broadly qualified as metabolic, trophic and protective (Falk et al. 1998; Guarner and Malagelada 2003; Vael and Desager 2009).

The gut microbiota has a significant impact on host metabolism, participating in microbial-mammalian co-metabolism. The microbiota is considered a multifunctional organ with metabolic capabilities that humans have not yet fully evolved into their own genomes (Xu and Gordon 2003). It has the ability to break down indigestible plant polysaccharides, termed dietary fibers and also plays an important role in the biotransformation of conjugated bile acids, described in more detail later in this review (Campbell et al. 1997; Hooper et al. 2002; Hylemon and Harder 1998; Martin et al. 2007). The importance of the gut microbiota in vitamin synthesis was demonstrated many years ago with the use of germ-free animals (Mickelsen 1956). Experiments on a chick animal model demonstrated the synthesis of riboflavin, vitamin B, pantothenic acid, vitamin B_{12}, folic acid, nicotinic acid, thiamine and biotin by the gut microbiota (Coates et al. 1968). Furthermore, *Pseudomonas* and *Klebsiella* sp., two resident organisms of the small intestine, were specifically shown to synthesize significant amounts of vitamin B_{12} (Albert et al. 1980). As described, the gut microbiota has extensive roles to play in normal human metabolism, and so its role in MetS is not far-fetched.

2.3.2 The gut microbiota of metabolic syndrome

Recent work suggests that the gastrointestinal microbiota is directly associated with MetS and its associated disorders, as exemplified in **Figure 2.3**. Backhed *et al.* demonstrated this principle using germ-free rodents, animals that are free of all microorganisms both in and on them (Backhed et al. 2004). When the microbiota of conventionally raised animals was transferred to germ-free rodents, the latter

demonstrated a 60% increase in adiposity and insulin resistance, despite decreased muscle mass and food intake (Backhed et al. 2004). Ley *et al.* investigated the gut microbiota composition of ob/ob mice, a model of obesity (Ley et al. 2006). The ob/ob mouse is a genetic model with a gene mutation coding for the leptin hormone, a hormone that is normally responsible for the regulation of food intake and appetite. Ley *et al.* demonstrated that ob/ob mice have a distinct microbiota when compared to their lean counterparts. Specifically, they have a 50% reduction in Bacteroidetes counts (Gram-negative) and a 50% increase in Firmicutes (Gram-positive) (Ley et al. 2006). Similarly, studies by Murphy *et al.* demonstrated that high-fat-fed conventional rodents also displayed a gut microbiota with elevated counts of Firmicutes and reduced counts of Bacteroidetes (Murphy et al. 2010). Indeed, Turnbaugh *et al.* observed similar changes in the gut microbiota when a high-fat/high-sugar Western diet was administered to wild-type mice (Turnbaugh et al. 2008a).

In humans, there have been conflicting results with respect to the changes in the two major bacterial phyla, Bacteroidetes and Firmicutes. Indeed, some clinical studies have observed increases in Firmicutes and decreases in Bacteroidetes in obese individuals (Armougom et al. 2009; Ley et al. 2006; Santacruz et al. 2010). Conversely, many studies have not observed any changes in the ratio of Firmicutes/Bacteroidetes (Duncan et al. 2008; Schwiertz et al. 2010; Zhang et al. 2009). The conflicting results observed may simply be due to differing factors such as age, degree of obesity, diet, population size, demography and gut microbiota analysis methodology. Nonetheless, significant changes in the microbiome have been observed in obese individuals. Turnbaugh *et al.* defined the "core microbiome" as a "set of features shared across all or the vast majority of gut

microbiomes" (Turnbaugh and Gordon 2009). Deviations away from this core were shown to be associated with obesity (Turnbaugh and Gordon 2009). Analysis methods based on metagenomics and systems biology approaches demonstrated that the microbiome of obese individuals have a reduced taxonomic diversity when compared to the microbiome of lean individuals (Greenblum et al. 2012; Turnbaugh et al. 2008b). Recently, research groups have demonstrated that human microbiota can be subdivided into three enterotype clusters, identified by the increased levels of *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3) (Arumugam et al. 2011). Interestingly, these enterotypes have been shown to be significantly modulated by diet, particularly with *Bacteroides* increases associated with protein and animal fat consumption and increases in *Prevotella* associated with carbohydrate consumption (Wu et al. 2011).

2.3.3 Metabolic syndrome, the gut microbiota and inflammation

A key component of MetS, aforementioned, is the presence of a chronic state of low-grade systemic inflammation. Research suggests that changes in the human gut microbiota can alter the intestinal epithelial barrier integrity, including changing the mucous thickness and altering the tight junctions of the epithelium. Tight junctions, also termed zonula occludens, are present between two intestinal epithelial cells creating an impermeable barrier, preventing the passage of molecules, ions and other intestinal luminal contents into the systemic circulation. Lipopolysaccharide (LPS), also termed lipoglycans, are endotoxins found in the outer membrane of Gram-negative bacteria and consist of a lipid and a polysaccharide that are covalently bound, and have been shown to play an important role in the inflammatory processes of MetS. LPS, in the systemic

circulation promotes the secretion of pro-inflammatory cytokines by macrophages and B lymphocytes. In recent work, Cani *et al.* demonstrated that a high-fat diet consumption increases LPS plasma concentrations, subsequently termed "metabolic endotoxemia" (Cani et al. 2007b). Indeed, Cani and his research group investigated this concept further using a continuous administration of low levels of LPS subcutaneously in mice and demonstrated that LPS administration gave rise to most of the features of metabolic diseases (Cani et al. 2007b). Indeed, knockout mice of cluster of differentiation 14 (CD14), a co-receptor for the detection of LPS, delayed the response of high-fat diet consumption on the development of insulin resistance, T2DM and obesity. In addition, Cani *et al.* correlated changes in the Gram negative and Gram positive ratio with LPS plasma concentrations upon high-fat diet consumption, although a causative link was not established (Cani et al. 2007b). Interestingly, oligofructose, a dietary fibre, administration in mice decreased the Gram-negative/Gram-positive ratio and reduced plasma endotoxemia (Cani et al. 2007a; Cani et al. 2006), suggesting that the intestinal microbiota is, indeed, responsible for the development of metabolic endotoxemia. Antibiotic (ampicillin and neomycin) treatment administered to ob/ob mice, prevented adipocyte hypertrophy, improved glucose tolerance, decreased insulin resistance, reduced body weight and decreased adipose weights, and also reduced metabolic endotoxemia and inflammatory markers (Cani et al. 2008). It clear from the current status that the gut microbiota and inflammation are closely associated with MetS.

2.3.4 Metabolic syndrome, the gut microbiota and atherogenic dyslipidemia

Atherogenic dyslipidemia, as aforementioned, is one of the risk factors of MetS, with increases in cholesterol, LDL and triglycerides, as well as a reduction in HDL.

Interestingly, as early as 1959, research was performed to elucidate the role of the gut microbiota in cholesterol homeostasis with researchers demonstrating that germ-free rats, administered a diet without significant amounts of cholesterol, nonetheless showed higher serum-cholesterol values than control rats administered the same diet (Danielsson and Gustafsson 1959). Several mechanisms have been proposed as methods by which the gut microbiota may modulate cholesterol levels within the host, including enzyme specific pathways, such as bile salt hydrolase activity (Delzenne et al. 2008; Jones et al. 2013). Recent developments have demonstrated that the composition of the microbiota and diet are directly correlated with cholesterol levels *in vivo,* specifically, the number of *Bifidobacteria* found in the gut is positively correlated with higher levels of serum HDL (Martinez et al. 2009; Vijay-Kumar et al. 2010; Xiao et al. 2003). In contrast, the number of *Coreobacteriaceae* is correlated with higher levels of non-HDL cholesterol (Martinez et al. 2009). Gut microbial activities influence lipid metabolism, bearing a significant impact on hypercholesterolemia, by the modification of bile acid metabolic patterns, by impacting the emulsification, absorption properties of bile acids, storage of fatty acids in the liver and by influencing the lipoperoxidation through bile acid signalling properties (Martin et al. 2007). It is clear that atherogenic dyslipidemia and the gut microbiota are closely associated, giving rise to a potential therapeutic target for the development of a MetS therapeutic.

2.4 Modulating the gut microbiota

The importance of the gut microbiota in health and disease has been clearly elucidated. The microbiota has become a therapeutic target, with a number of ways to

modify its contents to promote human health, including by the use of antibiotics, prebiotics, probiotics and synbiotics, shown in **Figure 2.4**.

2.4.1 Antibiotics

As the gut microbiota consists of a microbial system, it is evident that antibiotic therapy could be considered to selectively modify the contents of the human microbiota. However, it has become common knowledge, in recent years that although antibiotics can prove beneficial in short-term use their prolonged use may result in significant side effects. An important concern is the development of bacterial resistance which reduces the effectiveness of the therapy and further predisposes the patient to life-threatening illnesses caused by potential pathogens with increased resistance to the antibiotic. As such, the use of antibiotics is not a preferred method for the modulation of the contents of the gut microbiota.

2.4.2 Prebiotics

Prebiotic therapeutics have recently gained interest for modulating the gut microbiota. The Food and Agriculture Organization of the United Nations defines a prebiotic as a "non-viable food component that confers a health benefit on the host, associated with a modulation of the microbiota" (Food and Agriculture Organization of the United Nations (FAO) 2007). Prebiotic molecules consist of naturally occurring or synthetic sugars used by certain colonic bacteria, especially *Bifidobacteria*, as a carbon source for growth and metabolism (Gibson and Roberfroid 1995). Numerous prebiotics have demonstrated their beneficial effects on disease through modulation of the gut microbiota (Bezkorovainy 2001; Gibson et al. 1995; Rastall 2010; Sharma et al. 2008; Tuohy et al. 2005). Examples of prebiotics include trans-galactooligosaccharide, inulin,

fructooligosaccharide, lactulose and mannan oligosaccharides, most of which can be found in dietary sources such as soybeans, chicory root and unrefined wheat. Despite the beneficial effects of prebiotic therapeutics, they only act non-specifically when administered *in vivo* not allowing for a species-specific modulation of the human microbiota contents that probiotic formulations could provide.

2.4.3 Probiotics

Probiotic biotherapeutic formulations can also be used to modulate the intestinal microbiota. Probiotics, as compared to prebiotics, can more specifically modify the intestinal flora, by the delivery of defined microbes. The FAO and WHO define probiotics as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO and WHO 2001). Probiotic microorganisms are more commonly bacteria, but also yeasts and filamentous fungi. Probiotics, of which the most common type are the LAB and bifidobacteria, are naturally occurring organisms found in foods such as milk and yoghurt that are generally recognized as safe (GRAS) by the American Food and Drug Administration (FDA). Studies are also being performed to investigate probiotic safety for human clinical use (Branton et al. 2011; Jones et al. 2012d). Probiotics have been investigated in a number of health disorders including colorectal cancer (Davis and Milner 2009), allergies (Huffnagle 2010), inflammatory bowel disease (Keohane and Shanahan 2010), inflammatory bowel syndrome (Moayyedi et al. 2010), cardiovascular diseases (Jones et al. 2012a) and oral diseases such as periodontitis (Saha et al. 2012b). Of interest is also the use of probiotic bacteria for the treatment and prevention of acquisition of multidrug-resistant organisms including *Staphylococcus aureus*, *Streptococcus pneumonia* and vancomycin-resistant *Enterococci*

(Gluck and Gebbers 2003; Manley et al. 2007; Vandenbergh 1993). Probiotic bacteria have also been used in preterm neonates in the prevention of necrotizing enterocolitis, demonstrating the safety of probiotic bacteria for use in infants (Deshpande et al. 2010; Lin et al. 2008; Manzoni et al. 2006). There are a number of mechanisms by which probiotic bacteria may alter the intestinal flora. One mechanism involves the production of pathogen-inhibitory substances such as organic acids, hydrogen peroxide and bacteriocins. Bacteriocins are ribosomally-synthesized heat-stable antimicrobial peptides secreted by bacteria (termed bacteriocinogenic), including LAB, that have a narrow or extensive target spectrum (Dobson et al. 2012). Probiotic bacteria have also been shown to block pathogen adhesion sites on intestinal epithelial cells. Another proposed mechanism by which probiotic bacteria can alter the intestinal flora is by competing with potential pathogens for nutrients. Probiotic bacteria have been shown to alter the intestinal flora, specifically with an increase of the gram-positive to gram-negative ratio. Research has demonstrated that a decrease in this ratio is linked to increases in adiposity, hepatic triglyceride accumulation, diabetes and increased lipopolysaccharide levels (originating from gram-negative bacteria) that contribute to systemic inflammation (Cani et al. 2007b). *Lactobacillus rhamnosus* GG (American Type Culture Collection (ATCC) 53103), isolated from the intestinal tract of a healthy human being, is a commercial probiotic in a number of countries, including the United States, Netherlands, Finland and Croatia, and has been shown as beneficial for various health disorders. As well, VSL#3 is a commercially available probiotic made of *Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus bulgaricus* and *Streptococcus thermophilus*.

Research has demonstrated that *L. rhamnosus* GG can colonize the intestine and lead to a balance/re-balance of the intestinal flora. Specifically, it was shown to have antimicrobial effects on a number of bacterial species, including *Escherichia coli* and species of *Streptococcus*, *Salmonella*, *Pseudomonas* and *Clostridium*, while showing no inhibitory effect against other strains of *Lactobacillus* (Saxelin 1997).

2.4.4 Probiotic and antibiotic co-administration

The co-administration of probiotics and antibiotics has also demonstrated potential in altering the intestinal flora to promote health (Boyanova and Mitov 2012). It is well-accepted that oral antibiotic administration may lead to dysbiosis, with a commonly associated symptom of diarrhea. In previous years, re-colonization of the intestinal flora, following antibiotic administration, by consumption of foods high in probiotic content, such as yoghurt, were recommended. More recently, human trials have demonstrated the success of co-administration of probiotics with antibiotics for the treatment of bacterial infections, including *Clostridium difficile* nosocomial infections and *Helicobacter pylori* infections (Boyanova and Mitov 2012; Johnston 2005). The co-administration of probiotics with antibiotics may also allow for improved patient compliance.

2.4.5 Synbiotics

Synbiotics may also show important effects in modifying the intestinal flora (Shimizu et al. 2013). Synbiotics are formulations containing both probiotics and prebiotics that act in synergy to promote health. Synbiotics may prove useful in altering the intestinal flora by the effects of the prebiotic on the beneficial bacteria of the intestine, as well as by the delivery of a desired probiotic. Even more interesting is the

concept that the colonization and activity of the delivered probiotic may be enhanced and extended by the co-administration of a prebiotic. For instance, the delivered prebiotic may provide a specific substrate for the probiotic to ferment.

2.5 Ferulic acid as a metabolic syndrome therapeutic

Ferulic acid (FA, 4-hydroxy-3-methoxy cinnamic acid) is a natural phenol isolated from plant cell walls, as a product of the biosynthesis of lignin from phenylalanine and tyrosine (Srinivasan et al. 2007). FA is present in a number of different plants and in both monocots (Hartley and Ford 1989) and dicots (Hartley and Harris 1981). In plants, FA is typically found cross-linked with polysaccharides in the plant cell wall (Iiyama et al. 1994) but may also cross-link with proteins (Figueroa-Espinoza et al. 1999). Many commonly consumed foods contain FA, including, whole grains, citrus fruits, banana, coffee, and orange juice. Among agricultural plant products, FA is present in the highest concentration (3.1 % (w/w)) in maize bran (Saulnier et al. 1995). In plants, FA has been shown to play a number of roles, including providing structural rigidity and strength to the cell wall by the cross-linking of pentosan chains, hemicelluloses, and arabinoxylans, making these components more resistant to hydrolytic enzymes during germination (Graf 1992). In addition, FA is a precursor to various antimicrobial compounds, including phytoalexins, and signaling molecules that contribute to plant disease resistance (Dixon and Paiva 1995). *Cimicifuga heracleifolia* Komarov and *Cimicifuga dahurica* Maxim, frequently used in Japanese Oriental medicines as analgesics and anti-inflammatory drugs (Shibata et al. 1975; Shibata et al. 1977) have FA, and its isomer isoferulic acid, as the main active component (Shotani et al. 1993). FA has shown tremendous potential as an antioxidant, anti-inflammatory agent, antimicrobial

and cholesterol-lowering agent, interesting for the development of a MetS biotherapeutic. The following sections will provide an analysis of the specific properties of FA that make it promosing as a MetS biotherapeutic.

2.5.1 Ferulic acid as an antioxidant

Humans are susceptible to the attack of free radicals and reactive oxygen species (ROS), such as superoxide and hydroxyl radicals, which are present in high levels in a number of health disorders, including cardiovascular diseases, obesity, type 2 diabetes and atherosclerosis (Hulsmans et al. 2012). FA has been shown to scavenge hydrogen peroxide, superoxide, hydroxyl and nitrogen dioxide free radicals (Kayahara et al. 1999; Ketsawatsakul et al. 2000; Ou et al. 1999; Zhouen et al. 1998). FA's antioxidant activity in terms of free radical scavenging is strongly attributed to its structural characteristics (**Figure 2.5**). Potent antioxidant activity can be explained by its phenolic nucleus and unsaturated side chain which allow for the formation of a resonance stabilized phenoxy radical since the unpaired electron may be present on the oxygen but also delocalized across the FA molecule (Srinivasan et al. 2007). The resulting phenoxy radical cannot propagate a radical chain reaction, most often resulting in collision and condensation with another ferulate radical forming the curcumin dimer (Srinivasan et al. 2007). This may result in even greater free radical scavenging activity provided by enhanced resonance stabilization and o-quinone formation (Virgili et al. 2000). Furthermore, FA has three structural motifs that provide significant free radical scavenging properties. The presence of 3 methoxy and 4-hydroxyl on the benzene ring of FA contribute two electron donating groups that provide free radical scavenging activity. In addition, the carboxylic acid group adjacent to an unsaturated carbon-carbon double bond provides antioxidant

activity. This carboxylic acid group may also provide an anchor for FA to bind to a lipid bilayer, combatting lipid peroxidation (Srinivasan et al. 2006). Also of interest is the fact that FA has been shown to increase the activity of enzymes capable of scavenging free radicals, including hepatic glutathione S-transferase and hepatic and colonic quinone reductase in a rat model of colon cancer (Kawabata et al. 2000). Conversely, FA and its derivatives have also been shown to inhibit host enzymatic free radical producing activity. Specifically, Kayahara *et al* demonstrated inhibitory activity towards tyrosinase and superoxide dismutase (Kayahara et al. 1999).

2.5.2 Ferulic acid as an anti-inflammatory agent

Macrophages play a central role in chronic and acute inflammation, overproducing pro-inflammatory cytokines and inflammatory mediators, such as ROS, nitric oxide (NO), tumor necrosis factor (TNF)-α and prostaglandin E_2. FA has been shown to decrease the levels of prostaglandin E2, NO, macrophage inflammatory protein 2 (MIP-2) and tumor necrosis factor-alpha (TNF-α) (Ou et al. 2003). FA has shown the ability to inhibit the production of MIP-2, a polymorphonuclear leukocytes chemotactic molecule, in respiratory syncytial virus (RSV) infected (Sakai et al. 1999) and lipopolysaccharide (LPS) stimulated Raw 264.7 cells (Sakai et al. 1997). FA was also shown to reduce the production of interleukin-8 (IL-8), a neutrophil chemotactic factor, *in vitro* and *in vivo*, in response to influenza infections (Hirabayashi et al. 1995).

2.5.3 Ferulic acid as an antimicrobial

FA has demonstrated important antimicrobial effects, against bacteria, yeasts and viruses. FA has shown antimicrobial activity towards yeasts as well as both Gram-positive and Gram-negative bacteria (Jeong et al. 2000). FA has also shown to inhibit a

number of human gastrointestinal inhabitants, including *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Shigella sonnei* (Lo and Chung 1999; Tsou et al. 2000). The antimicrobial activity of FA has been correlated with bacterial arylamine *N*-acetyltransferase inhibition (Lo and Chung 1999). In terms of antiviral activity, Edeas *et al.* demonstrated the inhibition of the AIDS virus by FA using an infected cell line, *in vitro* (Edeas et al. 1995).

2.5.4 Ferulic acid as an antidiabetic agent

Of interest for the development of a MetS biotherapeutic, FA has also demonstrated antidiabetic effects. Indeed, FA (10-40 mg/kg animal mass) was shown to reduce blood glucose levels in streptozotocin (STZ)-induced diabetic female rats (Balasubashini et al. 2004). In addition, FA administration led to a significant expansion of pancreatic islets, increasing β-cell mass, thus promoting the secretion of insulin and the utilization of sucrose in the STZ-induced diabetic rats (Balasubashini et al. 2004). Indeed, Prabhakar *et al.* proposed that FA can act synergistically with currently available antidiabetic drugs to manage diabetes and its associated complications (Prabhakar et al. 2013).

2.5.5 Ferulic acid as a cholesterol-lowering agent

FA has also shown potential as a MetS biotherapeutic due to its significant cholesterol-lowering activity. When orally delivered to rats, FA significantly reduced LDL cholesterol and very-low-density lipoprotein (VLDL) cholesterol in rats, while also increasing high-density lipoprotein (HDL) cholesterol levels (Kamal-Eldin et al. 2000; Kim et al. 2003). Son *et al.* demonstrated that the administration of FA, incorporated in the diet of high-fat fed mice, demonstrated a hypolipidemic effect, promoting an

increased fecal excretion of cholesterol and triglycerides, as well as by inhibiting fatty acid biosynthesis (Son et al. 2010).

2.6 Ferulic acid esterase active probiotic as a metabolic syndrome biotherapeutic

In terms of oral delivery, orally-delivered FA is quickly absorbed in the upper gastrointestinal tract and quickly excreted, greatly reducing its residence time (Zhao et al. 2004). Interestingly, FA esterases (FAE) have been isolated from a wide range of fungi, bacteria and yeast, including in *Aspergillus niger*, *Pycnoporus cinnabarinus*, *Aureobasidium pullulans*, *Streptomyces avermitilis*, *Clostridium thermocellum*, *Bacillus spp.*, *Lactobacilli*, *Pseudomonas fluorescens* and *Brettanomyces anomalus* (de Vries and Visser 1999; Ou and Kwok 2004). FAE can release aromatic residues, such as FA from polymers present in plant cell walls and other esterified substances (De Vries et al. 1997; Kroon et al. 1999). **Figure 2.6** demonstrates the production of FA from a dietary food such as wheat bran, and from a simpler substrate, ethyl ferulate (EFA), used for *in vitro* screening assays. Interestingly, probiotic bacterial strains have been shown to possess FAE activity (Bhathena et al. 2007; Bhathena et al. 2008; Lai et al. 2009b). Oral administration of probiotic bacteria with FAE activity, with the availability of a dietary substrate, such as wheat bran, allows for the production of FA in the gastrointestinal tract. The probiotic cells can provide continuous low levels of FA, increasing its bioavailability. It is suggested that the beneficial effects of probiotic bacterial cells and FA can be formulated into a synergistic combination by formulating a FAE-active probiotic biotherapeutic.

2.7 Probiotic delivery

2.7.1 Currently available formulations for the delivery of probiotics

For centuries, live microorganisms have been consumed in fermented foods such as yoghurt, sauerkraut and cheeses, with Élie Metchnikoff pioneering the concept of probiotics in the early 1900s (Metchnikoff 1907). In recent years, various delivery systems have been used as probiotic carriers, including nutraceuticals and functional foods. A nutraceutical is a product that is isolated/purified from foods and sold in medicinal forms, that has a physiological benefit on the consumer. Probiotic supplement formulations are commonly referred to as nutraceuticals as the bacteria are typically isolated from foods such as cheese and yoghurt. A functional food is a conventional food consumed as part of a regular diet that has demonstrated physiological benefits. Common functional food products include yoghurts and drinks which have been supplemented with prebiotics, probiotics, antioxidant molecules or vitamins. Functional foods are often preferred over nutraceuticals as they may increase patient compliance. In short, prebiotics and probiotics can be delivered orally in the form of nutraceuticals and functional foods.

2.7.2 Requirement for an alternative probiotic delivery system

In terms of probiotic delivery, the probiotic cells are most frequently targeted to the colon, the important site of bacterial fermentation. Hence, an important concern is the delivery of both viable and metabolically active bacterial cells to the colon, following a transit through the harsh conditions of the upper gastrointestinal tract (acidic pH, digestive enzymes and bile).One commonly used delivery vehicle is the enteric coated capsules containing lyophilized (freeze-dried) bacterial cells. Enteric coated capsules are designed to release their contents in the small intestine, due to a more basic content. The

use of enteric coated capsules generally limits the delivery of probiotics by nutraceuticals rather than by functional foods. A more efficient delivery vehicle is, hence, required to ensure optimal probiotic delivery to the colon.

2.7.3 Microcapsules as a probiotic delivery system

Microencapsulation is a method defined as the "entrapment of a compound or a system inside a dispersed material for its immobilization, protection, controlled release, structuration and functionalisation" (Poncelet 2006). Microencapsulation has been widely used for the encapsulation and immobilization of microorganisms (Prakash et al. 2011b). Bacterial cell encapsulation is a process that can occur naturally as bacteria proliferate and produce exo-polysaccharides, high-molecular-weight polymers composed of sugar residues. The exo-polysaccharide structure can act as a protective capsule and reduce the permeability and bacterial exposure to potential adverse environmental factors. Recent research has led to the development of a great variety of microcapsules which can differ in size, composition and function, depending on the final goal of the encapsulated product. Microcapsules can be used to entrap all sorts of substances: solids, liquids, drugs, proteins, bacterial cells, stem cells, etc (Hernandez et al. 2010; Rokka and Rantamaki 2010; Song et al. 2009). With such a range of substances that can be entrapped, microcapsules can have an assortment of objectives and applications, whether for drug delivery, enzyme retrieval, artificial cell and artificial organ delivery and, as described in this review, for the delivery of live probiotic bacteria.

There are a number of microcapsule delivery systems that have been proposed for the oral delivery of live bacterial cells, as detailed in **Table 2.2**. Sun *et al.* investigated the use of acid-stable beads made of gellan and xantham gum for the immobilization of

Bifidobacterium (Sun and Griffiths 2000). The research group demonstrated that immobilized cells survived significantly better than free cells after refrigeration in pasteurized yogurt for a period of 5 weeks (Sun and Griffiths 2000). One common encapsulation method, for viable cell immobilization, utilizes calcium alginate as a polymer (Hansen et al. 2002). However, one prominent difficulty encountered with the use of alginate beads is that these, alone, are not acid resistant and upon exposure to the low pH conditions encountered in the stomach, display significant shrinkage and a decrease in mechanical strength (Krasaekoopt et al. 2004). A number of methods utilizing polymer cross-linking have been suggested, including formulations using carrageenan, alginate-poly-L-lysine, starch polyanhydrides, polymethacrylates and enteric coated polymers (Ouyang et al. 2004). Microencapsulation methods are still being developed and optimized to allow for increased gastrointestinal survival and immunoprotection. One newly developed type of microcapsule that shows promising results in terms of mechanical stability and pH resistance is the genipin-crosslinked-alginate-chitosan (GCAC) microcapsule (Chen et al. 2007a; Chen et al. 2010).

One of the most commonly utilized and characterized formulations for microencapsulation is the alginate-poly-L-lysine-alginate (APA) microcapsule (Prakash and Chang 1996). This type of microcapsule has been used for many applications including drug, stem cell and bacterial cell delivery. This method relies on a polyelectrolyte complexation mechanism for the association of the polymers, alginate and poly-L-lysine (PLL). Alginate is a naturally occurring biocompatible polymer, extracted from brown algae, that is increasingly being used in the biotechnology industry for a wide range of applications (Wee and Gombotz 1998). Alginate is an unbranched

polysaccharide which contains 1,4'-linked β-D-mannuronic acid and α-L-guluronic acid blocks which are interdispersed with regions of the alternating structure, β-L-mannuronic acid-α-L-guluronic acid blocks (Haug and Bjorn Larsen 1962). PLL is a polypeptide made up of the amino acid L-lysine that is available in a variable number of chain lengths, determined by its molecular weight. It is a polycationic polymer that can be used during the coating step of microencapsulation. The addition of this polymer leads to the formation of a capsule membrane that provides selective permeability and immunoprotection. The alginate bead could not withstand the harsh conditions of the GIT in the absence of PLL, which provides it with an increased mechanical stability. Microencapsulation has successfully been used for the oral delivery of probiotic cells in renal failure, cardiovascular diseases, and in colon disorders. To enhance the number of viable and FAE-active probiotic bacteria delivery to the lower gastrointestinal tract upon oral administration, APA microencapsulation is used as a vehicle (Tomaro-Duchesneau et al. 2013b).

2.8 Summary of the Literature and Thesis Research Goals

With the limitations and shortcomings associated with the current MetS therapeutics, it is clear that there is a need for the development of a novel therapeutic. Probiotic bacteria provide a safe, natural and cost-effective approach. Probiotic FAE activity can provide a synergistic approach to the development of a MetS probiotic biotherapeutic. Building on the already available research, this thesis project aims to develop a novel microencapsulated FAE-active *Lactobacillus* formulation for the prevention and treatment of MetS. The first research objective involves the screening,

selection and characterization of a FA-producing probiotic *Lactobacillus* bacterium using *in vitro* studies. Upon probiotic selection, the second goal focuses on the *in vitro* characterization of the APA microencapsulated probiotic for its viable delivery to the targeted site. To investigate the potential of the probiotic formulation as a MetS biotherapeutic, studies into the anti-inflammatory properties of the *Lactobacillus* probiotic were performed *in vitro* using a co-culture model of the intestinal epithelium. *In vitro* studies were also performed to investigate the potential of the probiotic formulation for cholesterol lowering. Finally, *in vivo* studies using both a dietary and genetic rodent models of MetS were undertaken to investigate the efficacy of the FA-producing probiotic formulation.

Table 2.1: MetS diagnosis guidelines by different organizations. The WHO and the NCEP/ATP III guidelines are the most used clinically.

World Health Organization (WHO) – 1998
Insulin resistance: Hyperinsulinemia or a fasting plasma glucose equal to or >6.1 mmol/L or a 2 h postprandial serum glucose >11.1mmol/L, plus any two of the following: • *Abdominal adiposity*: waist-to-hip ratio >0.90 in men and >0.85 in women or body mass index (BMI) > 30kg/m^2 • *Atherogenic dyslipidemia*: high-density lipoprotein (HDL) <0.9mmol/L for men, < 1.0 mmol/L for women; and serum triglyceride >1.7mmol/L • *Elevated blood pressure*: >140/90 mmHg
European Group for Study of Insulin Resistance (EGIR) - 1999
Insulin resistance: plasma insulin > 75th percentile, plus any two of the following: • *Abdominal adiposity*: Waist circumference equal to or >94cm in men or equal to or >80cm in women • *Atherogenic dyslipidemia*: Triglycerides ≥1.7mmol/L and/or HDL <1.0mmol/L • *Elevated blood pressure:* ≥140/90 mmHg
National Cholesterol Education Program's Adult Treatment Panel III (NCEP/ATP III) - 2001
Three or more of the following: • *Abdominal obesity*: waist circumference: >102 cm in men, > 88 cm in women • *Atherogenic dyslipidemia*: fasting blood triglycerides ≥ 1.7 mmol/L • *Atherogenic dyslipidemia*: Low levels of HDL cholesterol < 1.0 mmol/L for men, < 1.3 mmol/L for women • *Elevated blood pressure*: ≥ 130/85 mmHg • *Fasting blood glucose*: ≥ 6.1 mmol/L

Guidelines are referenced from: WHO (Alberti and Zimmet 1998), EGIR (Balkau and Charles 1999), NCEP/ATP III (2001).

Table 2.2: MetS therapeutics, mechanism(s) of action and their associated limitations.

MetS Therapeutic Methods	Mechanism(s) of Action	Limitations	Reference
Dyslipidemia			
Statins Simvastatin, Atorvastatin, Fluvastatin, Lovastatin	HMG-CoA reductase inhibitors	Do not target hypertension and insulin resistance Side effects related to hepatotoxicity and muscle toxicity	(Jacobson 2006)
Ezetimibe Ezedoc, Ezetib, Ezetrol, Maxetibe, Zemitra, Zetavim, Zetia, Zient	↓cholesterol absorption (NPC1L1)	Does not target hypertension and insulin resistance Reports of "no effects" in a number of clinical trials Adverse reactions related to myalgia, pancreatitis and myopathy	(Kastelein et al. 2008; Simard and Poirier 2006)
Fibrates Bezafibrate/ Bezalip, Ciprofibrate/ Modalim, Gemfibrozil/ Lopid, Fenofibrate/ TriCor	↑ lipoprotein lipolysis ↑ hepatic fatty acid uptake ↓ hepatic triglyceride production ↑ removal of LDL particles ↓ neutral lipid exchange between VLDL and HDL ↑ HDL production and reverse cholesterol transport	Do not target hypertension and insulin resistance Side effects include myopathy, renal failure, kidney toxicity	(Carvalho et al. 2004)
Nicotinic acid Vitamin B_3	↑ HDL cholesterol and reverse cholesterol transport ↓ lipolysis in adipocytes	Does not target hypertension and insulin resistance ↑ Hyperglycemia Adverse reactions include skin rashes, liver toxicity, birth defects and nausea	(Garg and Grundy 1990)
Bile acid sequestrants Cholestyramine/ Questran,	Bind to bile acids and sequester them	Do not target hypertension and	(Andrade et al. 1995)

Colesevelam/ Welchol, Colestipol/ Colestid	from the enterohepatic circulation	insulin resistance Not as efficacious as statins Side effects include vitamin deficiencies, gastrointestinal disruptions	
Hypertension			
Angiotensin-converting enzyme (ACE) inhibitors Perindopril, Captopril, Enalapril, Lisinopril, Ramipril	Block conversion of angiotensin I to angiotensin II ↓ renin-angiotensin-aldosterone system activity ↓ tension of blood vessels and blood volume	Do not target dyslipidemia and insulin resistance Adverse reactions incude renal impairment, hyperkalemia, angioedema and congenital malformations	(Schepkens et al. 2001)
Beta-blockers Acebutolol/ Sectral, Atenolol/ Tenormin, Bisoprolol/ Zebeta, Metoprolol, Nadolol/ Corgard, Nebivolol/ Bystolic, Propranolol/ Inderal LA	Block the effects of epinephrine (adrenaline)	Alter glucose and lipid metabolism Side effects include nausea, bradycardia, heart failure, fatigue, edema, hypoglycemia	(Rubin 1982)
Thiazides Bendroflumethiazide/ Naturetin, Chlorothiazide/ Diuril, Chlorthalidone/ Hygroton, Indapamide/ Lozol	↓ reabsorption of Na and Cl ions in the kidneys ↑ reabsorption of Ca ions in the kidneys	May worsen diabetes ↑ Hyperglycemia ↑ Hyperlipidemia Adverse effects include hypotension, gout, renal failure, hypokalemia	(Zillich et al. 2006)
Alpha-blockers Alfuzosin/ Uroxatral, Doxazosin/ Cardura, Prazosin/ Minipress, Terazosin, Tamsulosin/ Flomax	Block the effects of norepinephrine (noradrenaline), opening vessels	Do not target dyslipidemia and insulin resistance Not a first treatment option Adverse effects include hypotension, heart failure, weight gain and nausea	(Debruyne 2000)
Calcium-channel blockers Amlodipine/ Norvasc, Diltiazem/ Cardizem LA, Felodipine, Isradipine/ DynaCirc CR, Verapamil/	↓ Ca ion entry into heart cells and blood vessel walls	Do not target dyslipidemia and insulin resistance Not a first treatment option	(Buckley et al. 2007)

Calan		Side effects include constipation, nausea, swelling of lower legs, tachycardia	
<u>Insulin resistance</u>			
<u>Metformin</u> Glucophage, Glumetza, Fortamet, Riomet	↓ hepatic gluconeogenesis ↑ insulin sensitivity ↑ peripheral glucose uptake ↑ fatty acid oxidation ↓ gut glucose absorption	Does not target dyslipidemia and hypertension Adverse reactions include diarrhea, nausea, lactic acidosis and impaired liver and kidney functions	(Bolen et al. 2007)
<u>Thiazolidinediones/Glitazones</u> Rosiglitazone, Pioglitazone, Troglitazone	↑ peroxisome proliferator-activate receptor (PPARγ) receptor activity ↓ insulin resistance ↓ leptin levels ↑ adiponectin levels	Do not target dyslipidemia and hyptertension Side effects include edema, hepatitis, liver failure and heart failure	(Edwin et al. 2006)

Table 2.3: Types of microcapsules for the targeted delivery of probiotic bacteria.

Types of Microcapsules	Bacteria	Reference(s)
Alginate Beads	*L. rhamnosus* *B. longum* *L. salivarius* *L. plantarum* *L. acidophilus* *L. paracasei* *L. casei* *B. lactis* *L. reuteri*	(Capela et al. 2006; Chandramouli et al. 2004; Ding and Shah 2007; Krasaekoopt et al. 2004; Lee and Heo 2000; Mandal et al. 2006; Muthukumarasamy and Holley 2007)
Alginate-cellulose acetate phthalate	*B. lactis* *L. acidophilus*	(Favaro-Trindade and Grosso 2002)
Alginate-chitosan	*B. animalis* subsp. *lactis* *L. bulgaricus*	(Lee et al. 2004; Liserre et al. 2007)
Alginate-chitosan-Acryl-Eze	*B. animalis* subsp. *lactis*	(Liserre et al. 2007)
Alginate-chitosan-alginate	*B. bifidum* *L. casei*	(Krasaekoopt et al. 2004)
Alginate-chitosan-Sureteric	*B. animalis* subsp. *lactis*	(Liserre et al. 2007)
Alginate-coated gelatin	*B. adolescentis* *B. pseudolongum*	(Annan et al. 2008)
Alginate-poly-L-lysine-alginate	*B. bididum* *L. reuteri* *L. casei*	(Krasaekoopt et al. 2004; Martoni et al. 2008)
Alginate-starch	*L. acidophilus* *B. lactis* *B. infantis* *L. casei*	(Homayouni et al. 2008; Kailasapathy 2006; Sultana et al. 2000)
Gelatin-gum arabic-soluble starch	*B. infantis* *B. longum*	(Hsiao et al. 2004; Lian et al. 2002; Lian et al. 2003)
Gelatin-toluene-2-4-diisocyanate	*L. lactis*	(Hyndman et al. 1993)
Gellan-alginate	*B. bifidum*	(Chen et al. 2007b)
Gellan-xanthan	*B. adolescentis* *B. bifidum* *B. breve* *B. infantis* *B. lactis* *B. longum*	(McMaster et al. 2005; McMaster and Kokott 2005; Sun and Griffiths 2000)
Genipin-crosslinked-alginate-chitosan	*L. plantarum*	(Chen et al. 2007a)

Pectin-casein	*B. lactis* *L. acidophilus*	(Oliveira et al. 2007)
Potato starch granules-amylose	*B. longum*	(Lahtinen et al. 2007)
Whey protein	*B. breve* *B. longum* *L. rhamnosus*	(Ainsley Reid et al. 2005; Picot and Lacroix 2004)
κ-carageenan	*B. longum* *S. thermophilus* *L. bulgaricus* *S. lactis*	(Adhikari K. et al. 2002; Audet et al. 1988)

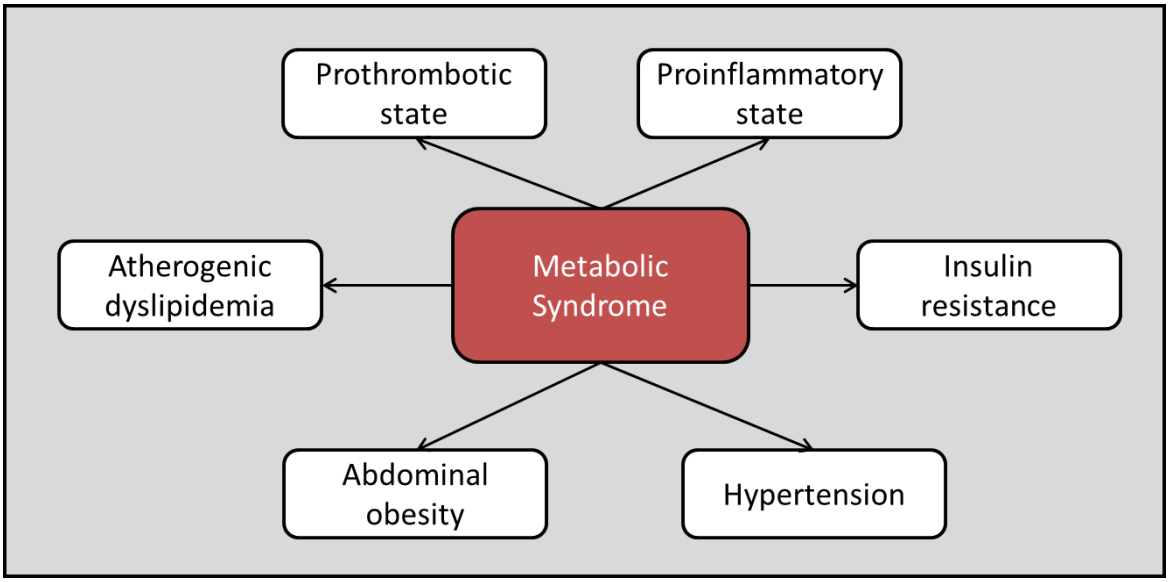
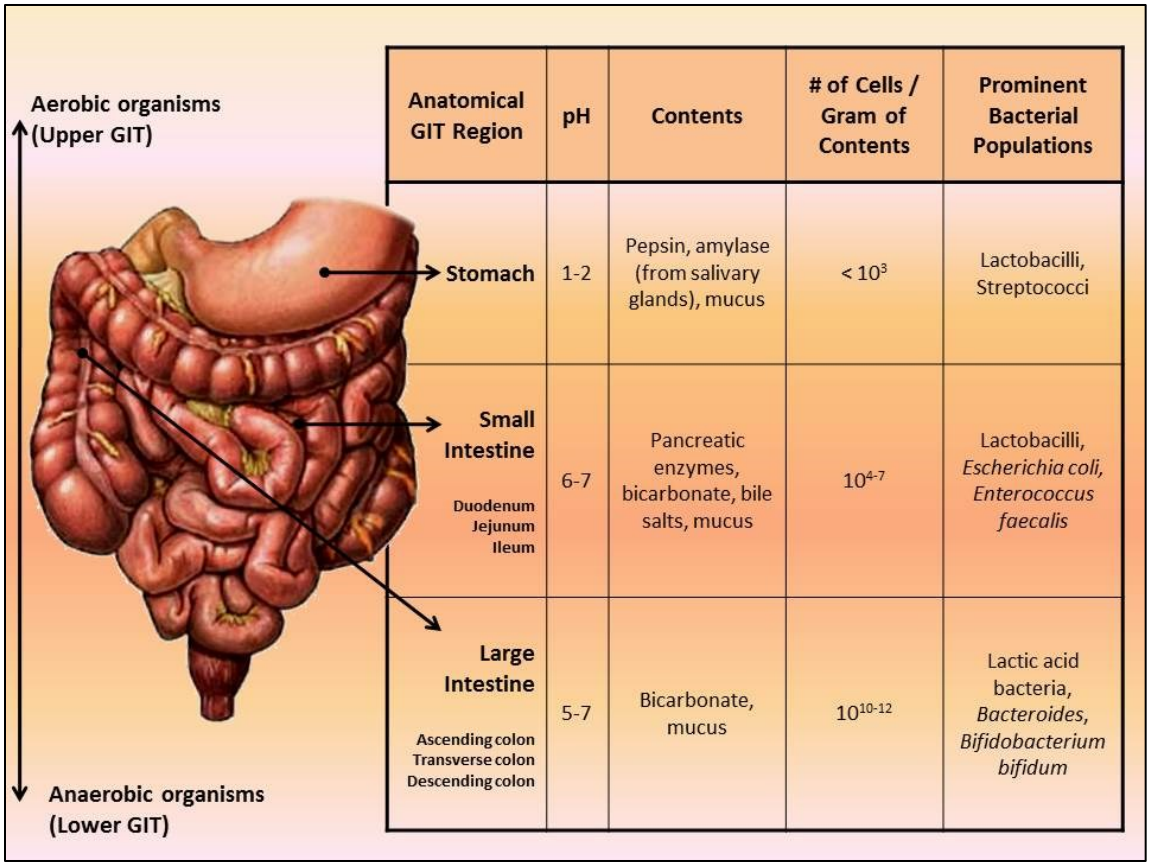


Figure 2.1: Metabolic Syndrome and its associated risk factors.



Anatomical GIT Region	pH	Contents	# of Cells / Gram of Contents	Prominent Bacterial Populations
Stomach	1-2	Pepsin, amylase (from salivary glands), mucus	$< 10^3$	Lactobacilli, Streptococci
Small Intestine (Duodenum, Jejunum, Ileum)	6-7	Pancreatic enzymes, bicarbonate, bile salts, mucus	10^{4-7}	Lactobacilli, *Escherichia coli*, *Enterococcus faecalis*
Large Intestine (Ascending colon, Transverse colon, Descending colon)	5-7	Bicarbonate, mucus	10^{10-12}	Lactic acid bacteria, *Bacteroides*, *Bifidobacterium bifidum*

Figure 2.2: The gastrointestinal tract (GIT) characteristics (oxygen distribution, pH, bacterial populations, and bacterial cell counts) and the localization of the various gut bacterial populations, termed microbiota (Prakash et al. 2011b).

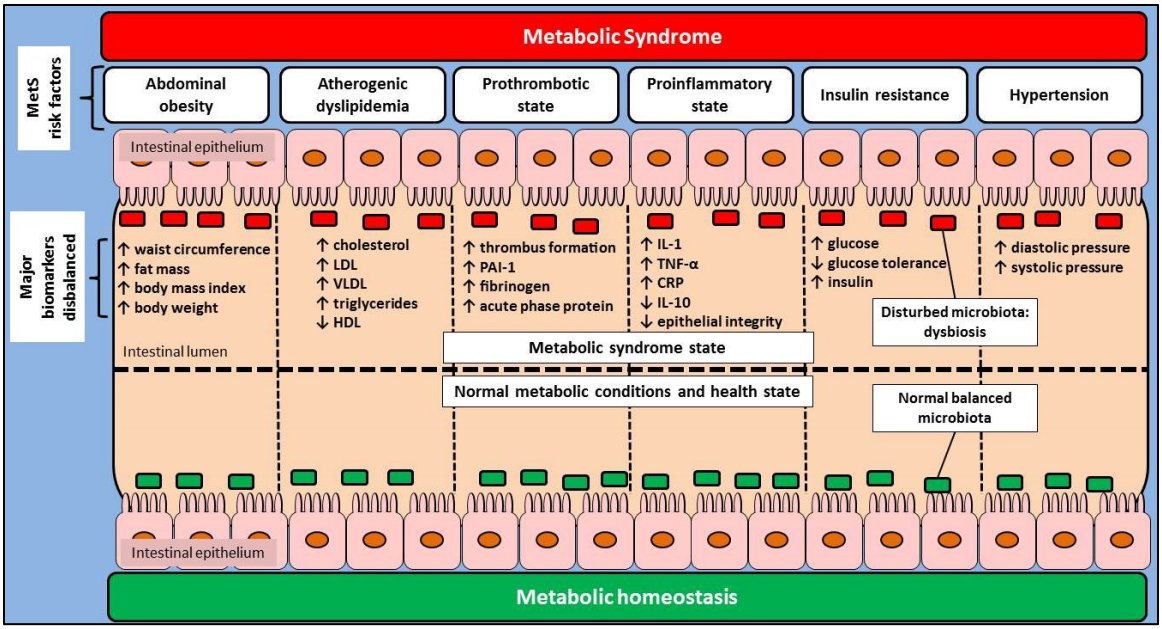


Figure 2.3: The gut microbiota in metabolic syndrome and metabolic homeostasis.

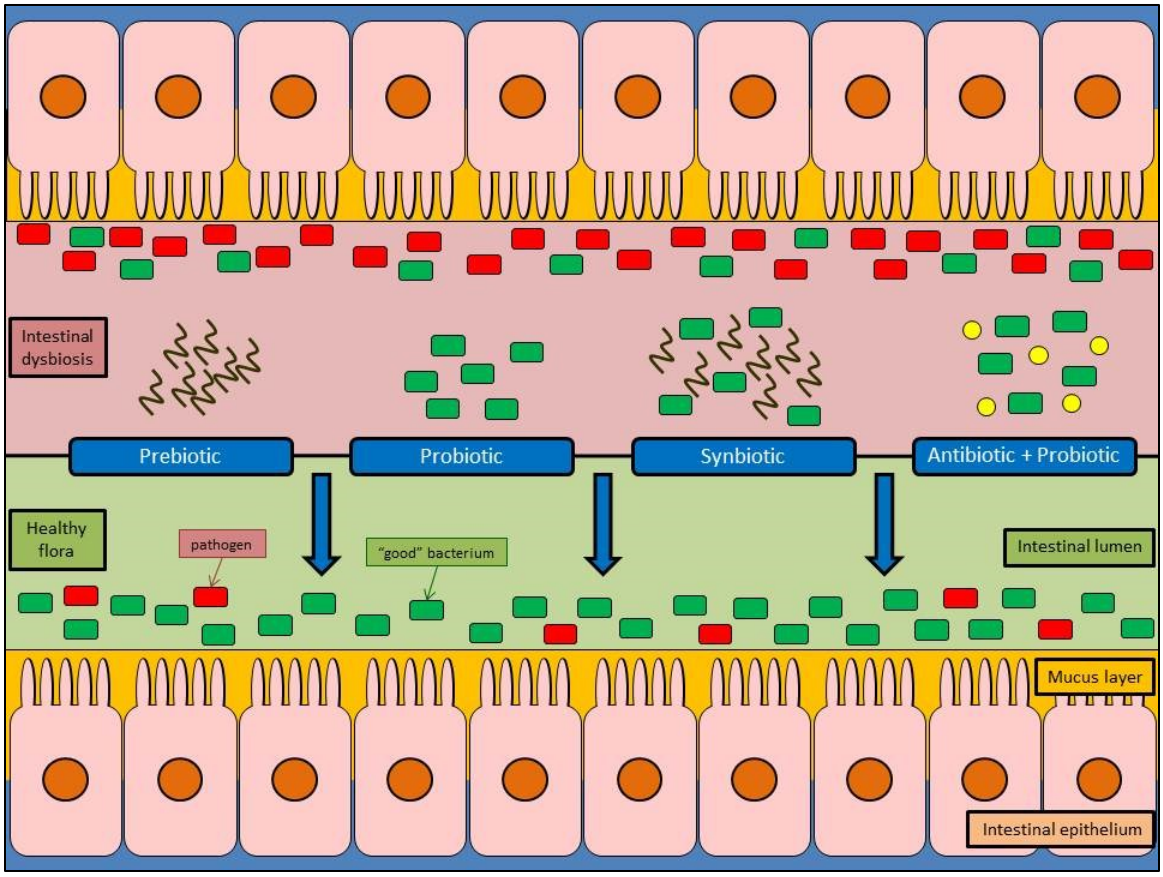


Figure 2.4: Modulation of the gut microbiota contents using prebiotics, probiotics, synbiotics and antibiotic-probiotic combination.

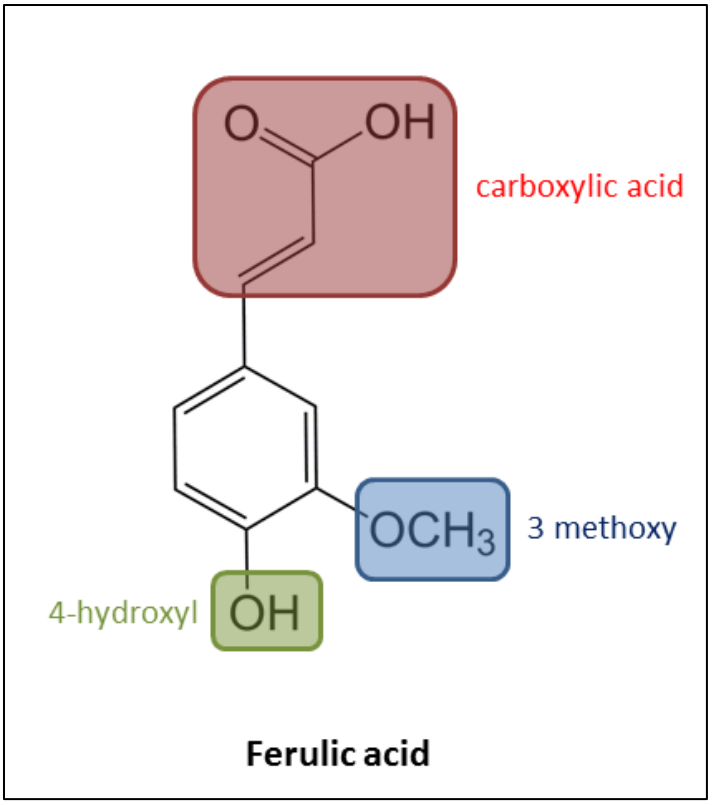


Figure 2.5: Ferulic acid structure and associated antioxidant free radical scavenging functional groups, highlighted in dark.

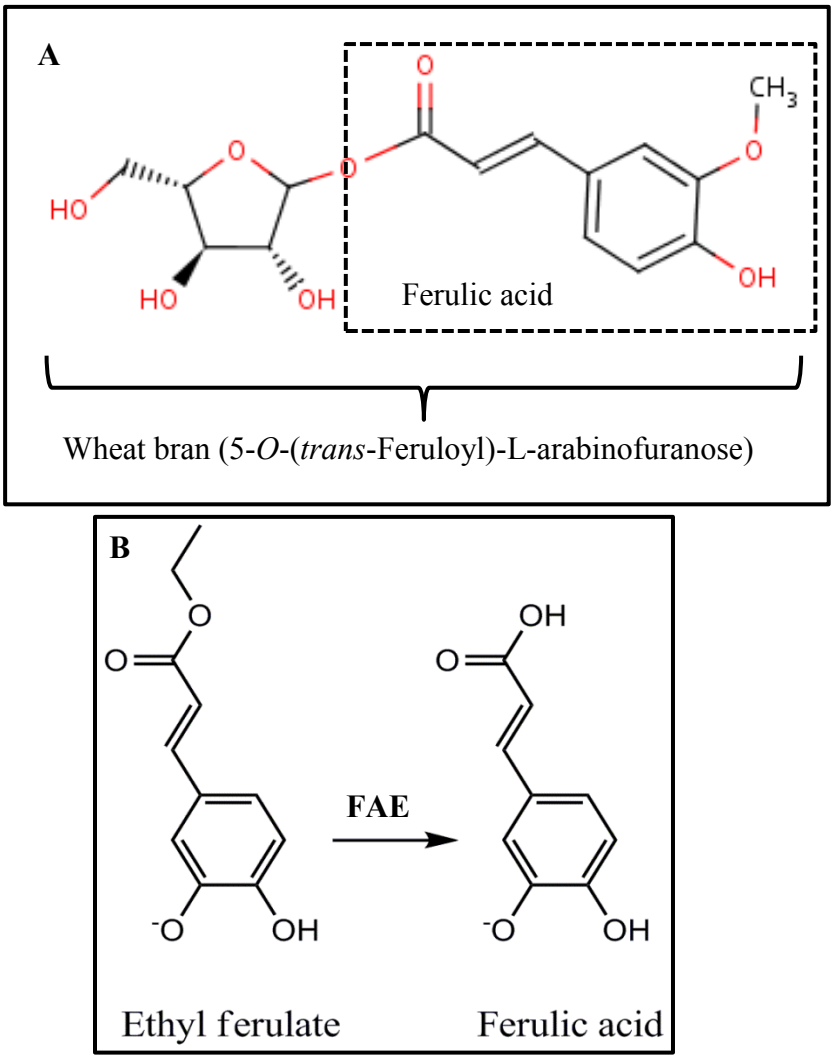


Figure 2.6: Ferulic acid esterase activity on (A) wheat bran and (B) ethyl ferulate.

PREFACE TO CHAPTERS 3 - 8

Presented in the following chapters are the studies performed in order to investigate the stated research hypothesis and to achieve the stated research objectives. Each chapter discusses various important aspects of the thesis research project.

Chapter 3: There is a great need for the development of a novel therapeutic to manage MetS, as the literature suggests. Ferulic acid-producing probiotic bacteria possess important potential for the development of such a biotherapeutic. The goal of this chapter was to select a *Lactobacillus* bacterium that produces ferulic acid, based on their ferulic acid esterase enzymatic activity, as well as other chacteristics. Probiotic bacteria were screened for ferulic acid production using both qualitative and quantitative assays. The selected probiotic strain was then further investigated for growth and enzymatic kinetics, as well as antioxidant activity.

Chapter 4: Following the selection of a ferulic acid-producing probiotic strain, in Chapter 3, there was a need to microencapsulate the strain for successful oral delivery of viable and active probiotic cells. Microencapsulation, as demonstrated in the literature, has been used to encapsulate drugs, bacterial and mammalian cells. The *L. fermentum* probiotic strain, selected Chapter 3, was microencapsulated using alginate-polylysine-alginate microencapsulation. The microencapsulation size and efficiency, in terms of probiotic viability, was determined. The ferulic acid esterase enzymatic activity of *L. fermentum* was investigated to determine whether the microcapsule would impede the uptake of the ethyl ferulate substrate and the release of the desired product, ferulic acid. The protective capability of the alginate-polylysine alginate microcapsule was then demonstrated using simulated gastrointestinal conditions.

Chapter 5: MetS is characterized by low-grade chronic systemic inflammation. Hence, following the selection and microencapsulation of the probiotic strain, in Chapters 3 and 4, investigations into the anti-inflammatory properties of the probiotic were undertaken, *in vitro*, by measurements of ferulic acid production and antioxidant activity. Probiotic nitric oxide, a mediator of inflammation, production was also quantified. Macrophage cells stimulated by lipopolysaccharide were used to represent the inflammation present in the gastrointestinal tract in MetS. Specifically, pro-inflammatory molecule, Tumor Necrosis Factor-α and nitric oxide, production by the macrophages was determined with and without probiotic treatment. A co-culture model of an inflamed intestinal epithelium was established using colon epithelial and macrophage cells, and used to show the effect of *L. fermentum* on lipopolysaccharide-induced intestinal epithelial permeability.

Chapter 6: MetS is also characterized by dyslipidemia. The goal of the research presented in this chapter was to investigate, *in vitro*, the cholesterol-lowering properties of probiotic *L. fermentum*, selected in Chapter 3. The cell surface hydrophobicity of the probiotic was also determined by hydrocarbon adhesion. *L. fermentum* showed no significant impact on Caco-2, colon epithelial cell viability. In addition, colon epithelial cells pre-exposed to *L. fermentum* significantly decreased cholesterol uptake compared to the untreated cells. These results demonstrate that *L. fermentum* has important properties for the successful development of a cholesterol-lowering biotherapeutic and potentially a therapeutic for MetS.

Chapter 7: Following characterization *in vitro*, as presented in Chapters 3, 4, 5 and 6, of the probiotic formulation for its potential as a MetS therapeutic, the aim of this chapter was to investigate the role of alginate-polylysine alginate microencapsulated *L.*

fermentum to modulate markers of MetS *in vivo*, using a high-fat fed $BioF_1B$ Golden Syrian hamster model. We investigated the effects on the adiposity index, serum insulin, insulin resistance, glycosylated albumin, serum leptin, serum uric acid, serum total cholesterol, serum esterified cholesterol and free fatty acid levels in the treated animals. This research indicates that the probiotic *L. fermentum* formulation may significantly delay the onset of insulin resistance, hyperglycemia, hyperinsulinemia, dyslipidemia and obesity, indicating a lower risk of diabetes and cardiovascular disease.

Chapter 8: Following investigations using a diet-induced model of MetS, presented in Chapter 7, we investigated the formulation's effect on a genetic model of MetS, the Zucker Diabetic Fatty rat, presented in this chapter. We investigated the probiotic effect on insulin levels, insulin resistance, serum triglycerides, serum low-density lipoprotein cholesterol, serum cholesterol, serum high-density lipoprotein cholesterol and the atherogenic and atherosclerosis index. This research indicates that the administration of the ferulic acid-producing *L. fermentum* can reduce insulin resistance, hyperinsulinemia, hypercholesterolemia, and other markers involved in the pathogenesis of MetS, in a genetic model of the syndrome.

Chapter 9: This chapter provides a summary of the findings of the thesis work.

Chapter 10: This chapter details the claims of the original contributions to knowledge and conclusions

Chapter 11: This chapter provides the recommendations for future research.

During this thesis research period, I contributed to **32** original research articles/reviews/book chapters, of which **11** are as first author and **28** are currently published or in press. I also contributed to **36** research abstracts or proceedings, of which **8** are as presenting author. In this thesis, I have elected to use **6** articles, of which I am the first author.

Original research articles included in the thesis:

1. **C Tomaro-Duchesneau**, S Saha, M Malhotra, M Coussa-Charley, H Al-Salami, ML Jones, A Labbé and S Prakash. (2012) *Lactobacillus fermentum* NCIMB 5221 has a greater potential for the production of ferulic acid when compared to other ferulic acid esterase active Lactobacilli. *International Journal of Probiotics and Prebiotics.* 7(1), 23-32.
2. **C Tomaro-Duchesneau**, S Saha, M Malhotra, M Coussa-Charley, I Kahouli, ML Jones, A Labbé, S Prakash. Probiotic Ferulic Acid Esterase Active *Lactobacillus fermentum* NCIMB 5221 APA Microcapsules for Oral Delivery: Preparation and *in Vitro* Characterization. (2012) *Pharmaceuticals.* 5(2), 236-248.
3. **C Tomaro-Duchesneau**, S Saha, L Rodes, M Malhotra, S Prakash. (2013) Anti-inflammatory properties of *Lactobacillus fermentum* NCIMB 2797: an *in vitro* investigation. *Journal of Molecular Biology* (*submitted*)
4. **C Tomaro-Duchesneau**, S Saha, M Malhotra, ML Jones, S Prakash. (2013) Ferulic acid producing *L. fermentum* as cholesterol-lowering probiotic biotherapeutics. *British Journal of Nutrition* (*submitted*)
5. J Bhathena*, **C Tomaro-Duchesneau* (*co-first)**, C Martoni, M Malhotra, A Kulamarva, AM Urbanska, A Paul, S Prakash. (2012) Effect of orally administered microencapsulated FA-producing *L. fermentum* on markers of metabolic syndrome: an *in vivo* analysis. *Journal of Diabetes & Metabolism*. S2-009.
6. **C Tomaro-Duchesneau**, S Saha, M Malhotra, ML Jones, A Labbé, L Rodes, I Kahouli, Satya Prakash. (2013) Effect of orally administered *L. fermentum* NCIMB 5221 on markers of metabolic syndrome: an *in vivo* analysis using ZDF rats. *Applied Microbiology and Biotechnology* (*in press*)

Original articles not included in the thesis (published/accepted/submitted):

7. **C Tomaro-Duchesneau**, D Shah, P Jain, S Saha, S Prakash (2013) Cholesterol assimilation by *Lactobacillus* probiotic bacteria: an *in vitro* investigation (*to be submitted*)

8. **C Tomaro-Duchesneau**, S Saha, M Malhotra, I Kahouli, S Prakash. (2013) Microencapsulation for the therapeutic delivery of drugs, live mammalian and bacterial cells and other biopharmaceutics: current status and future directions. *Journal of Pharmaceutics*.

9. ML Jones*, **C Tomaro-Duchesneau* (*co-first)**, C Martoni, S Prakash. (2013) Cholesterol-lowering with Bile Salt Hydrolase (BSH) active probiotic bacteria, mechanism of action, clinical evidence and future direction for heart health applications. *Expert Opinion on Biological Therapy*. 13(5): 631-642.

10. S Saha*, **C Tomaro-Duchesneau* (*co-first)**, JT Daoud, M Tabrizian, S Prakash. (2013) Novel probiotic dissolvable carboxymethyl cellulose films as oral health biotherapeutics: *in vitro* preparation and characterization. *Expert Opinion on Drug Delivery* 10(11): 1471-1482.

11. S Prakash, **C Tomaro-Duchesneau**, S Saha, L Rodes, I Kahouli, M Malhotra. (2013) Probiotics for the prevention and treatment of allergies, with an emphasis on mode of delivery and mechanism of action. *Current Pharmaceutical Design*. (*in press*)

12. M Malhotra, **C Tomaro-Duchesneau**, S Prakash. (2013) Synthesis of TAT peptide-tagged PEGylated chitosan nanoparticles for siRNA delivery targeting neurodegenerative diseases. *Biomaterials*. 34(4), 1270-1280.

13. M Malhotra, **C Tomaro-Duchesneau**, S Saha, I Kahouli, S Prakash. (2013) Development and characterization of novel chitosan-PEG-TAT nanoparticles for the intracellular delivery of siRNA. *International Journal of Nanomedicine* 8: 2041-2052.

14. I Kahouli, **C Tomaro-Duchesneau**, S Prakash. (2013) Probiotics in colorectal cancer (CRC) with emphasis on mechanisms of actions and current prospectives. *Journal of Medical Microbiology* 62(Pt 8): 1107-1123.

15. L Rodes, **C Tomaro-Duchesneau**, S Saha, A Paul, M Malhotra, D Marinescu, W Shao, S Prakash. (2013) Enrichment of Bifidobacterium longum subsp. infantis ATCC 15697 within the human gut microbiota using alginate-poly-L-lysine-alginate microencapsulation oral delivery system: an in vitro analysis using a computer-controlled dynamic human gastrointestinal model. *Journal of Microencapsulation* (*in press*)

16. S Saha, **C Tomaro-Duchesneau**, L Rodes, M Malhotra, M Tabrizian, S Prakash. Characterization of probiotic bacteria as oral health biotherapeutics: an *in vitro* analysis. *Research in Microbiology* (*submitted*)

17. M Malhotra, **C Tomaro-Duchesneau**, S Saha, S Prakash. (2013) Systemic siRNA delivery via peptide tagged polymeric nanoparticles, targeting PLK1 gene in a mouse xenograft model of colorectal cancer. *International Journal of Biomaterials* (*in press*)

18. D Marinescu, **C Tomaro-Duchesneau**, M Malhotra, Imen Kahouli, ML Jones, L Rodes, S Prakash. Effect of orally administered APA microencapsulated BSH-

active *Lactobacillus reuteri* NCIMB 30242 on markers of obesity: an *in vivo* analysis. *Journal of Medical Microbiology* (*in press*)

19. J Bhathena, C Martoni, A Kulamarva, **C Tomaro-Duchesneau**, M Malhotra, A Paul, S Prakash. (2013) Oral probiotic microcapsule formulation ameliorates non-alcoholic fatty liver disease in Bio F1B Golden Syrian hamsters. *PLOS ONE* 8(3): e58394.
20. L Rodes*, M Coussa-Charley*, D Marinescu, A Paul, M Fakhoury, S Abbasi, A Khan, **C Tomaro-Duchesneau**, S Prakash. (2013) Design of a novel gut bacterial adhesion model for probiotic applications. *Artificial Cells, Blood Substitutes, and Biotechnology: An International Journal.* 41(2): 116-124.
21. L Rodes, A Khan, A Paul, M Coussa-Charley, D Marinescu, **C Tomaro-Duchesneau**, W Shao, I Kahouli, S Prakash. (2013) Effect of probiotics *Lactobacillus* and *Bifidobacterium* on gut derived-lipopolysaccharides and inflammatory cytokines: an *in vitro* study using a human colonic microbiota model. *Journal of Microbiology and Biotechnology.* 23(4): 518-526.
22. M Malhotra, **C Tomaro-Duchesneau**, S Saha, S Prakash. (2013) Intranasal siRNA delivery to the brain by TAT/MGF tagged PEGylated chitosan nanoparticles. *Journal of Pharmaceutics* (*in press*)
23. S Saha, **C Tomaro-Duchesneau,** M Tabrizian, S Prakash. (2012) Probiotics as oral health biotherapeutics. *Expert Opinion on Biological Therapy*. 12(9), 1207-1220.
24. S Saha, **C Tomaro-Duchesneau**, M Malhotra, M Tabrizian, S Prakash. (2012) Suppression of *Streptococcus mutans* and *Candida albicans* by probiotics: an *in vitro* study. *Dentistry*.
25. S Prakash, **C Tomaro-Duchesneau**, S Saha, A Cantor. (2011) The Gut Microbiota and Human Health with an Emphasis on the Use of Microencapsulated Bacterial Cells. *Journal of Biomedicine and Biotechnology*.
26. WB Branton, ML Jones, **C Tomaro-Duchesneau**, CJ Martoni, S Prakash. (2011) In-vitro characterization and safety of the probiotic strain *Lactobacillus reuteri cardioviva* NCIMB 30242. *International journal of probiotics and prebiotics*. 6(1), 1-12.
27. M Malhotra, C Lane, **C Tomaro-Duchesneau**, S Saha, S Prakash. (2011) A novel method for synthesizing PEGylated chitosan nanoparticle: strategy, preparation and in-vitro analysis. *International Journal of Nanomedicine*. 6, 485-494.
28. S Prakash, M Malhotra, W Shao, **C Tomaro-Duchesneau**, Sana Abbasi. (2011) Polymeric nanohybrids and functionalized carbon nanotubes as drug delivery carriers for cancer therapy. *Advanced Drug Delivery Reviews.* 63(14-15), 1340-1351.
29. L Rodes, A Paul, M Coussa-Charley, H Al-Salami, **C Tomaro-Duchesneau**, M Fakhoury, S Prakash. (2011) Transit time affects the community stability of Lactobacillus and Bifidobacterium species in an in-vitro model of human colonic

microbiotia. *Artificial Cells, Blood Substitutes, and Biotechnology: An International Journal.* 39(6), 351-356.

30. S Prakash, L Rodes, M Coussa-Charley, **C Tomaro-Duchesneau**. (2011) Gut microbiota: next frontier in understanding human health and development of biotherapeutics. *Biologics: Targets and Therapy.* 2011(5), 71-86.

Book chapters:

31. **C Tomaro-Duchesneau**, S Saha, S Prakash. (2013) Chapter 2: Modification of the intestinal flora to promote human health. *In: Ask the experts: Probiotics, prebiotics and gut health*. (*in press*)
32. M Malhotra, C Tomaro-Duchesneau, S Saha, S Prakash (2014) Intranasal delivery of chitosan-siRNA nanoparticle formulation to the brain. *In: Drug Delivery Systems*. (*in press*)

Oral presentations:

1. **C Tomaro-Duchesneau** (2013). Health benefits of ferulic acid-producing probiotic bacteria. McGill University Biomedical Engineering Seminar Series. Oct 23. Montreal, QC, Canada
2. **C Tomaro-Duchesneau.** Rat Metabolic 3-plex: Insulin, Glucagon, GLP-1. (2012) *2nd Meso Scale Discovery Multiplexing Symposium*. Nov 29. McGill University, Montreal, Canada
3. M Malhotra, **C Tomaro-Duchesneau**, S Saha, S Prakash. (2013) Peptide-tagged nanoparticle formulation for intranasal, siRNA delivery to the brain. Society for Brain Mapping and Therapeutics. 10th Annual World Congress of SBMT. Baltimore Convention Centre. May 12-14. Baltimore, MD, USA
4. M Malhotra, **Catherine Tomaro-Duchesneau**, S Saha, S Prakash. (2012) Novel nanoparticles for targeted delivery of siRNA in CNS diseases. *TechConnect World Submit & Innovation Showcase*. June 18-21. Santa Clara, California, USA
5. M Malhotra, **C Tomaro-Duchesneau**, S Saha, R Chemali, S Prakash. (2011) Nanocarriers for siRNA brain delivery to silence neurodegenerative diseases in pets and humans. *38th Annual Meeting and Exposition of the Controlled Release Society – Delivering Bioactives 2011*. July 30 - August 3. National Harbor, MD, U.S.A
6. S Saha, **C Tomaro-Duchesneau**, M Malhotra, M Tabrizian, Satya Prakash (2011). Role of Probiotics in Dental Caries Causing Organism *Streptococcus mutans* and Other Bacterial Systems: In-Vitro Analysis. McGill *University-Faculty of Dentistry–6th Annual Research Day*, Montreal, QC, Canada. February 21.

Poster presentations:

7. **C Tomaro-Duchesneau**, S Saha, M Malhotra, ML Jones, A Labbé, L Rodes, S Prakash. (2013). Ferulic acid-producing probiotic *Lactobacillus fermentum* as a metabolic syndrome biotherapeutic. *Biomedical Engineering Symposium 2013*. Sept 13. McGill University, Montreal, QC, Canada.
8. **C Tomaro-Duchesneau**, S Saha, M Malhotra, ML Jones, A Labbé, L Rodes, I Kahouli, S Prakash. (2013) Oral administration of probiotic *L. fermentum* NCIMB 5221 in ZDF rats alleviates markers of metabolic syndrome. *Probiotics, Prebiotics, and the Host Microbiome: The Science of Translation*. June 12. NY, NY, USA.
9. **C Tomaro-Duchesneau**, S Saha, M Malhotra, ML Jones, A Labbé, L Rodes, I Kahouli, S Prakash. Modulation of markers of metabolic syndrome by oral administration of FA-producing *L. fermentum* NCIMB 5221: an *in vivo* study. (2013) *The 10th Annual NHP Conference and Trade Show: The Best of Both Worlds- Tradition & Modern Approaches*. May 12-15. Windsor, ON, Canada.
10. **C Tomaro-Duchesneau**, S Saha, J Daoud, M Tabrizian, S Prakash. (2013) Development of a novel dissolvable carboxymethyl cellulose film for the oral delivery of probiotic bacteria. *McGill University-Faculty of Dentistry – 8th Annual Research Day*, Montreal, QC. March 28.
11. **C Tomaro-Duchesneau**, S Saha, ML Jones, A Labbé, M Malhotra, I Kahouli, L Rodes, S Prakash. APA microcapsules for the delivery of *Lactobacillus fermentum* NCIMB 5221: an in-vitro study. (2012) *Canadian Society for Pharmaceutical Sciences, Canadian Society of Pharmacology and Therapeutics, Controlled Release Society and the Natural Health Products Research Society of Canada–Modern Therapeutics 2012: Advances in Physiology, Pharmacology and Pharmaceutical Sciences.* June 12-15. Toronto, ON, Canada.
12. **C Tomaro-Duchesneau**, S Saha, M Malhotra, H Al-Salami, I Kahouli, S Prakash. (2011) Artificial cell APA microcapsules for the delivery of a *Lactobacillus fermentum* based therapeutic. *Canadian Society for Pharmaceutical Sciences, Canadian Society of Pharmacology and Therapeutics, Controlled Release Society and the Natural Health Products Research Society of Canada–Multidisciplinary Approaches to Modern Therapeutics: Joining Forces for a Healthier Tomorrow*. May 24 – 27. Montreal, QC.
13. L Rodes, **C Tomaro-Duchesneau**, S Saha, W Shao, I Kahouli, S Prakash. (2013). Anti-inflammatory properties of probiotics: an *in vitro* investigation using a human colonic microbiota model and RAW 264.7 macrophage cells. *1st International Center for Infection and Immunity of Lille Symposium: Revisiting Paradigms in Innate Immunity*. Oct 7-9. Lille, France.
14. S Saha, **C Tomaro-Duchesneau**, M Malhotra, L Rodes, M Tabrizian, S Prakash. (2013). *In vitro* screening and characterization of probiotic bacteria for the prevention and treatment of oral diseases. *Biomedical Engineering Symposium 2013*. Sept 13. McGill University, Montreal, QC, Canada.

15. L Rodes, **C Tomaro-Duchesneau**, S Saha, W Shao, I Kahouli, S Prakash. (2013). Investigation of anti-inflammatory properties of probiotics: an *in vitro* study using a human colonic microbiota model and RAW 264.7 macrophage cells. *Biomedical Engineering Symposium 2013*. Sept 13. McGill University, Montreal, QC, Canada.

16. S Saha, **C Tomaro-Duchesneau**, L Rodes, M Malhotra, M Tabrizian, S Prakash. (2013) Probiotic bio-therapeutic for the prevention and treatment of dental caries. *Probiotics, Prebiotics, and the Host Microbiome: The Science of Translation*. June 12. NY, NY, USA.

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CONTRIBUTIONS OF CO-AUTHORS

As the first author of the original research articles included in this thesis, I was responsible for the planning and execution of each study, including a review of the literature and the specific research objectives and methodology to be implemented, as well as the data analysis. Dr. Satya Prakash, who is the last author in each of the research articles, is the corresponding author. Specific co-author contributions for each chapter are provided below.

Chapter 3: Shyamali Saha and Meenakshi Malhotra helped with the cell culture experiments and in the writing of the manuscript. Michael Coussa-Charley provided assistance for the HPLC analysis. Hani Al-Salami, Mitchell L. Jones and Alain Labbé helped with troubleshooting at the various stages of the experiments as well as analyzing and discussing the results and the manuscript.

Chapter 4: Shyamali Saha and Meenakshi Malhotra provided assistance with the cell culture experiments, data analysis and writing of the manuscript. Michael Coussa-Charley provided assistance for the HPLC analysis. Imen Kahouli helped proofreading the manuscript. Mitchell L. Jones and Alain Labbé helped with troubleshooting at the various stages of the experiments as well as in the preparation of the manuscript.

Chapter 5: Shyamali Saha provided assistance with all of the *in vitro* experiments and preparation of the manuscript. Laetitia Rodes and Meenakshi Malhotra provided assistance in data interpretation and manuscript preparation.

Chapter 6: Shyamali Saha and Meenakshi Malhotra provided intellectual and technical assistance with all of the *in vitro* experiments and preparation of the manuscript. Mitchell

L. Jones provided technical assistance. Laetitia Rodes helped to prepare and proofread the manuscript.

Chapter 7: Jasmine Bhathena worked on all aspects of this chapter, in collaboration with me. Christopher Martoni, Arun Kulamarva and Aleksandra Malgorzata Urbanska provided help with the animal studies. Meenakshi Malhotra helped with the animal sample analysis and the preparation of the manuscript.

Chapter 8: Shyamali Saha helped with the animal studies, animal sample analysis and manuscript preparation. Meenakshi Malhotra helped to analyze the animal samples and proofreading the manuscript. Mitchell L. Jones and Alain Labbé provided guidance and troubleshooting throughout the various stages of the animal work and to proofread the manuscript. Laetitia Rodes and Imen Kahouli provided assistance with the animal work.

Original Research Article 1

CHAPTER 3: LACTOBACILLUS FERMENTUM NCIMB 5221 HAS A GREATER FERULIC ACID PRODUCTION COMPARED TO OTHER FERULIC ACID ESTERASE PRODUCING LACTOBACILLI

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Preface: There is a great need for the development of a novel therapeutic to manage MetS. Ferulic acid-producing probiotic bacteria possess important potential for the development of such a biotherapeutic. The goal of this chapter was to select a *Lactobacillus* bacterium that produces ferulic acid, as a result of ferulic acid esterase enzymatic activity. Probiotic bacteria were screened for ferulic acid production using both qualitative and quantitative assays. The selected probiotic strain was then further investigated for growth and enzymatic kinetics, as well as antioxidant activity.

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3.1 Abstract

Ferulic acid (FA) is an antioxidant compound known to neutralize free radicals, such as reactive oxygen species (ROS). These free radicals have been shown to be involved in DNA damage, cancer and aging. The administration of FA, as an oral therapeutic is hampered by its absorption in the small intestine followed by its quick excretion. Colonic microbial enzymes have been shown to produce FA. In this article, selected Lactobacillus strains were screened for FA production by Ferulic Acid Esterase (FAE), as determined by the release of free FA from a natural substrate, ethyl ferulate (EFA). Using a MRS-EFA plate clearing assay, *L. fermentum* ATCC 11976, *L. reuteri* ATCC 23272 and *L. fermentum* NCIMB 5221 all showed clearance zones of 10 mm in diameter, confirming FAE activity. Results show that *L. fermentum* NCIMB 5221 is the most efficient FA producing strain, producing 0.168±0.001mg/mL FA following 48 hours of incubation in 0.296 mg/mL EFA. We also investigated the total antioxidant capacity of *L. fermentum* NCIMB 5221 when grown in culture. Results suggest that, due to its FA production, *L. fermentum* NCIMB 5221 has potential for use as a future therapeutic.

3.2 Introduction

Antioxidants are therapeutically interesting molecules capable of neutralizing free radicals, such as Reactive Oxygen Species (ROS). ROS have been implicated in DNA damage, cancer and accelerated cell aging (Lombard et al. 2005a). Ferulic acid (FA), a phenolic acid, is a potent antioxidant able to neutralize these free radicals (Rice-Evans et al. 1996). Recent studies suggest that FA can have direct antitumor activity against breast cancer (Chang et al. 2006; Kampa et al. 2003) and liver cancer (Lee 2005; Taniguchi et al. 1999) and is also effective at preventing cancer induced by the exposure to various carcinogenic compounds such as benzopyrene (Lesca 1983) and 4-nitroquinoline 1-oxide (Tanaka et al. 1993). Along with its health beneficial properties, FA also exhibits a number of potential commercial applications in food preservation and in the production of vanillin, making it a compound of economic interest (Ou and Kwok 2004). Currently, FA is being produced industrially by batch fermentation using fungal ferulic acid esterases (FAE) (Bartolome et al. 1997; Bonnin et al. 1999). It has been proposed that gut bacterial cells also produce FAE, an enzyme that has the inherent capacity to produce FA from available substrates in the gastrointestinal tract **Figure 3.1**.

In terms of a therapeutic, the oral delivery of free FA is limited due to its quick absorption in the small intestine, specifically in the jejunum, followed by its rapid excretion (Spencer et al. 1999; Zhao et al. 2003). The oral delivery of conjugated FA, in the form of a dietary source such as wheat bran, is a feasible alternative, with the release of free FA by microbial FAE present in the lower human digestive tract, giving rise to a constant and controlled release of FA (Spencer et al. 1999; Zhao et al. 2003). Attempts have also been made to develop probiotic therapeutic formulations to enhance the FA

bioavailability for the treatment of inflammatory metabolic disorders (Bhathena et al. 2009). This article investigates Lactobacilli for FA production from a dietary substrate, explores the correlation between biomass production and FAE activity and investigates the antioxidant production of the most FAE-active strain.

3.3 Materials and Methods

3.3.1 Chemicals

Ethyl ferulate (ethyl 4-hydroxy-3-methoxycinnamate, EFA) and ferulic acid (*trans*-4-hydroxy-3-methoxycinnamate, FA) were purchased from Sigma-Aldrich (Oakville, ON, Canada). De Man, Rogosa, Sharpe (MRS) broth and Methanol of high-performance liquid chromatography (HPLC) grade were obtained from Fisher Scientific Canada (Ottawa, ON, Canada). Water was purified with an EasyPure reverse osmosis system and a NanoPure Diamond Life Science (UV/UF) ultrapure water system from Barnstead (Dubuque, IA, USA). The QuantiChrom™ Antioxidant Assay Kit was purchased from BioAssay Systems (Hayward, California, USA). All other chemicals were of analytical or HPLC grade and purchased from commercial sources.

3.3.2 Bacterial strains and culture conditions

The bacterial strains *Lactobacillus reuteri* ATCC 23272, *Lactobacillus rhamnosus* ATCC 53103 and *Lactobacillus fermentum* ATCC 11976 were purchased from Cedarlane Laboratories (Burlington, ON, Canada). The bacterial strain *Lactobacillus fermentum* NCIMB 5221 was purchased from NCIMB (Aberdeen, Scotland, UK). All bacterial strains were stored at -80°C in MRS containing 20% (v/v) glycerol. An MRS-agar plate was streaked for isolation from the frozen stock and

incubated at 37°C with 5% CO_2 for 24 hours to ensure purity. One colony from the MRS-agar plate was inoculated into 5mL of MRS broth and incubated at 37°C for 24 hours. A 1% (v/v) inoculum was then used for subculturing and incubated at 37°C for 24 hours immediately before use.

3.3.3 Ferulic acid esterase activity method: plate assay

Prior to the assay, the bacterial strains were subcultured from MRS broth at 1% (v/v) to MRS-EFA broth at an EFA concentration of 1.33mM and incubated at 37°C for 24 hours. MRS-EFA agar was prepared at 1.5% (w/v) of agar in MRS broth at pH 6.5 and autoclaved at 121°C for 15 minutes. The MRS agar was placed in a water bath to cool down to 55°C. 0.3mL of sterile EFA, prepared as a 10% (w/v) solution in dimethylformamide, was added per 20mL of agar mix. Using the poured and dried MRS-EFA agar plates, bacterial strains were impregnated, using three sterile Whatman #3 filter disks, in the culture from the MRS-EFA broth, placed on the MRS-EFA agar and then incubated at 37°C and 5% CO_2 for 48 hours. Each strain was impregnated on filter disks in triplicates to measure accuracy and reproducibility.

3.3.4 Ferulic acid esterase activity and FA production method: HPLC assay

All bacterial strains were subcultured from MRS broth at 1% (v/v) to MRS-EFA broth at an EFA concentration of 1.33mM (0.2956mg/mL). Uninoculated MRS-EFA broth was used as a negative control and treated in the exact same way. Each sample was treated in triplicate and incubated at 37°C during the course of the experiment. An HPLC assay, modified from Mastihuba et al., was used to measure FAE activity (Mastihuba et al. 2002). At every time point, 500µL of each sample was added to centrifuge tubes and centrifuged at 10,000rpm for 7 min at 4°C. The resulting supernatant (300µL) was

acidified with 0.35M H_2SO_4 (100µL) and briefly vortexed. To the acidified solution, 1mM benzoic acid (300µL) was added, as an internal standard, to each tube followed by the addition of 0.7M NaOH (100µL) to neutralize the pH. The processed samples were then stored at -20°C prior to HPLC analysis.

For HPLC analysis, the samples were thawed to room temperature and filtered with a 0.45µm syringe filter. The HPLC analysis was performed on a reverse-phase C-18 column: LiChrosorb RP-18, 25 x 0.46cm (Grace Davison Discovery Sciences, ON, Canada). The HPLC system consists of a ProStar 335 diode array detector (DAD) set at 280nm and 320nm, a ProStar 410 autosampler, and the software Star LC workstation version 6.41. 25µL was injected for each sample. The mobile phase (solvent A) consisted of 37% (v/v) methanol and 0.9% (v/v) acetic acid in water (HPLC grade). Solvent B consisted of 100% (v/v) methanol. The HPLC run was initiated with solvent A at 100% for 16 minutes. This was then followed by a 1 minute linear gradient to reach 100% of solvent B, attained at the 17^{th} minute. Solvent B was isocratically held at 100% for 12 minutes, until the 29^{th} minute. This was then followed by a 1 minute linear gradient to reach 100% of solvent A by the 30^{th} minute. Standard curves of FA and EFA, using peak area quantification, were generated for quantifying the test samples' FA and EFA concentrations. The FA standard curve was generated using triplicates and the concentrations 100, 300, 500, 960 and 1100µM were plotted against peak area (R^2=0.9869). The EFA standard curve was generated using triplicates and the concentrations 100, 300, 500, 700, 1000, 1400 and 1800µM were plotted against peak area (R^2=0.9785). Standards and quality control samples were prepared and analyzed in the exact same way as the test samples.

3.3.5 Method for correlating *L. fermentum* NCIMB 5221 growth with FAE activity

MRS-agar plates were streaked, inoculated and subcultured, as described above. A 1% (v/v) (350µL) inoculum was used for subculturing into 35mL of MRS broth and incubated at 37°C throughout the experiment. MRS-broth containing no inoculum was used as a negative control. At each time point, the culture tubes were shaken gently to ensure homogeneity and 200µL from each tube was pipetted into a 96-well plate and read using a UV spectrophotometer Victor^{3}V 1420 Multilabel Counter (Perkin Elmer, Boston, MA), at a wavelength of 620nm, with the readout correlated to a standard curve of absorbance vs. cell count. At each time point, samples were also prepared for a FAE HPLC assay, as described above. Each analysis was carried out in triplicate.

3.3.6 Total antioxidant capacity of L. fermentum NCIMB 5221

The total antioxidant production of *L. fermentum* NCIMB 5221 was measured using a QuantiChrom™ Antioxidant Assay Kit, a spectrophotometric assay based on the reduction of Cu^{2+} to Cu^{+}. The protocol provided with the assay kit was followed. A standard curve was generated for Trolox, a standard provided with the kit, at concentrations of 0, 300, 600 and 1000 µM and plotted against absorbance at 570nm (R^2=0.9970). A standard curve was also generated with FA at concentrations of 0.3, 0.6, 0.9, 1.2 and 1.5mM and plotted against absorbance at 570nm (R^2=0.9715). *L. fermentum* NCIMB 5221 was subcultured from MRS broth at 1% (v/v) to MRS-EFA broth at an EFA concentration of 1.5mM. Uninoculated MRS-EFA broth was used as a negative control. Each sample was treated in triplicate and incubated at 37°C during the course of

the experiment. Samples were removed at every time point and were stored at -20°C until the assay was performed.

3.3.7 Statistical analysis

The experimental results are expressed as means ± Standard Deviation. Statistical analysis was carried out using Minitab (Minitab, Version 14, Minitab Inc, Pennsylvania, USA). Statistical comparisons between EFA/FA concentrations in media were carried out by using the general linear model (GLM). Statistical significance was set at $p < 0.05$. All interaction terms were treated as fixed terms and p-values less than 0.01 were considered highly significant.

3.4 Results

3.4.1 Ferulic acid esterase activity by a plate assay

Ethyl ferulate (EFA) clearing zones, due to FAE hydrolysis, were visible for *L. fermentum* ATCC 11976 and *L. reuteri* ATCC 23272 (positive controls), but also for the test strain, *L. fermentum* NCIMB 5221 **Figure 3.2**. The zones of clearance were 10 mm in diameter with no significant difference between the strains. The negative control strain, *L. rhamnosus* ATCC 53103, and the unimpregnated disk showed no visible clearance on the MRS-EFA agar.

3.4.2 Ferulic acid esterase activity and FA production by HPLC

MRS-EFA at a concentration of 1.33mM (0.2956 mg/mL) was used. Following 24 hours of incubation, *L. reuteri* ATCC 23272 (0.01625±0.00673 mg/mL) and *L. fermentum* NCIMB 5221 (0.02297±0.00232 mg/mL) had the lowest concentrations of unhydrolysed EFA remaining in solution when compared to *L. fermentum* ATCC 11976

(0.04611±0.00561 mg/mL) **Figure 3.3**. Based on these results, *L. fermentum* NCIMB 5221 and *L. reuteri* ATCC 23272 were chosen for further FAE activity characterisation by HPLC.

The HPLC screening method was repeated, with only the two strains aforementioned. The hydrolysis of EFA by *L. fermentum* NCIMB 5221 and *L. reuteri* ATCC 23272 followed a similar pattern for both bacterial strains, through the 48 hours of the assay. Following 48 hours of incubation, *L. reuteri* ATCC 23272 was observed to have a lower concentration of unhydrolysed EFA in the solution at 0.00358±0.00030 mg/mL, when compared to *L. fermentum* NCIMB 5221 with 0.03917±0.00320 mg/mL remaining **Figure 3.4a**. FA quantification was performed for a direct measurement of the desired end-product of FAE activity. Surprisingly, FA quantification demonstrated the absence of any FA production by *L. reuteri* ATCC 23272, despite its efficient EFA hydrolysis **Figure 3.4b**. *L. fermentum* NCIMB 5221 had a final production, following 48 hours, of 0.16847±0.00122 mg/mL FA. The summary of the results, following 24 hours of incubation of the strains in MRS-EFA, is presented in **Table 3.1**.

3.4.3 Correlating *L. fermentum* NCIMB 5221 growth with FAE activity

To investigate the FAE enzyme kinetics, with relation to *L. fermentum* NCIMB 5221's growth kinetics, growth and FA production were investigated in parallel. Results in **Figure 3.5a** show a lag phase between 0 and 6 hours, the exponential phase from 6 hours ($3.27x10^8$ cfu/mL) to 16 hours ($1.32x10^9$ cfu/mL) and the stationary phase starting at 16 hours. The correlated FA production curve, generated by an HPLC assay, elucidates the direct proportionality between bacterial cell growth and FAE enzyme kinetics **Figure**

3.5b. The higher the growth rate of *L. fermentum* NCIMB 5221, exemplified by the logarithmic scale of its growth curve, the greater was its rate of FA production.

3.4.4 Total antioxidant capacity of *L. fermentum* NCIMB 5221

To investigate the antioxidative potential of the FA produced by *L. fermentum* NCIMB 5221, the strain was incubated in MRS-EFA at an initial concentration of 1.5mM and supernatant was collected at various time points. The supernatant samples were measured using an antioxidant measurement kit. Following 48 hours of incubation, *L. fermentum* NCIMB 5221 had a production of 509.58±13.23 μM Trolox, equivalent to 857.16±22.25μM FA **Figure 3.6**. It can also be concluded that the levels of antioxidant compounds increased for the first 16 hours and then reached a plateau. This is the same trend observed with the HPLC results aforementioned.

3.5 Discussion

Probiotics are dietary supplements containing bacteria which, when administered in adequate amounts, confer a health benefit on the host (FAO and WHO 2001). Due to the proven safety of many bacterial strains present in current probiotic formulations, their clinical applications have gained wide interest. Various bacterial strains have been shown to be beneficial in a wide range of conditions including infections, allergies and metabolic disorders such as ulcerative colitis and Crohn's disease (Al-Salami et al. 2008a; Floch 2010; Jankovic et al. 2010; Luoto et al. 2010; Wolvers et al. 2010). The most common microorganisms used in probiotic formulations are the lactic acid bacteria (LAB), which are important components of the healthy gut microbiota and have been regarded as safe by the American FDA (Parvez et al. 2006). Lactobacilli have shown

unique advantages in metabolic diseases through the production of useful enzymes that exert desirable biological activities in the host (Al-Salami et al. 2008b; Guglielmetti et al. 2008). Current research focuses on using specific bacterial strains that secrete or produce useful biologically active enzymes and proteins (Azcarate-Peril et al. 2004; Guglielmetti et al. 2008). These enzymes include ornithine decarboxylase, used as a potent antioxidant for the treatment of oxidative stress associated autoimmune diseases and accelerated cell apoptosis (Mates et al. 2002), bile salt hydrolase for hypercholesterolemia and cholesterolosis (Martoni et al. 2007; Martoni et al. 2008; Tanaka et al. 1999a), and bile transport and tolerance proteins for efficient delivery of probiotics (Pfeiler and Klaenhammer 2009). Another type of enzyme that recently gained interest in food production is cinnamoyl esterases (Guglielmetti et al. 2008). In recent studies, the products of cinnamoyl esterase have shown remarkably high levels of antioxidant activity (Srinivasan et al. 2007) and other health-promoting effects, including stimulation of insulin secretion (Adisakwattana et al. 2008; Sri Balasubashini et al. 2003), prevention of oxidative stress (Srinivasan et al. 2007), lipid peroxidation (Balasubashini et al. 2004), cholesterol lowering capabilities (Bhathena et al. 2009) and inhibition of diabetic nephrophathy progression (Atsuyo et al. 2008). Cinnamoyl esterase enzymatic activities include the production of caffeic, ferulic, sinapic and *p*-coumaric acids (Fazary and Ju 2007). FAE are a subclass of cinnamoyl esterases, found mainly in plants, but also in some microbes, capable of hydrolysing the ester bond between hydroxyl cinnamic acids and sugars (Crepin et al. 2004). FA, a well-characterised antioxidant, is one of the desired products of hydrolysis by FAE. Gut bacteria have been described as having FAE activity, with probiotic bacteria already demonstrating health beneficial properties through FA

production (Bhathena et al. 2009). In this research, we focus on probiotic Lactobacilli, specifically investigating the strains for inherent FA production.

In recently published articles, the FAE precipitation plate assay was used to screen for FAE and a strong activity was shown by *L. fermentum* ATCC 11976 and *L. reuteri* ATCC 23272 (Bhathena et al. 2007). The plate screening assay was performed with *L. fermentum* ATCC 11976 and *L. reuteri* ATCC 23272 as positive controls, with *L. rhamnosus* ATCC 53103 as a negative control and with *L. fermentum* NCIMB 5221 as the strain to be screened for FAE activity. As expected, EFA clearing zones, due to FAE activity, were obtained for the positive control strains and no clearing zone was observed for the negative control strain. *L. fermentum* NCIMB 5221 tested positive for FAE activity, with a clearing size approximately equivalent to the two positive control strains. The EFA plate assay remains qualitative, so further investigation into the strains' FAE activity was necessary.

An HPLC assay was used for the quantification of the FAE activity, in terms of EFA hydrolysis, of *L. fermentum* ATCC 11976, *L. reuteri* ATCC 23272 and *L. fermentum* NCIMB 5221. Following 24 hours of incubation, *L. reuteri* ATCC 23272 and *L. fermentum* NCIMB 5221 had the lowest concentrations of unhydrolysed EFA in comparison *to L. fermentum* ATCC 11976. *L. reuteri* ATCC 23272 had a slightly lesser concentration of unhydrolysed EFA than did *L. fermentum* NCIMB 5221. For further investigation into the different FAE activity profiles of *L. reuteri* ATCC 23272 and *L. fermentum* NCIMB 5221, quantification was performed for EFA hydrolysis and FA production, in parallel. Following 48 hours of incubation, *L. reuteri* ATCC 23272, as the previous assay results, was observed to have a lower concentration of unhydrolysed EFA

than *L. fermentum* NCIMB 5221. Unexpectedly, the quantification of FA production by *L. reuteri* ATCC 23272 demonstrated the absence of any FA, even lower than the background values of the control. This suggests enzymatic activity of *L. reuteri* ATCC 23272 which can use FA as a precursor for the production of other compounds. It has been shown that, through microbial transformation, vanillic acid can be produced from FA, a conversion that occurs by the removal of the phenyl-propenoic C_2-side chain from FA (Bloem et al. 2007; Ghosh et al. 2007). Vanillic acid has, as well, been shown to have antioxidant activity, although not as effective as that of FA (Baublis et al. 2000). Hence, *L. fermentum* NCIMB 5221 was used for further investigations, although further work is required to characterize the end-product of *L. reuteri* ATCC 23272 metabolism of EFA.

There was a need to further characterize *L. fermentum* NCIMB 5221 in terms of its growth kinetics with relation to FAE activity. It was demonstrated that the FA production rate is maximum between 6 and 16 hours, correlating with the exponential phase of *L. fermentum* NCIMB 5221. This is important for future studies, *in vitro* and *in vivo*, in terms of timing bacterial cell cultivation at the appropriate stage of culture growth. In the case of *L. fermentum* NCIMB 5221, the bacterial cells should be cultivated at the point of highest cell turbidity, at the late exponential or early stationary phase, where enzyme activity is also greatest.

Investigations into the antioxidant potential of FA produced by *L. fermentum* NCIMB 5221 was also performed using an antioxidant kit which measures the total antioxidant potential present in solutions, based on the reduction of Cu^{2+} to Cu^{+}. It was clearly demonstrated that, when incubated with EFA, an FAE substrate, *L. fermentum* NCIMB 5221 produced a significant level of antioxidants, correlating with the levels of

FA quantified using the HPLC methods. These results prove the functionality of the FA molecule rather than just its presence in solution.

Future work should involve additional characterisation of a final formulation for potential preclinical use, in terms of the mechanisms of action and safety of the probiotic strain and the produced FA with *in vitro* and *in vivo* studies. FAE activity also needs to be tested using more complex substrates than EFA, such as wheat bran, to more closely resemble the dietary intake of FA precursors. The successful delivery of bacteria through the upper gastrointestinal tract remains an issue, and this strain needs to be investigated for characteristics such as bile resistance. Techniques such as microencapsulation may also prove useful for efficient delivery to the lower gastrointestinal tract, the desired site of action of probiotics (Bhathena et al. 2009; Chen et al. 2010; Urbanska et al. 2009). In terms of the gastrointestinal tract, FAE activity should be characterized in terms of FA production in a gastrointestinal system and *in vivo* in appropriate animal models to ensure enzyme activity remains stable and efficient under potentially harsh conditions. There still remains a great deal of work to be done before a FAE active bacterial strain, such as *L. fermentum* NCIMB 5221, can be developed into an efficient therapeutic.

In summary, the presented work successfully screened for FAE active bacteria using a qualitative precipitation assay on MRS-EFA agar and a quantitative HPLC assay. The most active FAE bacterial strain in terms of FA production, from the selected bacteria, was shown to be *L. fermentum* NCIMB 5221. The FAE activity of this strain was correlated with its growth curve, demonstrating a direct relationship between bacterial cell count and FA production. The total antioxidant capacity of the strain was also investigated. This work opens up future potentials for using a synergistic formulation

of *L. fermentu*m NCIMB 5221 with its intrinsic microbial FAE activity for both industrial and therapeutic applications.

3.6 Acknowledgements

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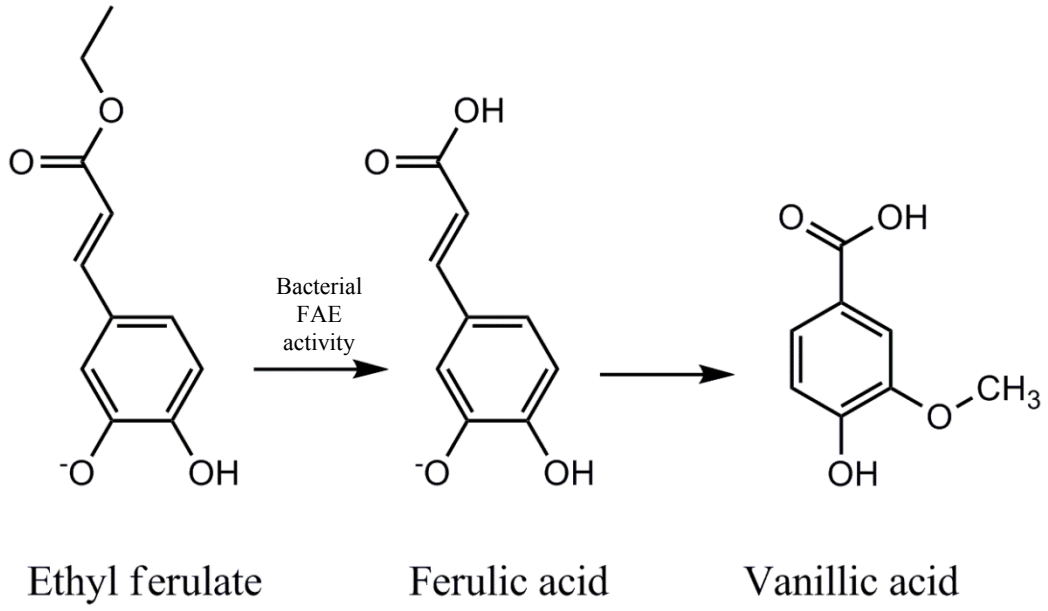

Figure 3.1: Ferulic acid esterases (FAE) are a subclass of cinnamoyl esterases, found in some microbes, capable of hydrolysing the ester bond between hydroxyl cinnamic acids and sugars. EFA is one of the natural substrates of FAE. EFA hydrolysis by FAE can give rise to FA as one of the desired products, a compound with a number of health-promoting benefits. Vanillic acid may also be produced from FA through bacteria biotransformations.

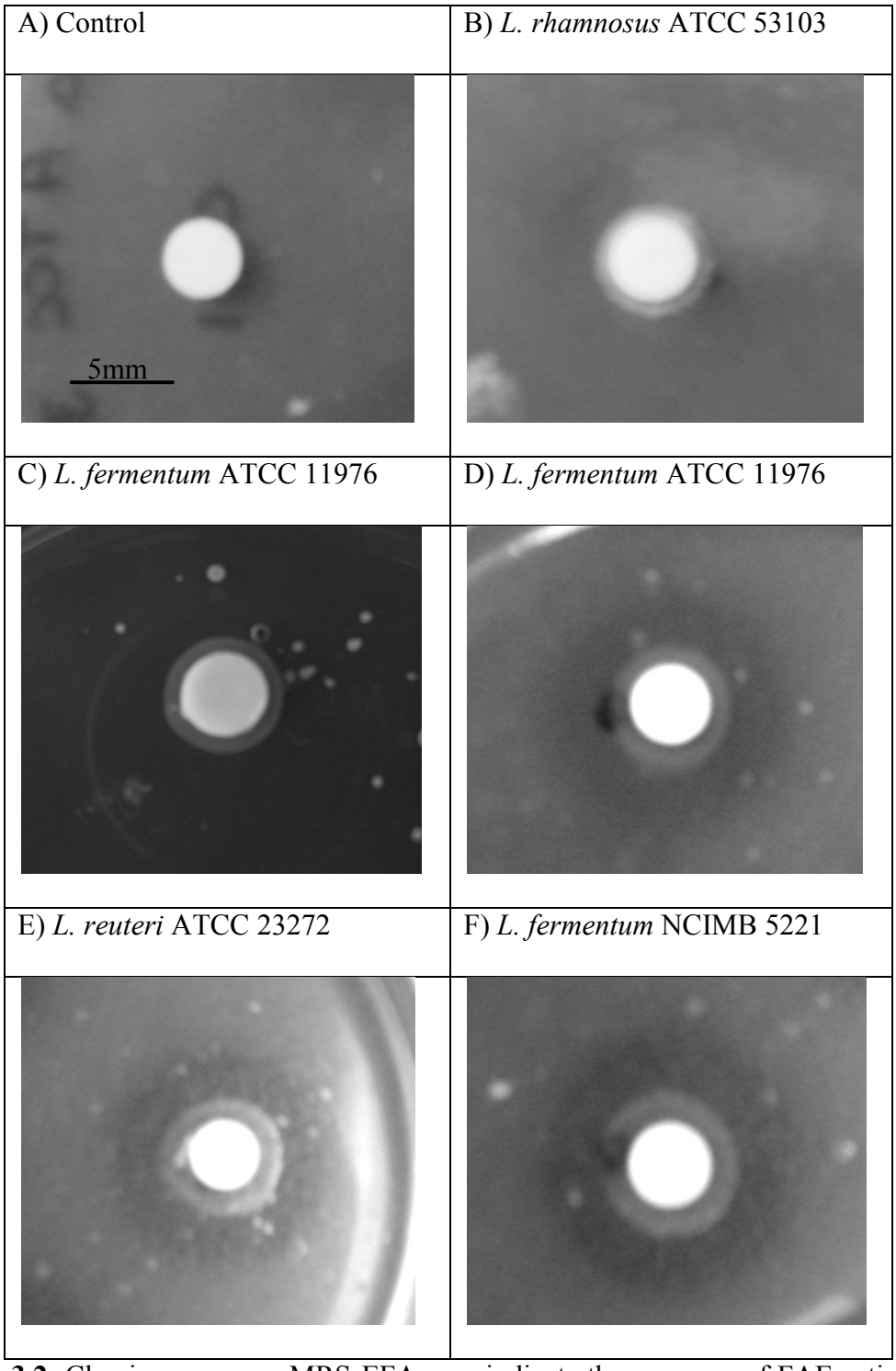

Figure 3.2: Clearing zones on MRS-EFA agar indicate the presence of FAE activity. A) MRS-EFA agar with no bacteria; B) MRS-EFA agar with *L. rhamnosus* ATCC 53103; C) MRS agar with *L. fermentum* ATCC 11976; D) MRS-EFA agar with *L. fermentum* ATCC 11976; E) MRS-EFA agar with *L. reuteri* ATCC 23272; F) MRS-EFA agar with *L. fermentum* NCIMB 5221. The zones of clearance are 10mm in diameter, with no significant difference between positive strains.

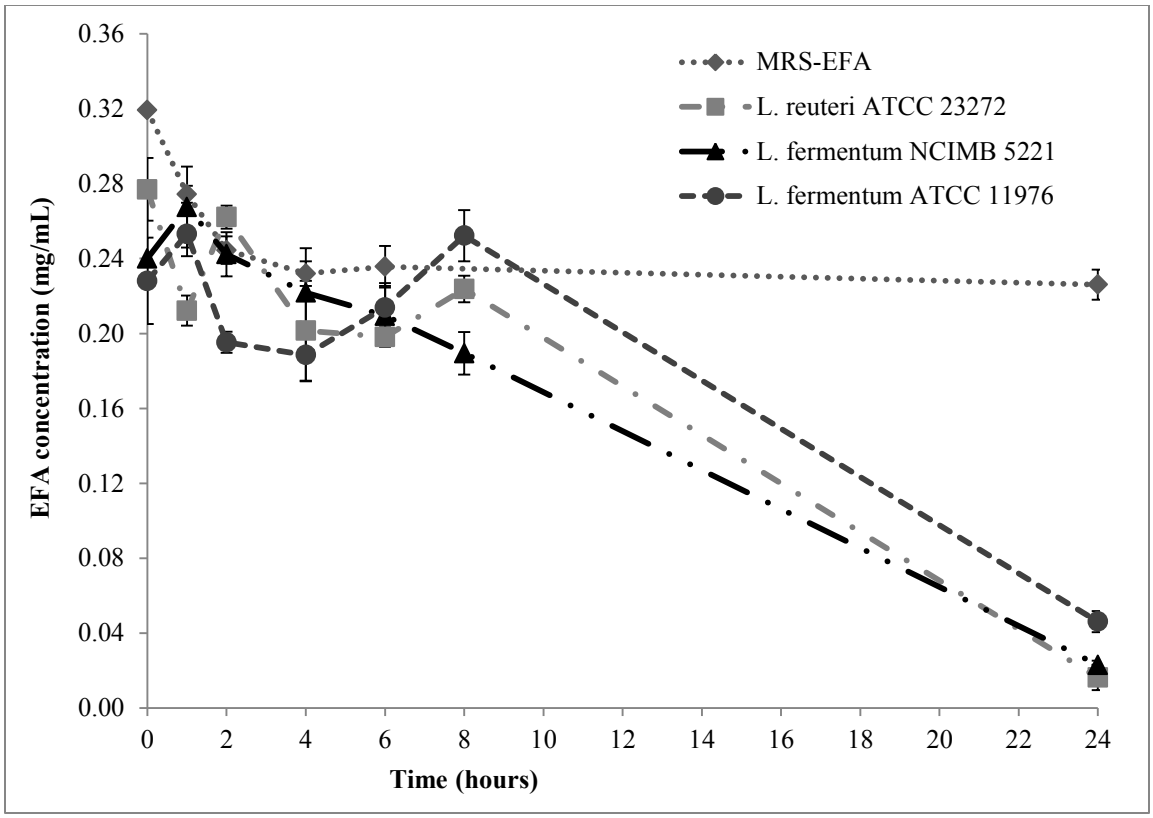

Figure 3.3: FAE quantitative HPLC assay for *L. fermentum* ATCC 11976, *L. reuteri* ATCC 23272 and *L. fermentum* NCIMB 5221. Uninoculated MRS-EFA was used as a negative control. The data represents the amount of EFA, the FAE substrate, remaining in solution, as measured by HPLC peak area data converted to EFA concentration values using a standard curve for EFA concentration vs. peak area. The initial concentration of MRS-EFA was 1.33 mM (0.2956 mg/mL). Following 24 hours of incubation, *L. reuteri* ATCC 23272 (0.01625 ± 0.00673 mg/mL) and *L. fermentum* NCIMB 5221 (0.02297 ± 0.00232 mg/mL) had the lowest concentrations of unhydrolysed EFA when compared to *L. fermentum* ATCC 11976 (0.04611 ± 0.00561 mg/mL). Each point represents the mean of triplicates and the error bars represent the standard deviations.

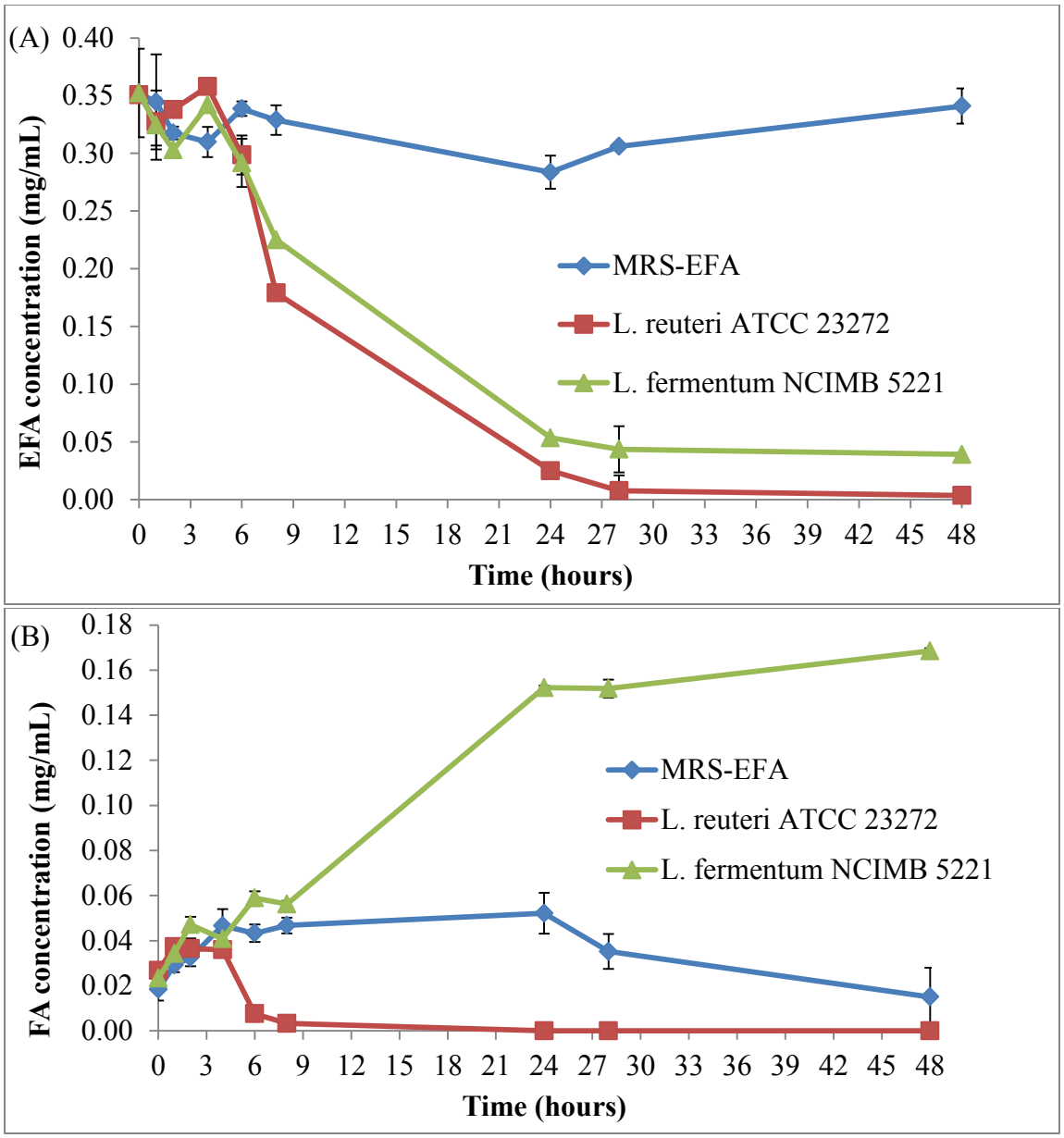

Figure 3.4: FAE quantitative HPLC assay for *L. reuteri* ATCC 23272 and *L. fermentum* NCIMB 5221. Uninoculated MRS-EFA was used as a negative control. The presented data represents the amount of unhydrolysed EFA remaining in solution (A) and the amount of FA produced (B), as measured by HPLC peak area data converted to EFA and FA concentration values using standard curves for EFA and FA concentration vs. peak area. The initial concentration of MRS-EFA was 1.33 mM (0.2956 mg/mL). (A) Following 48 hours of incubation, *L. reuteri* ATCC 23272 had 0.00358 ± 0.00030 mg/mL and *L. fermentum* NCIMB 5221 had 0.03917 ± 0.00320 mg/mL unhydrolysed EFA. (B) FA quantification demonstrates the absence of any FA production by *L. reuteri* ATCC 23272 throughout the experiment. *L. fermentum* NCIMB 5221 had a final production, following 48 hours, of 0.16847 ± 0.00122 mg/mL FA. Each point represents the mean of triplicates and the error bars represent the standard deviations.

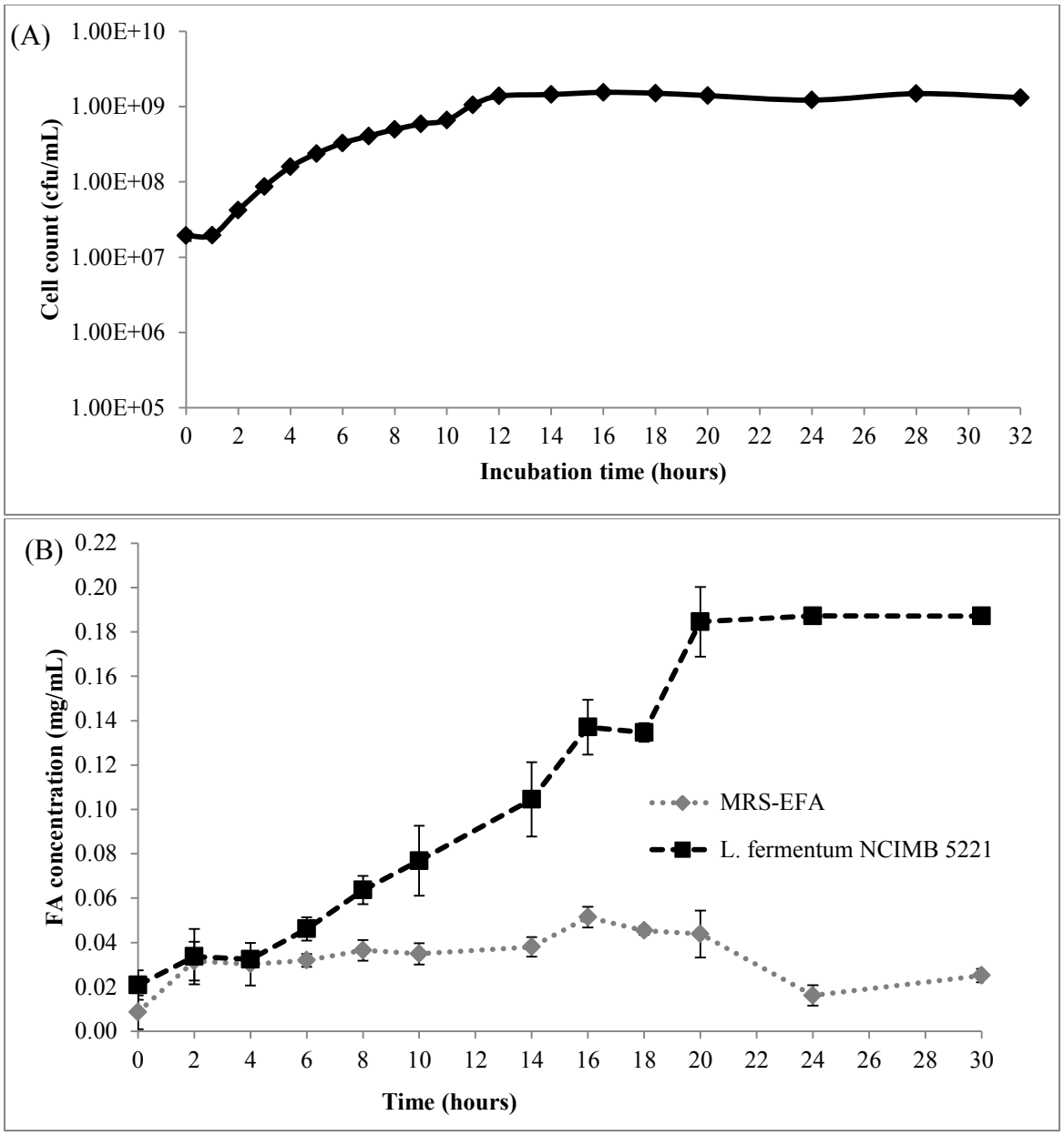

Figure 3.5: The growth curve (A) of *L. fermentum* NCIMB 5221 correlated with FA production (B). (A) The growth curve was generated by absorbance at 620nm over time and correlation to a standard curve of absorbance vs. colony forming units. The growth curve is characterized by the lag phase between 0 and 6 hours, the exponential phase between 6 ($3.27x10^8$ cfu/mL) and 16 hours ($1.32x10^9$ cfu/mL) and the stationary phase from 16 hours on. (B) The correlated FA production data represents the amount of FA produced, as measured by HPLC peak area data converted to FA concentration values using a standard curve for FA concentration vs. peak area. The initial concentration of MRS-EFA was 1.33 mM (0.2956 mg/mL). These results elucidate the direct relationship between bacterial cell growth and enzyme kinetics. The FA production rate is maximal during the exponential phase of growth of *L. fermentum* NCIMB 5221. Each point represents the mean of triplicates and the error bars represent the standard deviations.

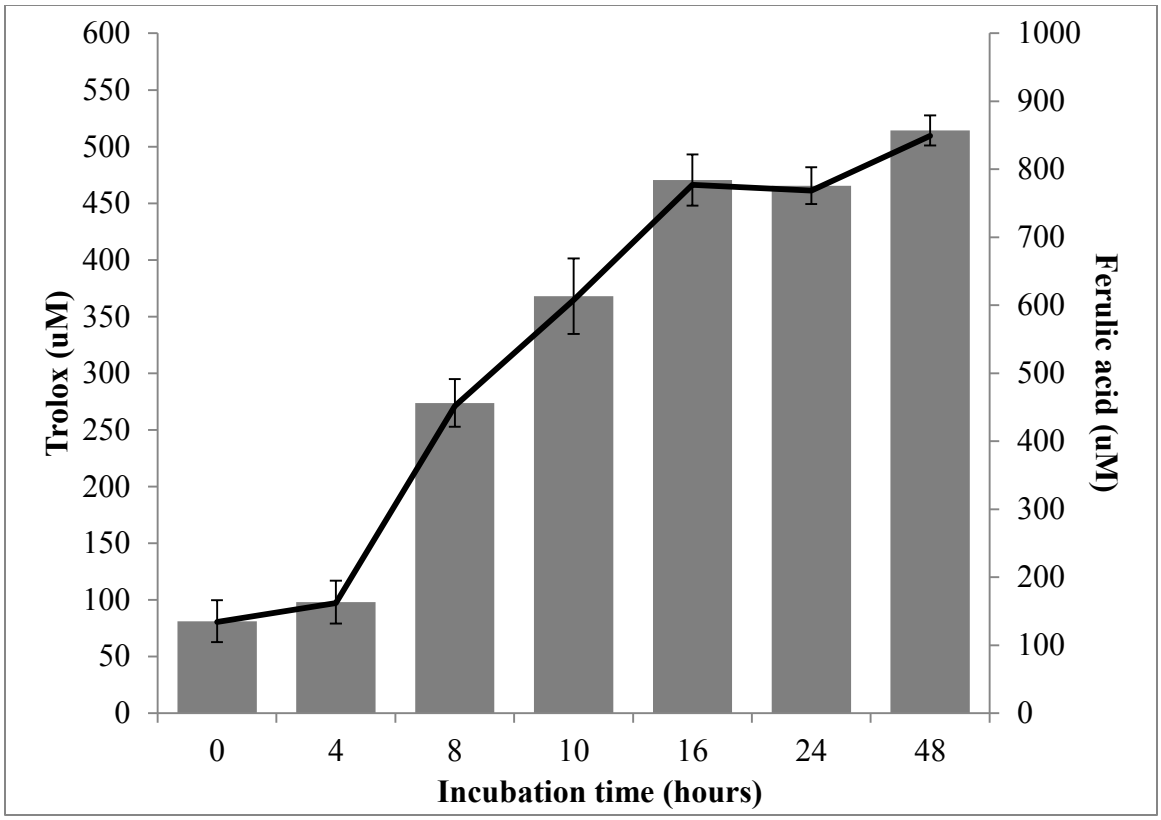

Figure 3.6: Measurement of total antioxidant production by *L. fermentum* NCIMB 5221 incubated in an initial concentration of MRS-EFA of 1.5 mM. Standard curves for Trolox and FA were generated and used to determine antioxidant concentration. Following 48 hours of incubation, *L. fermentum* NCIMB 5221 had a production of 509.58 ± 13.23 μM Trolox, equivalent to 857.16 ± 22.25 μM FA. Each point represents the mean of triplicates and the error bars represent the standard deviations.

Bacterial strain	24 hr EFA (mg/mL)	24 hr EFA hydrolysis (%)	24 hr FA production (mg/mL)
L. fermentum ATCC 11976	0.04611 ± 0.00561	84.4±1.9	0.10348 ± 0.00442
L. reuteri ATCC 23272	0.01625 ± 0.00673	94.5±2.3	Not detectable
L. fermentum NCIMB 5221	0.02297 ± 0.00232	92.2±0.8	0.15221 ± 0.00101

Table 3.1: A comparison of EFA hydrolysis and FA production for *L. fermentum* NCIMB 5221, *L. reuteri* ATCC 23272 and *L. fermentum* ATCC 11976, following 24 hours of incubation in MRS-EFA (0.2956 mg/mL EFA). The data represents the amount of unhydrolysed EFA, also expressed as efficacy of EFA hydrolysis and FA production. FA quantification demonstrates the absence of any FA production by *L. reuteri* ATCC 23272 throughout the experiment. *L. fermentum* ATCC 11976 (results not shown in the HPLC graphs), had a production of 0.10348 ± 0.00442 mg/mL FA. *L. fermentum* NCIMB 5221 had a production of 0.15221 ± 0.00101 mg/mL FA. The data values represent the mean of triplicates with the standard deviation associated with the measurements.

Original Research Article 2

CHAPTER 4: PROBIOTIC FERULIC ACID ESTERASE ACTIVE *LACTOBACILLUS FERMENTUM* NCIMB 5221 APA MICROCAPSULES FOR ORAL DELIVERY: PREPARATION AND *IN VITRO* CHARACTERIZATION

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Preface: Following the selection of a ferulic acid-producing probiotic strain, there was a need to microencapsulate the strain for successful oral delivery of viable and active probiotic cells. The *L. fermentum* probiotic strain, selected in the previous chapter, was microencapsulated using alginate-polylysine-alginate microencapsulation. The microencapsulation size and efficiency, in terms of probiotic viability, was determined. The ferulic acid esterase enzymatic activity of *L. fermentum* was investigated to determine whether the microcapsule would impede the uptake of the ethyl ferulate substrate and the release of the desired product, ferulic acid. The protective capability of the alginate-polylysine alginate microcapsule was then demonstrated using simulated gastrointestinal conditions.

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4.1 Abstract

Probiotics possess potential therapeutic and preventative effects for various diseases and metabolic disorders. One important limitation for the oral delivery of probiotics is the harsh conditions of the upper gastrointestinal tract (GIT) which challenge bacterial viability and activity. One proposed method to surpass this obstacle is the use of microencapsulation to improve the delivery of bacterial cells to the lower GIT. The aim of this study is to use alginate-poly-L-lysine-alginate (APA) microcapsules to encapsulate *Lactobacillus fermentum* NCIMB 5221 and characterize its enzymatic activity and viability through a simulated GIT. This specific strain, in previous research, was characterized for its inherent ferulic acid esterase (FAE) activity which could prove beneficial in the development of a therapeutic for the treatment and prevention of cancers and metabolic disorders. Our findings demonstrate that the APA microcapsule does not slow the mass transfer of substrate into and that of the FA product out of the microcapsule, while also not impairing bacterial cell viability. The use of simulated gastrointestinal conditions led to a significant 2.5 log difference in viability between the free ($1.10 \times 10^4 \pm 1.00 \times 10^3$ cfu/mL) and the microencapsulated ($5.50 \times 10^6 \pm 1.00 \times 10^5$ cfu/mL) *L. fermentum* NCIMB 5221 following exposure. The work presented here suggests that APA microencapsulation can be used as an effective oral delivery method for *L. fermentum* NCIMB 5221, a FAE-active probiotic strain.

4.2 Introduction

Ferulic acid (FA), a naturally found phenolic acid, is a potent antioxidant able to neutralize free radicals, such as Reactive Oxygen Species (ROS) (Rice-Evans et al. 1996). ROS have been implicated in DNA damage, cancer and accelerated cell aging (Lombard et al. 2005a). Recent studies suggest that FA has antitumor activity against breast cancer (Chang et al. 2006; Kampa et al. 2003) liver cancer (Lee 2005; Taniguchi et al. 1999) and is effective at preventing cancer induced by the exposure to various carcinogenic compounds such as benzopyrene (Lesca 1983) and 4-nitroquinoline 1-oxide (Tanaka et al. 1993). The oral delivery of free FA is hampered by its quick absorption in the small intestine, specifically in the jejunum, followed by its rapid excretion (Spencer et al. 1999; Zhao et al. 2003). However, it has been proposed, and shown in previous studies that some GIT bacterial strains produce FAE, an enzyme that has the inherent capability to produce FA from available substrates in the GIT (see Figure 4.1).

The oral delivery of conjugated FA, in the form of a dietary source such as wheat bran, is a feasible alternative, with the release of free FA by microbial FAE present in the lower human digestive tract, giving rise to a constant and controlled release of FA (Spencer et al. 1999; Zhao et al. 2003). The development of probiotic formulations to enhance the FA bioavailability should prove beneficial for the treatment and prevention of inflammatory metabolic disorders (Bhathena et al. 2009).Previous research by our group has demonstrated the use of *Lactobacillus fermentum* NCIMB 5221 as a superior microbial producer of FA (Tomaro-Duchesneau et al. 2012a). The oral delivery of probiotics, however, is impeded by the harsh conditions of the upper GIT, specifically the presence of bile and a detrimentally acidic pH (Prakash et al. 2011b).

Microencapsulation, a method defined as the "entrapment of a compound or a system inside a dispersed material for its immobilization, protection, controlled release, structuration and functionalisation" has been used to overcome the challenge of delivering bacterial cells through the GIT (Poncelet 2006). This article investigates the use of APA microencapsulation for the viable delivery of the FAE active *Lactobacillus fermentum* NCIMB 5221 to the colon. The results should demonstrate the efficiency of APA microcapsules to increase the viability of this specific probiotic strain in the GIT while preserving enzymatic activity in terms of FA production.

4.3 Experimental Methods and Materials

4.3.1 Bacterial Growth Media and Chemicals

Ethyl ferulate (ethyl 4-hydroxy-3-methoxycinnamate, EFA) and ferulic acid (*trans*-4-hydroxy-3-methoxycinnamate, FA) were purchased from Sigma-Aldrich (Oakville, ON, Canada). De Man, Rogosa, Sharpe (MRS) broth and Methanol of high-performance liquid chromatography (HPLC) grade were obtained from Fisher Scientific (Ottawa, ON, Canada). Water was purified with an EasyPure reverse osmosis system and a NanoPure Diamond Life Science (UV/UF) ultrapure water system from Barnstead (Dubuque, IA, USA). All other chemicals were of analytical or HPLC grade and purchased from commercial sources.

4.3.2 Bacterial Strains and Culture Conditions

Lactobacillus fermentum NCIMB 5221 was purchased from NCIMB (Aberdeen, Scotland, UK). The bacterial strain was stored at −80 °C in MRS containing 20% (v/v) glycerol. An MRS-agar plate was streaked for isolation from the frozen stock and

incubated at 37 °C with 5% CO_2 for 24 h to ensure purity. One colony from the MRS-agar plate was inoculated into 5 mL of MRS broth and incubated at 37 °C for 24 h. A 1% (v/v) inoculum was then used for subculturing and incubated at 37 °C for 24 h immediately before use.

4.3.3 APA Microencapsulation

The microencapsulation of *L. fermentum* NCIMB 5221 was performed according to the standard protocol, with slight modifications to the flow rate, vibration frequency and voltage (Chang and Prakash 2001). The microcapsules were loaded with 8% (w/v) bacterial pellet. A sodium-alginate solution (1.75% w/v) containing the *L. fermentum* NCIMB 5221 was extruded into a stirred $CaCl_2$ solution (0.1 M) using a microencapsulator and a 200 μm nozzle (Inotech Corp.). The formed calcium-alginate beads were washed in a physiological solution (PS) followed by their immersion in a poly-L-lysine (PLL) solution (0.1% w/v) for 20 min. Another wash in PS was performed for 5 min followed by immersion in a sodium-alginate solution (0.1% w/v) for 20 min. The resulting microcapsules were stored in minimal storage media at 4 °C for further testing. Viability on microcapsules was performed by exposure to 0.1 M sodium citrate until disruption of the microcapsule was observed. Ten-fold serial dilutions in physiological saline followed by plating on MRS-agar plates were then performed to determine the colony forming units.

4.3.4 Ferulic Acid Esterase HPLC Assay to Measure FA Production

The bacterial strain was subcultured from MRS broth at 1% (v/v) to MRS-EFA broth at an EFA concentration of 1.33 mM (0.2956 mg/mL). For the APA microcapsules these were added at 10% (w/v) into MRS-EFA broth. Uninoculated MRS-EFA broth was

used as a negative control and treated in the exact same way. Each sample was treated in triplicate and incubated at 37 °C during the course of the experiment. An HPLC assay, modified from Mastihuba *et al.*, was used to measure FAE activity (Mastihuba et al. 2002). At each time point, 500 µL of sample was added to tubes and centrifuged at 10,000 rpm for 7 min at 4 °C. The resulting supernatant (300 µL) was acidified with 0.35 M H_2SO_4 (100 µL) and vortexed. 1mM benzoic acid (300 µL) was added, as an internal standard, followed by the addition of 0.7 M NaOH (100 µL) to neutralize the pH. The samples were then stored at −20 °C until all of the samples were collected for the HPLC analysis.

For HPLC analysis, the samples were thawed to room temperature and filtered with a 0.45 µm filter. The analysis was performed on a reverse-phase C-18 column: LiChrosorb RP-18, 25 × 0.46 cm (Grace Davison Discovery Sciences, ON, Canada). The HPLC system consists of a ProStar 335 diode array detector (DAD) set at 280 nm and 320 nm, a ProStar 410 autosampler, and the software Star LC workstation version 6.41. 25 µL was injected for each sample. The mobile phase (solvent A) consists of 37% (v/v) methanol and 0.9% (v/v) acetic acid in water (HPLC grade). Solvent B consisted of 100% (v/v) methanol. The run was initiated with solvent A at 100% for 16 minutes. This was followed by a 1 minute linear gradient to reach 100% of solvent B, attained at the 17th minute. Solvent B was isocratically held at 100% for 12 minutes, until the 29th minute. This was followed by a 1 minute linear gradient to reach 100% of solvent A by the 30th minute. Standard curves of FA and EFA, using peak area quantification, were generated for quantifying the test sample FA and EFA concentrations. The FA standard curve was generated using triplicates and the concentrations 100, 300, 500, 960 and 1,100 µM were

plotted against peak area (R^2 = 0.9869). The EFA standard curve was generated using triplicates and the concentrations 100, 300, 500, 700, 1,000, 1,400 and 1,800 μM were plotted against peak area (R^2 = 0.9785). Standards and quality control samples were prepared and analyzed in the same way as the test samples.

4.3.5 Simulated Gastrointestinal Conditions to Determine the Stability and Viability of Microencapsulated L. fermentum NCIMB 5221 Delivered Orally

Simulated gastric (SGF) and intestinal fluids (SIF) were prepared according to the U.S. Pharmacopeia, with some minor modifications (U.S.Pharmacopeia 2010). SGF consisted of NaCl (2 g/L), glucose (3.5 g/L) and pepsin (3.2 g/L) in deionized water. The pH of the SGF was adjusted to 1.5 using 2 M HCl. SIF consisted of monobasic potassium phosphate (6.8 g/L), pancreatin (10 g/L), Oxgall (1.5 g/L) and glucose (3.5 g/L) in deionized water. 20 g of APA microcapsules or 2 g of free *L. fermentum* NCIMB 5221 were added to 200 mL SGF and incubated at 37 °C on a rotary shaker at 75 rpm for 2 h. Following the 2 h, 200 mL of SIF was added into each flask and the pH was increased to 6.8 using 2 M NaOH. At each time point, 1 mL of the solution was sampled into 9 mL of 0.1 M sodium citrate. This was serially diluted in 10-fold dilutions in 0.85% (w/v) NaCl and plated on MRS agar in triplicates. These MRS-agar plates were incubated at 37 °C and 5% CO_2, followed by colony counting following 48 h of incubation.

4.3.6 Statistical Analysis

Values are expressed as means ± Standard Deviation. Statistical analysis was carried out using Minitab (Minitab, Version 14, Minitab Inc., State College, PA, USA). Statistical comparisons between EFA/FA concentrations were carried out by using the general linear model (GLM) and post-hoc analysis. Statistical significance was set at $p < 0.05$.

All interaction terms were treated as fixed terms and *p*-values less than 0.01 were considered highly significant.

4.4 Results and Discussion

4.4.1 Results

L. fermentum NCIMB 5221 microcapsules were formed to investigate the suitability of the APA microcapsule for the oral delivery of an FAE active probiotic. *L. fermentum* NCIMB 5221 microencapsulation was optimized—by controlling the flow rate, stirring time, stirring speed, coating time, vibration frequency, and voltage—to obtain monodispersed and spherical microcapsules. The obtained microcapsules were observed under light microscopy at magnifications of 40×, 100× and 200×. The APA microcapsules obtained were monodispersed, as seen in **Figure 4.2A**, with an approximate size of 400 ± 25 μm in diameter. **Figure 4.2B** demonstrates the spherical shape of the microcapsules and **Figure 4.2C** allows for visualisation of the outer coat of the APA microcapsule loaded with *L. fermentum* NCIMB 5221 at 8% of the total microcapsule weight. The viability of the bacteria prior to microencapsulation, in the alginate solution, was determined to be $1.95 \times 10^9 \pm 2.1 \times 10^8$ cfu/mL and the microcapsules had a final viability of $1.21 \times 10^9 \pm 9.54 \times 10^7$ cfu/g, suggesting no significant loss of bacterial viability due to the process of APA microencapsulation.

The FAE activity of APA microencapsulated *L. fermentum* NCIMB 5221 was then determined to ensure that the APA microcapsule does not impede the uptake of the substrate, EFA, and the release of the desired product, FA. For this, the viability of free

and microencapsulated *L. fermentum* NCIMB 5221 during the FAE assay was determined, as can be observed in **Figure 4.3**.

At the start of the assay, the free cells had a viability of $1.53 \times 10^9 \pm 9.02 \times 10^7$ cfu/mL and the microcapsules a viability of $6.77 \times 10^8 \pm 7.77 \times 10^7$ cfu/mL. Following 24 h of incubation, the free cells had a viability of $3.27 \times 10^8 \pm 1.00 \times 10^3$ cfu/mL and the microcapsules a viability of $7.72 \times 10^7 \pm 1.00 \times 10^5$ cfu/mL.

In terms of FAE activity, the initial EFA concentration was 0.2956 mg/mL. Following 30 h of incubation, the free cells had 0.0382 ± 0.0011 mg/mL (12.92 ± 0.37%) and the microcapsules had 0.057 ± 0.0054 mg/mL (19.28 ± 1.83%) EFA remaining in solution, as seen in **Figure 4.4A**. At this point, the free cells had a total production of 0.1872 ± 0.0033 mg/mL FA and APA microcapsules had a final production of 0.1760 ± 0.0149 mg/mL FA, as seen in **Figure 4.4B**.

The viability of *L. fermentum* NCIMB 5221 in free and encapsulated form was determined upon exposure to the simulated gastrointestinal conditions, **Figure 4.5**. The initial viability of *L. fermentum* NCIMB 5221 was $1.53 \times 10^9 \pm 9.02 \times 10^7$ cfu/mL for the free cells and $6.77 \times 10^8 \pm 7.77 \times 10^7$ cfu/mL for the microencapsulated cells. Following 2 h of exposure to the simulated conditions of the stomach the viability was $2.60 \times 10^8 \pm 1.22 \times 10^8$ cfu/mL for the free cells and $4.73 \times 10^8 \pm 4.93 \times 10^7$ cfu/mL for the encapsulated cells. Following the further exposure to simulated intestinal conditions for 24 h, the free *L. fermentum* NCIMB 5221 demonstrated a viability of $1.10 \times 10^4 \pm 1.00 \times 10^3$ cfu/mL and the microencapsulated *L. fermentum* NCIMB 5221 had a viability of $5.50 \times 10^6 \pm 1.00 \times 10^5$ cfu/mL. The viability of *L. fermentum* NCIMB 5221, through the *in vitro* gastrointestinal passage, and the associated conditions of the gastric and intestinal

exposure, are summarized in **Table 4.1**. It is also noted that, following the transition to the intestinal conditions, the microcapsules lost some of their integrity due to a change in pH and osmotic conditions.

4.4.2 Discussion

Past and recent research has looked at probiotics for use as therapeutics. These formulations are defined as dietary supplements containing bacteria which, when administered in adequate amounts, confer a health benefit on the host (FAO and WHO 2001; Prakash et al. 2011a; Prakash et al. 2011b). Probiotics, as natural compounds, are generally considered safe, but can also be tested for set-out safety parameters (Branton et al. 2011). A number of studies have investigated bacterial strains for a range of conditions, including infections, allergies and metabolic disorders such as ulcerative colitis and Crohn's disease (Al-Salami et al. 2008a; Floch 2010; Jankovic et al. 2010; Luoto et al. 2010; Wolvers et al. 2010). Promising research focuses on the microbial secretion and production of beneficial biologically active enzymes and proteins (Azcarate-Peril et al. 2004; Guglielmetti et al. 2008). These include the use of ornithine decarboxylase as a powerful antioxidant for the treatment of autoimmune diseases and accelerated cell apoptosis (Mates et al. 2002), the use of bile salt hydrolase for hypercholesterolemia (Martoni et al. 2007; Martoni et al. 2008; Tanaka et al. 1999a), and the use of bile transport and tolerance proteins for the efficient delivery of probiotics (Pfeiler and Klaenhammer 2009). In recent studies, the products of, another microbial protein, cinnamoyl esterase, have shown significant levels of antioxidant activity (Srinivasan et al. 2007; Tomaro-Duchesneau et al. 2012a) and other effects, including stimulation of insulin secretion (Adisakwattana et al. 2008; Sri Balasubashini et al. 2003),

prevention of oxidative stress (Srinivasan et al. 2007), lipid peroxidation (Balasubashini et al. 2004), cholesterol-lowering capabilities (Bhathena et al. 2009) and inhibition of diabetic nephropathy progression (Atsuyo et al. 2008). FA, a well-characterised antioxidant, is one of the desired products of hydrolysis by FAE (Tomaro-Duchesneau et al. 2012a).

Previously we have screened strains for FAE activity and selected *L. fermentum* NCIMB 5221 as the best FA producer of the investigated Lactobacilli strains (Tomaro-Duchesneau et al. 2012a). For the development of an efficient probiotic therapeutic formulation there is a requirement for a carrier system. The delivery of bacterial cells through the GIT is impaired by the harsh conditions of the upper GIT. Microencapsulation, specifically APA has been suggested as a method to overcome this obstacle (Prakash et al. 2011b). This method is investigated in the presented research, specifically with relation to *L. fermentum* NCIMB 5221 and its FA producing capabilities.

This work investigated the FA production of *L. fermentum* NCIMB 5221 in both the microencapsulated and the free form. The FAE activity of *L. fermentum* NCIMB 5221 free and encapsulated was determined by HPLC, to ensure that the microcapsule does not hamper the flow of substrate into and the FA product out of the microcapsule. Cell viability was determined during the assay to ensure that the cell count remained equal. Although FA production was non-significantly higher for free cells, this is explained by slightly higher free cell viability during the course of the experiment **Figure 4.3**. This is supported by the previous research that directly correlates bacterial cell counts of *L. fermentum* NCIMB 5221 with its FA production (Tomaro-Duchesneau et al.

2012a). Keeping this in mind, the higher the cell count delivered to the colon, the higher the FA production. The successful delivery requires a carrier method such as microencapsulation.

Research has previously been presented on the use of microencapsulated cells for FA production, but fails to demonstrate a comparison between free and microencapsulated bacterial cells under the same conditions, as investigated in the presented research (Bhathena et al. 2008). In terms of microencapsulation technology for the storage of microcapsules, Kailasapathy has demonstrated a significant increase in viability of microencapsulated cells in yoghurt cultures stored over 7 weeks, at a pH as low as 4 (Kailasapathy 2006). However, in terms of oral delivery, a pH of approximately 1.5 is encountered in the stomach. Hence, a microcapsule capable of delivering optimal numbers of bacteria through the GIT needs to sustain viability at such a low pH. In this flask study, the exposure to the simulated gastrointestinal conditions clearly illustrates the requirement for a carrier system when delivering live bacterial cells to the lower GIT **Figure 4.5**. A significant 2.5 log difference in viability following exposure to the simulated conditions could be detected between the free $1.10 \times 10^4 \pm 1.00 \times 10^3$ cfu/mL and the microencapsulated $5.50 \times 10^6 \pm 1.00 \times 10^5$ *L. fermentum* NCIMB 5221.

Work is being undertaken to determine the FAE activity of free vs. microencapsulated *L. fermentum* NCIMB 5221 under GIT conditions. Unfortunately, the simple substrate EFA is labile under the enzymatic and pH conditions used in this study, which quickly resulted in EFA degradation when added to the SGF and SIF solutions. Normally, in the diet, the FA substrate would be present in a more complex form, such as wheat bran, which would permit FA release in the colon due to fermentation processes.

This type of food matrix, however, is undefined, rendering it difficult to quantify the low levels of FA that are released. Our research continues to explore the enzymatic process, looking at other *in vitro* and, potentially, *in vivo* methods to comprehend the FAE activity in the GIT.

4.5 Conclusions

This work supports the use of APA microencapsulation for the oral delivery of the investigated probiotic. The presented work successfully demonstrated the advantage of using APA microencapsulation for *L. fermentum* NCIMB 5221 for use in oral delivery. The FAE enzymatic activity and bacterial viability were maintained post-encapsulation and the viability of encapsulated cells was greater than free cells in simulated gastrointestinal conditions.

Future work should involve further optimisation of the microencapsulation process, since a significant loss of cell count was still evident with the microencapsulated formulation. Additional characterisation of a final formulation, in terms of the mechanisms of action and safety of the probiotic strain, the produced FA, and the use of the microencapsulation with *in vitro* and *in vivo* studies should be performed. In terms of the gastrointestinal tract, FAE activity should be characterized *in vivo* in appropriate animal models to ensure enzyme activity remains stable and efficient under potentially harsh conditions. The fate of the APA microcapsule should also be investigated *in vivo*. This work, nonetheless, opens up future potentials for the use of a synergistic formulation of microencapsulated *L. fermentu*m NCIMB 5221 with its intrinsic microbial FAE activity for both industrial and therapeutic applications.

4.6 Acknowledgments

The authors would like to acknowledge Micropharma Limited grants and a Canadian Institute of Health Research (CIHR) grant (MOP 264308) to Satya Prakash, the support of the Industrial Innovation Scholarship (IIS) BMP Innovation – NSERC, FQRNT and Micropharma Limited to Catherine Tomaro-Duchesneau, a FRSQ Master's award to Michael Coussa-Charley and a FRSQ Doctoral award to Meenakshi Malhotra.

4.7 Conflict of Interest

The authors declare that no financial support or other compensation has been received relating to any aspect of this research or its publication that could be construed as a potential conflict of interest.

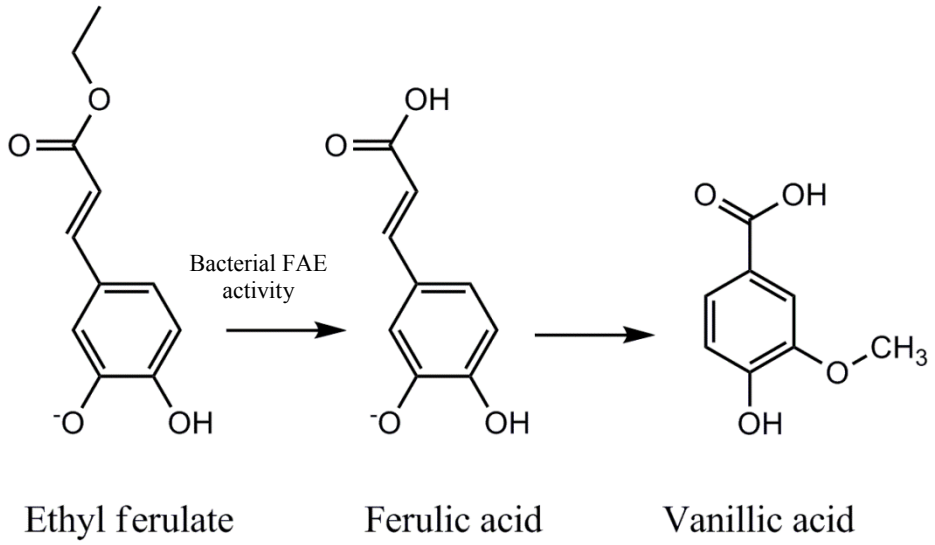

Figure 4.1: Ferulic acid esterases (FAE) enable microbes to hydrolyse the ester bond between hydroxyl cinnamic acids and sugars. The hydrolysis of ethyl ferulate by FAE gives rise to ferulic acid a compound with a number of health-promoting benefits.

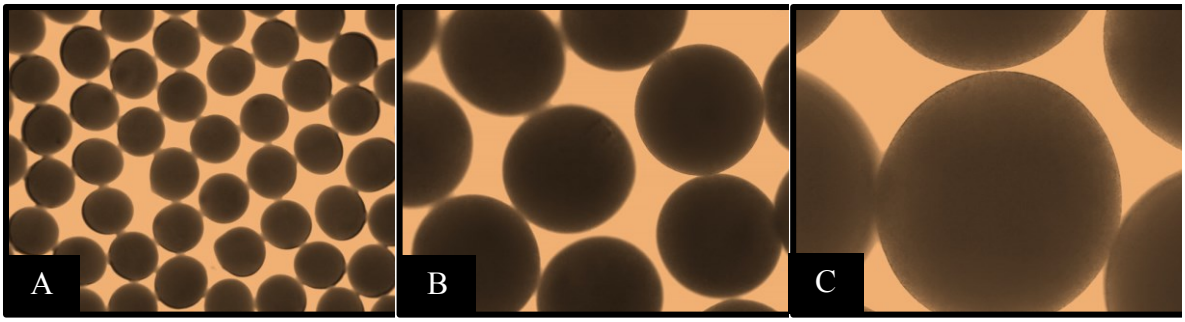

Figure 4.2: Morphology of APA microcapsule containing *L. fermentum* NCIMB 5221 taken by light microscope (A) 40× (B) 100× (C) 200×. The approximate diameter of the microcapsules was 400 ± 25 μm.

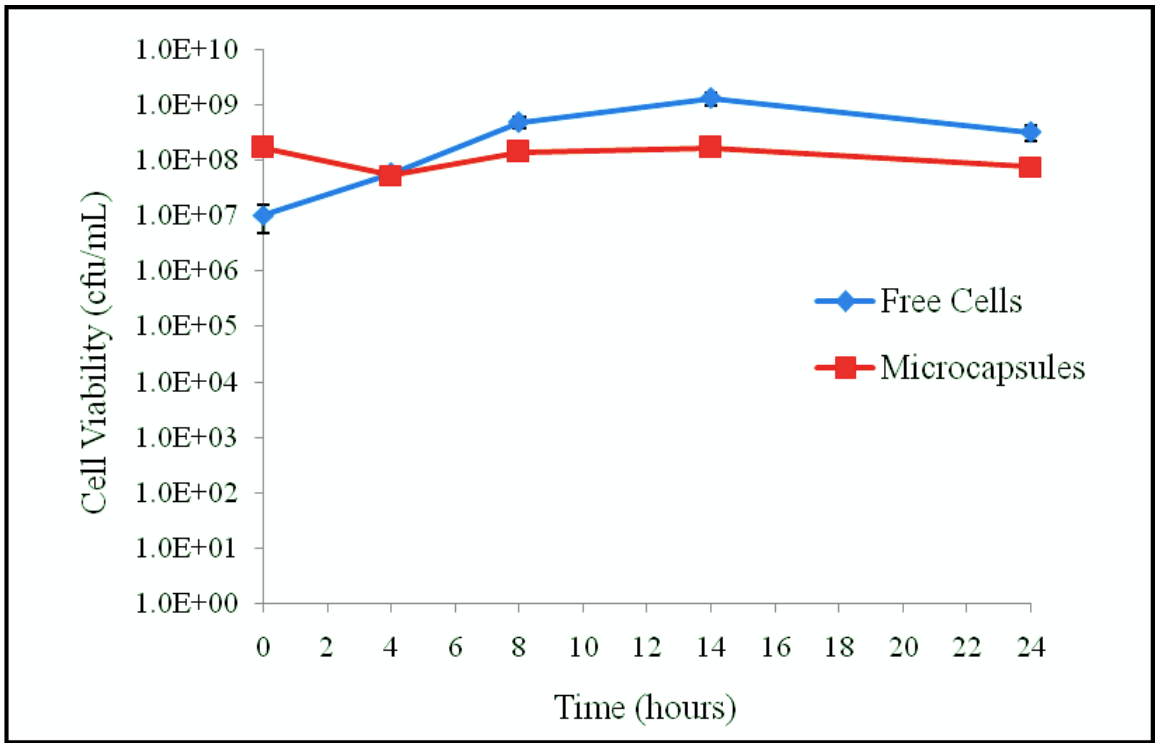

Figure 4.3: The viability of the free and microencapsulated *L. fermentum* NCIMB 5221 during the FAE assay (MRS-EFA 0.2956 mg/mL at 37 °C). Each point represents the mean of triplicates and the error bars the standard deviation. During the assay, there was no significant difference in viability between the free and microencapsulated bacteria.

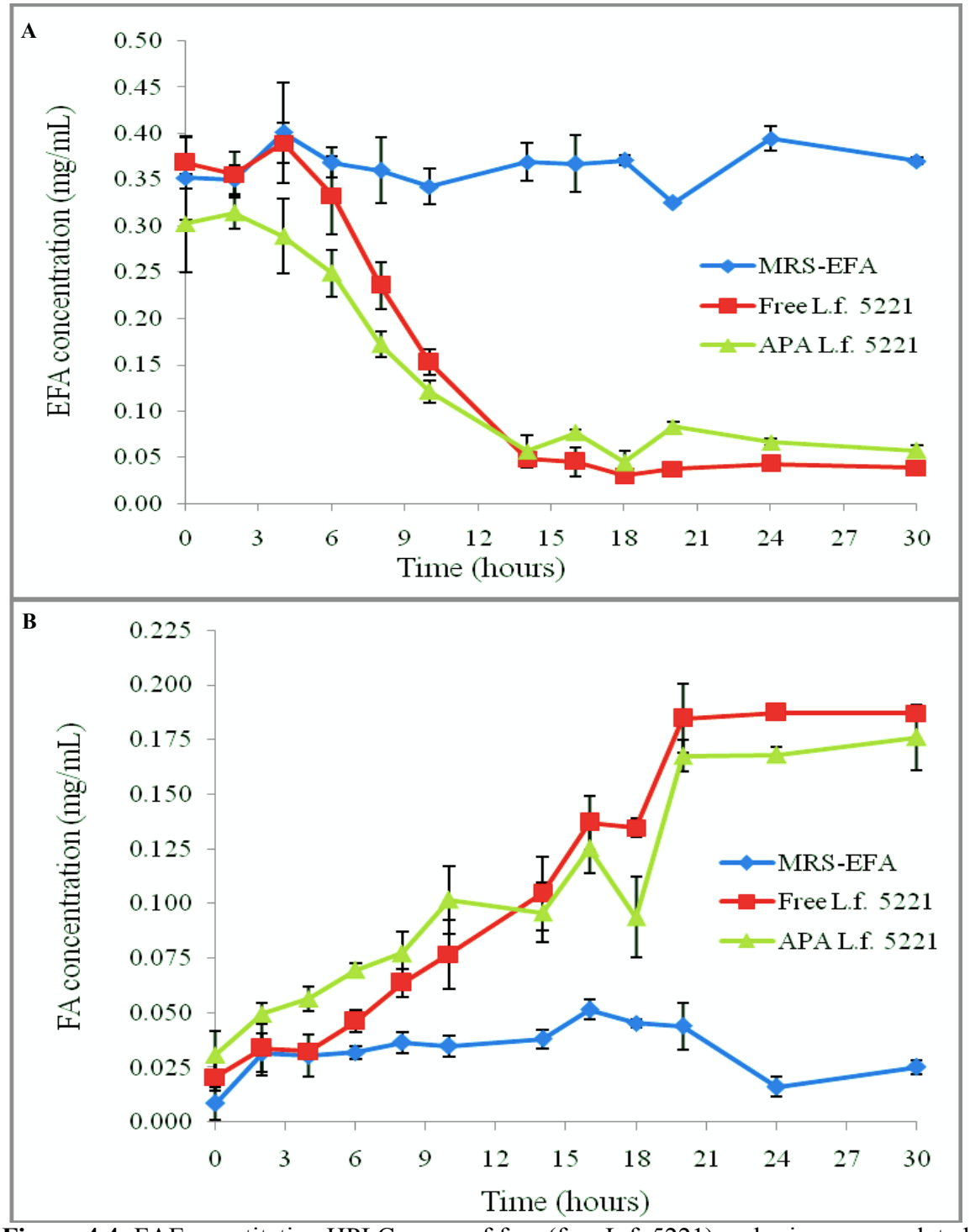

Figure 4.4: FAE quantitative HPLC assay of free (free L.f. 5221) and microencapsulated *L. fermentum* NCIMB 5221 (APA L.f. 5221). Uninoculated MRS-EFA was used as a negative control. The presented data represents the amount of unhydrolysed EFA remaining in solution (**A**) and the amount of FA produced (**B**), as measured by HPLC peak area data. Each point represents the mean of triplicates and the error bars represent the standard deviations. These results demonstrate no significant difference in FA production between the free and encapsulated *L. fermentum* NCIMB 5221.

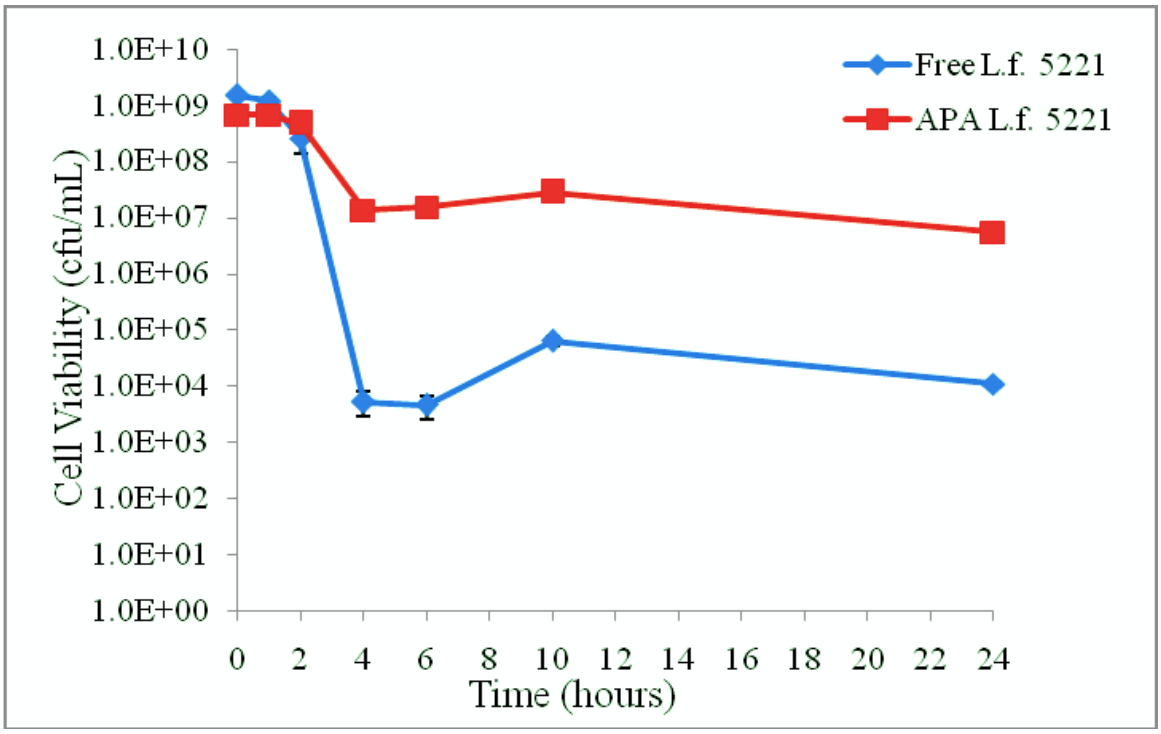

Figure 4.5: The viability of *L. fermentum* NCIMB 5221 in free and encapsulated form was determined upon exposure to the simulated GI conditions using standard colony counting methods. Each point represents the mean of triplicates and the error bars the standard deviations. A significant difference in viability between free and microencapsulated bacterial cells following simulated GI exposure was observed ($p < 0.001$; Tukey's HSD).

Time (h)	GIT section	pH	Solutes	Viability (cfu/mL)		Viability (%)	
				Free	APA	Free	APA
0	Stomach	1.5	Sodium chloride Peptic enzymes Glucose	$1.53 \times 10^9 \pm 9.02 \times 10^7$	$6.77 \times 10^8 \pm 7.77 \times 10^7$	100.00 ± 0.059	100.00 ± 0.115
1				$1.18 \times 10^9 \pm 2.04 \times 10^8$	$6.83 \times 10^8 \pm 4.51 \times 10^7$	77.51 ± 13.365	100.99 ± 6.664
2				$2.60 \times 10^8 \pm 1.22 \times 10^8$	$4.73 \times 10^8 \pm 4.93 \times 10^7$	17.03 ± 7.969	69.95 ± 7.290
4	Small / Large intestines	6.8	Potassium phosphate Pancreatic enzymes Bile Glucose	$5.33 \times 10^3 \pm 2.52 \times 10^3$	$1.35 \times 10^7 \pm 1.12 \times 10^6$	0.0004 ± 0.0002	2.00 ± 0.165
6				$4.67 \times 10^3 \pm 2.08 \times 10^3$	$1.53 \times 10^7 \pm 1.15 \times 10^6$	0.0003 ± 0.0001	2.26 ± 0.170
10				$6.37 \times 10^4 \pm 1.33 \times 10^4$	$2.82 \times 10^7 \pm 1.23 \times 10^6$	0.0042 ± 0.0009	4.17 ± 0.182
24				$1.10 \times 10^4 \pm 1.00 \times 10^3$	$5.50 \times 10^6 \pm 1.00 \times 10^5$	0.0007 ± 0.0001	0.813 ± 0.015

Table 4.1: The viability of *L. fermentum* NCIMB 5221, through the *in vitro* GI passage and the associated conditions of the gastric and intestinal exposure. Free = free *L. fermentum* NCIMB 5221; APA = microencapsulated *L. fermentum* NCIMB 5221. The data values represent the mean of triplicates ± SD. These results demonstrate a significant difference in viability between the free and microencapsulated cells ($p < 0.001$; Tukey's HSD).

Original Research Article 3

CHAPTER 5: ANTI-INFLAMMATORY PROPERTIES OF PROBIOTIC *L. FERMENTUM* NCIMB 2797: AN *IN VITRO* INVESTIGATION

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Preface: MetS is characterized by low-grade chronic systemic inflammation. Hence, following the selection and microencapsulation of the probiotic strain, investigations into the anti-inflammatory properties of the probiotic were undertaken, *in vitro*. Ferulic acid production was correlated to antioxidant activity. Probiotic nitric oxide, a mediator of inflammation, production was also quantified. Macrophage cells stimulated by lipopolysaccharide were used to represent the inflammation present in the gastrointestinal tract in MetS. Specifically, pro-inflammatory molecule, Tumor Necrosis Factor-α and nitric oxide, production by the macrophages was determined with and without probiotic treatment. A co-culture model of an inflamed intestinal epithelium was established using colon epithelial and macrophage cells, and used to show the effect of *L. fermentum* on lipopolysaccharide-induced intestinal epithelial permeability.

Article submitted: **Journal of Molecular Biology**

5.1 Abstract

Gastrointestinal and chronic systemic inflammation contributes to the pathogenesis of a number of health disorders. Microbial-originating toxins such as lipopolysaccharide from Gram-negative organisms in the intestinal lumen contribute to the inflammatory state and the loss in intestinal epithelial integrity, promoting an increased translocation of toxins and microbes into the systemic circulation. Probiotic bacteria, health-promoting microorganisms, have shown potential as biotherapeutics for a number of health disorders, including inflammatory conditions. In previous work, we investigated *Lactobacillus fermentum* probiotic bacteria for the production of ferulic acid (FA), a compound with health-promoting and antioxidant properties. In the presented research we investigate *L. fermentum* NCIMB 2797, as an anti-inflammatory probiotic. We demonstrate that this probiotic can produce high levels of FA from a natural substrate, 262.38 ± 39.48 μM, as well as significant levels of antioxidants, 595.71 ± 23.58 Trolox equivalents, in 24 h. In addition, *L. fermentum* NCIMB 2797 produced 144.83 ± 7.95 μM nitric oxide. Interestingly, *L. fermentum* NCIMB 2797 cell-free supernatant, but not whole cells, significantly decreased TNF-α ($p < 0.0001$) and NO ($p < 0.0001$) production by lipopolysaccharide-stimulated RAW 264.7 macrophages. Indeed, only the cell-free supernatant prevented ($p < 0.0001$) lipopolysaccharide-induced intestinal epithelial permeability using an *in vitro* colon epithelium model. These results demonstrate the potential of a *L. fermentum* NCIMB 2797 biotherapeutic for managing inflammatory and other disorders. Future studies should focus on the presence of other probiotic-produced anti-inflammatory compounds as well as the probiotic effect on other inflammatory markers, using both *in vitro* and *in vivo* studies.

5.2 Introduction

The human gastrointestinal tract (GIT) houses a large microbial ecosystem, termed the microbiota, containing approximately 10^{14} bacterial cells, ten-fold the number of human cells found in the body (Prakash et al. 2011c; Savage 1977). Lactic acid bacteria (LAB), including the *Bifidobacterium* and *Lactobacillus* genera, are commensal inhabitants of the microbiota that are commonly considered as probiotic. Probiotic bacteria are "live microorganisms which when administered in adequate amounts confer health benefits to the host" (FAO and WHO 2001). In recent years, probiotic bacteria have demonstrated great potential as biotherapeutics for a number of health disorders (Prakash et al. 2011c; Saha et al. 2013; Tomaro-Duchesneau et al. 2013b) including, metabolic syndrome (Bhathena et al. 2012; Tomaro-Duchesneau et al. 2013a), obesity (Takemura et al. 2010), hypercholesterolemia (Jones et al. 2013), inflammatory bowel disease (Jonkers et al. 2012), non-alcoholic fatty liver disease (Bhathena et al. 2013), colon cancer (Kahouli et al. 2013), allergies (Prakash et al. 2013) and oral disorders (Saha et al. 2012a; Saha et al. 2012b). Specifically, probiotic bacteria have been shown to have anti-inflammatory properties (LeBlanc et al. 2013), including antioxidant production (Tomaro-Duchesneau et al. 2012a).

Chronic systemic and GIT inflammation play important roles in the pathogenesis of a number of health disorders, including those previously mentioned as modulated by probiotic LAB. The GIT contains a crucial part of the human immune system, termed the gut-associated lymphoid tissue (GALT) belonging to the mucosa-associated lymphoid tissue (MALT). Specifically, intestinal epithelial and antigen-presenting (dendritic and macrophage) cells express pattern-recognition receptors which can trigger inflammatory

signalling cascades upon recognition of microbial components. For example, Gram-negative bacterial lipopolysaccharide (LPS) is recognized by toll-like receptor 4 (TLR 4) located on a number of cells, including macrophages. Upon recognition of LPS, a number of inflammatory mediators are secreted by macrophages, including tumour necrosis factor-α (TNF-α), reactive oxygen species (ROS) and nitric oxide (NO) which contribute to the establishment of an inflammatory state.

The intestinal epithelium, under healthy conditions, provides a barrier preventing the translocation of microbes, microbial products and other antigens from the blood stream which, if permitted, would contribute to a systemic inflammatory state. Under chronic inflammatory conditions due to macrophage activation, the colon epithelium is "leaky," characterized by an increased permeability, due to altered tight-junction barrier functions, resulting in a systemic inflammatory response which may involve other organs, such as the liver. Probiotic bacteria can reduce the secretion of pro-inflammatory markers and can thus promote the maintenance of the epithelial barrier function. Indeed, previous studies have demonstrated that treatment with probiotic bacteria can reduce the effect of pro-inflammatory cytokines on epithelial tight junctions (Ewaschuk et al. 2008; Madsen et al. 2001; Madsen 2012).

In previous work we demonstrated that certain probiotic *Lactobacillus*, specifically *Lactobacillus fermentum*, have anti-inflammatory properties linked to intrinsic antioxidant activity and ferulic acid (FA) production due to a ferulic acid esterase (FAE) (Bhathena et al. 2012; Tomaro-Duchesneau et al. 2012a; Tomaro-Duchesneau et al. 2012b). FAE-active *L. fermentum*, in previous studies by our group, have shown great potential for the treatment of metabolic syndrome, cholesterol and non-

alcoholic fatty liver disease (Bhathena et al. 2009; Bhathena et al. 2012; Bhathena et al. 2013; Tomaro-Duchesneau et al. 2013a). The anti-inflammatory and other health properties of FA have been demonstrated. However, the anti-inflammatory effect of a FA-producing probiotic has yet to be examined.

The aim of the presented work is to investigate the anti-inflammatory properties of *L. fermentum* NCIMB 2797, a FAE-active probiotic strain. We first quantify FA production by this strain when incubated in a natural substrate and correlate this with antioxidant activity. The production of probiotic NO, as another molecule having anti-inflammatory properties is also investigated. The effects of *L. fermentum* NCIMB 2797 formulations, both whole cell and cell –free supernatant preparations, are investigated on LPS-inflamed RAW 264.7 macrophage cells. An *in vitro* colon epithelium co-culture model, consisting of Caco-2 colon epithelial cells and RAW 264.7 macrophage cells, is then used to study the effects of the probiotic formulations on colon epithelial permeability, induced by LPS stimulation.

5.3 Materials and methods

5.3.1 Bacterial and mammalian growth media and chemicals

Ethyl ferulate (ethyl 4-hydroxy-3-methoxycinnamate, EFA), ferulic acid (*trans*-4-hydroxy-3-methoxycinnamate, FA) and lipopolysaccharide (LPS from *Escherichia coli* O55:B5) were purchased from Sigma-Aldrich (Oakville, ON, Canada). De Man-Rogosa-Sharpe (MRS) broth and methanol of high-pressure liquid chromatography (HPLC) grade were obtained from Fisher Scientific (Ottawa, ON, Canada). Water was purified with an EASYpure Reverse Osmosis System and a NANOpure Diamond Life Science (UV/UF)

ultrapure water system from Barnstead (Dubuque, IA, USA). The QuantiChrom™ Antioxidant Assay Kit was purchased from BioAssay Systems (Haiward, CA, USA) and the Enzyme-linked immunosorbent assay (ELISA) for tumor necrosis factor-alpha (TNF-α) was purchased from Cedarlane Laboratories (Burlington, ON, Canada). All other chemicals were of analytical or HPLC grade and purchased from commercial sources.

5.3.2 Bacterial strain and culture conditions

The probiotic bacterial strain *Lactobacillus fermentum* NCIMB 2797 was purchased from NCIMB (Aberdeen, Scotland, UK) and stored at -80°C in MRS broth containing 20% (v/v) glycerol. An MRS-agar plate was streaked with *L. fermentum* NCIMB 2797 for isolation and to ensure purity from the frozen stock and incubated at 37°C with 5% CO_2 for 24 h. Following incubation, one colony from the agar plate was inoculated into 5 mL MRS broth and incubated at 37°C for 24 h. A 1% (v/v) bacterial inoculum was then used for sub-culturing and incubated at 37°C for 24 h immediately prior to use. The bacterial viability of *L. fermentum* NCIMB 2797 was determined using standard colony counting methods. Briefly, overnight cultures were diluted in 0.85% (w/v) NaCl using 10-fold dilutions which were plated on MRS-agar incubated at 37°C and 5% CO_2 for 48 h, followed by colony counting. All viability assays were performed in triplicate to ensure accuracy and reproducibility.

5.3.3 Quantifying FA production

L. fermentum NCIMB 2797 was subcultured from an overnight MRS broth culture at 1% (v/v) into MRS-EFA broth of a final EFA concentration of 1330 μM, with uninoculated MRS-EFA as a control. The cultures were incubated at 37°C for 24 h, with triplicate samples following 0, 4, 8, 16 and 24 h of incubation. An HPLC assay,

previously described, was used to measure FA production (**Chapter 3** and **Chapter 4**) (Tomaro-Duchesneau et al. 2012a; Tomaro-Duchesneau et al. 2012b). Briefly, at every time point, 500 µL of sample were collected and centrifuged at 10,000 rpm for 7 min at 4°C to remove bacterial cells (Napco 2028R centrifuge, Fisher Scientific, Ottawa, ON, Canada). 100 µL of 0.35 M H_2SO_4 were added to 300 µL of the resulting cell-free supernatant and the acidified solution was briefly vortexed. 300 µL of 1 mM benzoic acid was added as an internal standard as well as 100 µL of 0.7 M NaOH to neutralize the pH. Samples were filtered with a 0.45 µm filter prior to analysis.

The HPLC system consisted of a ProStar 335 diode array detector set at 280 nm and 320 nm, a ProStar 410 autosampler, a reverse-phase C-18 column: LiChrosorb RP-18, 25 x 0.46 cm (Grace Davison Discovery Sciences, ON, Canada) and the Star LC workstation version 6.41. The injection volume was set at 25 µL, the mobile phase (solvent A) was 37% (v/v) methanol and 0.9% (v/v) acetic acid in HPLC water and solvent B consisted of 100% (v/v) methanol. The HPLC run was initiated with 100% solvent A for 16 min followed by a 1 min linear gradient to reach 100% solvent B. Solvent B was held isocratically at 100% for 12 min followed by a 1 min linear gradient to reach 100% of solvent A. A FA standard curve was generated using peak area quantification and triplicate samples of the concentrations 100, 300, 500, 960 and 1100 µM FA concentrations in MRS plotted against peak area (R^2 = 0.987). The EFA standard curve was generated using the concentrations 100, 300, 500, 700, 1000, 1400 and 1800 µM were plotted against peak area (R^2=0.9785). Standards and quality control samples were prepared and analyzed exactly as the test samples, in triplicate.

5.3.4 Quantifying total antioxidant capacity

L. fermentum NCIMB 2797 was subcultured from an overnight MRS broth culture at 1% (v/v) into MRS-EFA broth of a final EFA concentration of 1330 μM, with uninoculated MRS-EFA as a control. The cultures were incubated at 37°C for 24 h, with triplicate samples taken following 0, 4, 8, 16 and 24 h of incubation. At each time point, samples were centrifuged at 10,000 rpm for 7 min at 4°C to collect the probiotic cell-free supernatant. The total antioxidant capacity of *L. fermentum* NCIMB 2797 was measured, following the manufacturer's instructions, using a spectrophotometric assay kit based on Cu^{2+} to Cu^{+} reduction. Trolox was used as a standard with concentrations 0, 300, 600 and 1000 μM plotted against absorbance at 570 nm (R^2 = 0.997) using a UV spectrophotometer Victor^{3}V 1420 Multilabel Counter (Perkin Elmer, Boston, MA). Each sample and control was treated in triplicate to ensure accuracy and reproducibility.

5.3.5 Quantifying NO production by *L. fermentum* NCIMB 2797

L. fermentum NCIMB 2797 was subcultured from an overnight MRS broth culture at 1% (v/v) into fresh MRS broth and incubated at 37°C, with triplicate samples taken following 0, 4, 8, 16 and 24 h of incubation. Uninoculated MRS was used as a negative control and treated the exact same as the probiotic samples. At each time point, the viability of the culture was taken using standard colony counting methods, as aforementioned. For NO quantification, at each time point, the samples were centrifuged at 10,000 rpm for 7 min at 4°C to collect the probiotic cell-free supernatant. NO was quantified using the Griess assay. Briefly, 60 μL of a solution containing 1% (w/v) sulfanilamide and 0.5% (v/v) phosphoric acid was added to 60 μL of the supernatant and incubated for 10 min at 25°C. 60 μl of 0.1% (w/v) N-(1-Naphthyl)ethylenediamine

dihydrochloride were then added to the solution and incubated for 30 min at 25°C protected from light. Following the incubation, the absorbance was measured at 570 nm using a UV spectrophotometer Victor^{3}V 1420 Multilabel Counter. A standard curve was generated using sodium nitrite concentrations of 0, 1.46, 2.93, 5.86, 11.72, 23.44, 46.88, 93.75, 187.5, 375, 750, 1500 and 3000 μM ($R^2 = 0.998$) in MRS.

5.3.6 Macrophage culture conditions

RAW 264.7 murine macrophages were purchased from Cedarlane Laboratories (Burlington, ON, Canada). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% (v/v) fetal bovine serum (FBS), with no antibiotics, and incubated at 37°C and 5% CO_2 with passages every 5-7 days. The RAW 264.7 macrophage cells were used for experiments at passages 15-20. The cells were sub-cultured into 24-well plates, at a cell concentration of 5 x 10^5 cells/mL, as determined by cell counting using a haemocytometer and trypan blue staining. The cells were allowed to attach for 24 h prior to the assay. The RAW 264.7 macrophages were incubated for 24 h at 37°C and 5% CO_2 with 500 μL of each treatment, detailed in the next section. Cell viability was determined using a Promega CellTiter-Glo Luminescent Cell Viability assay purchased from Fisher Scientific (Ottawa, ON, Canada). The manufacturer's instructions were followed and the luminescence of the samples was measured using a UV spectrophotometer Victor^{3}V 1420 Multilabel Counter.

5.3.7 Preparation of probiotic whole cell and probiotic cell-free supernatant formulations

L. fermentum NCIMB 2797 whole cell extract was prepared by centrifuging 1 mL of an overnight culture grown in MRS-EFA at 4000 rpm for 7 min at 4°C. The

supernatant was discarded and the pellet re-suspended in phosphate buffered saline (PBS), followed by dilution in DMEM and the pH neutralized using 1 M NaOH. LPS was added to the DMEM-probiotic suspension at a concentration of 1 μg/mL. 500 μL of the whole cell probiotic suspension, containing $6.95 \times 10^5 \pm 2.57 \times 10^4$ probiotic *L. fermentum* NCIMB 2797, was added to the RAW 264.7 macrophages wells.

L. fermentum NCIMB 2797 cell-free supernatant was prepared by centrifuging 1 mL of an overnight culture grown in MRS-EFA at 4000 rpm for 7 min at 4°C. The supernatant was collected, filtered using a 0.22 μm filter and diluted in DMEM to the desired cell count. The pH of the probiotic supernatant solution was neutralized using 1 M NaOH. LPS was added to the supernatant solution at a final concentration of 1 μg/mL. 500 μL of the probiotic cell-free supernatant suspension, from a total of $4.24 \times 10^6 \pm 1.60 \times 10^5$ probiotic *L. fermentum* NCIMB 2797 cells, was added to the RAW 264.7 macrophages in the 24-well plates. Experimental controls included DMEM and DMEM with 1 μg/mL LPS. All treatments and controls were added to the cells in triplicate to ensure accuracy and reproducibility.

5.3.8 Effect of probiotic on macrophage nitric oxide production

Nitric oxide (NO) production by the RAW 264.7 macrophages exposed to the different treatments was quantified using the Griess assay. Following the 24 h incubation with probiotic treatment, the supernatant from the RAW 264.7 macrophages was collected and used for NO measurement by the Griess assay, previously described. A standard curve was generated using sodium nitrite concentrations of 0, 1.46, 2.93, 5.86, 11.72, 23.44, 46.88, 93.75, 187.5, 375, 75-, 1500 and 3000 μM ($R^2 = 0.996$) in DMEM plotted against absorbance.

5.3.9 Effect of probiotic on macrophage TNF-α production

TNF-α production by the RAW 264.7 macrophages exposed to the different treatments was quantified using an ELISA. Following the 24 h incubation with probiotic treatment, the supernatant from the RAW 264.7 macrophages was collected and used for TNF-α measurement. The manufacturer's instructions were followed. A standard curve was generated using known TNF-α concentrations of 15.6, 31.3, 62.5, 125, 250, 500 and 1000 pg/mL ($R^2 = 0.998$) plotted against absorbance.

5.3.10 Investigating probiotic effect on Caco-2 colonic epithelial cell viability

Caco-2 human colon epithelial cells were purchased from Cedarlane Laboratories (Burlington, ON, Canada). The cells were maintained in DMEM with 10% (v/v) FBS, with no antibiotics, and incubated at 37°C and 5% CO_2 with passages every 5-7 days. The Caco-2 epithelial cells were used for experiments at passages 20-25. The cells were sub-cultured into 12-well plates, at a cell concentration of 5 x 10^5 cells/mL, as determined by cell counting using a haemocytometer and trypan blue. The cells were allowed to attach for 48 h prior to the assay. The Caco-2 epithelial cells were then incubated with 500 µL of the different treatments for 24 h at 37°C and 5% CO_2. Cell viability was determined using the luminescence assay, described previously.

5.3.11 Investigating probiotic effect on colonic epithelial integrity using the co-culture model

A colonic epithelium model was developed based on previous research by Tanoue *et al.* (**Figure 5.1**) (Tanoue et al. 2008). Caco-2 cells (2 x 10^7 cells/mL) were seeded onto transwell inserts (0.3 cm^2, 8 µm pore size) purchased from Fisher Scientific (Ottawa, ON, Canada). The cells were maintained in DMEM with 10% (v/v) FBS, with no antibiotics,

and incubated at 37°C and 5% CO_2 with a change in media every 2-3 days. The integrity of the Caco-2 epithelial monolayer was monitored regularly by transepithelial electrical resistance (TER) measurements, using a Millicell®-ERS Instrument from Millipore (Billerica, MA, USA). A TER value of > 1000 Ω/cm^2 represented a Caco-2 epithelium with the desired integrity. RAW 264.7 macrophages were seeded in 24-well plates at a concentration of 5 x 10^5 cells/mL and allowed to attach for 24 h at 37°C and 5% CO_2. The macrophages were stimulated with 1 μg/mL LPS and the Caco-2 epithelial inserts were added to the stimulated macrophages. TER was measured following 6 h and 24 h of co-incubation at 37°C and 5% CO_2.

To investigate the effect of the probiotic on the LPS-activated macrophage induced loss of colonic epithelial integrity, the probiotic treatments were added on the apical side of the inserts concurrent with the Caco-2 exposure to the LPS-activated macrophage cells. The epithelial integrity was monitored following 24 h of incubation at 37°C and 5% CO_2 by TER.

5.3.12 Statistical analysis

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, USA). Linear regression was performed for generating standard curves. Statistical comparisons were carried out using the general linear model, followed by multiple comparisons of the means using Tukey's post-hoc analysis. 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 FA production by *L. fermentum* NCIMB 2797

FA production and EFA hydrolysis by *L. fermentum* NCIMB 2797 were monitored by HPLC during its growth in MRS-EFA (**Figure 5.2**). The initial viability of the probiotic culture was $7.67 \times 10^5 \pm 3.33 \times 10^4$ colony forming units (cfu)/mL and reached $1.39 \times 10^8 \pm 5.13 \times 10^6$ cfu/mL following 24 h of incubation, following the conventional bacterial growth curve kinetics. Following 8 h of *L. fermentum* NCIMB 2797 incubation in MRS-EFA, there was 848.09 ± 36.52 μM EFA in solution, demonstrating significant ($p = 0.030$) EFA hydrolysis by this point. *L. fermentum* NCIMB 2797 demonstrated significant ($p < 0.0001$) EFA hydrolysis with 207.51 ± 14.57 μM EFA remaining in solution following 24 h of incubation. Conversely, the FA production by *L. fermentum* NCIMB 2797 increased steadily throughout the growth of the probiotic cells, with a final FA production of 532.91 ± 13.14 μM following 24 h of incubation in MRS-EFA with significant ($p < 0.0001$) production of 262.38 ± 39.48 μM FA as of 4 h. Both EFA hydrolysis and FA production were closely associated with the growth kinetics of *L. fermentum* NCIMB 2797. In addition, EFA hydrolysis and FA production were clearly inversely correlated, an increase in FA concentration can be attributed to a decrease in EFA levels.

5.4.2 *L. fermentum* NCIMB 2797 FA production linked to its antioxidant capacity

FA production by *L. fermentum* NCIMB 2797 showed a close association with antioxidant production during growth in MRS-EFA (**Figure 5.3**). As FA production increased over the incubation period, so did the antioxidant activity of the bacterial

supernatant. A FA production of 532.91 ± 13.14 μM demonstrated a total antioxidant activity of 595.71 ± 23.58 μM Trolox equivalents, following 24 h of incubation.

5.4.3 *L. fermentum* NCIMB 2797 NO production

L. fermentum NCIMB 2797 produced significant levels of NO when grown in MRS broth (**Figure 5.4**). The initial probiotic viability at 0 h of growth was 1.2 x10^7 ± 1.53 x 10^6 cfu/mL with 6.70 x 10^8 ± 4.58 x 10^7 cfu/mL following 24 h of growth, following the growth curve previously shown. Following 8 h of growth, *L. fermentum* NCIMB 2797 had the maximum NO production of 144.83 ± 7.95 μM, a significant (p < 0.0001) production. Probiotic NO production did not increase significantly following 16 h and 24 h of incubation. In addition, following 16 h and 24 h of incubation, the viability of *L. fermentum* NCIMB 2797 did not significantly increase as compared to the bacterial cell count following 8 h, suggesting a cell growth dependent NO production.

5.4.4 LPS-stimulated macrophages treated with *L. fermentum* NCIMB 2797

LPS-stimulated RAW 264.7 macrophages demonstrated no significant loss (p > 0.05) in cell viability when exposed to *L. fermentum* NCIMB 2797 whole cell and cell-free supernatant probiotic extracts for 24 h (**Figure 5.5A**).

In terms of NO production (**Figure 5.5B**), LPS-stimulated RAW 264.7 macrophages demonstrated a significant increase (p < 0.0001) in NO concentration compared to non-activated macrophages, with a production of 26.57 ± 1.42 μM NO after 24 h. *L. fermentum* NCIMB 2797 whole cells had no significant effect (p = 0.968) on NO production by LPS-activated macrophages with a concentration of 27.04 ± 0.36 μM of NO following 24 h. Conversely, treatment with *L. fermentum* NCIMB 2797 cell-free

supernatant significantly decreased ($p < 0.0001$) NO production, with a concentration of 5.28 ± 0.056 μM NO following 24 h of incubation.

TNF-α production of the LPS-activated RAW 264.7 macrophages treated with *L. fermentum* NCIMB 2797 was also monitored (**Figure 5.5C**). Following exposure to LPS, the macrophages demonstrated a significant increase ($p < 0.0001$) in TNF-α production following 24 h of incubation, with a concentration of 10 330 ± 12.71 pg/mL of TNF-α. Treatment with the probiotic whole cell extract of *L. fermentum* NCIMB 2797 had no significant effect ($p = 0.936$) on TNF-α production by the macrophages, with a production of 10 466.5 ± 36.54 pg/mL of TNF-α. Treatment with *L. fermentum* NCIMB 2797 cell-free supernatant significantly reduced ($p < 0.0001$) TNF-α production by the LPS-activated macrophages with a production of 6974 ± 329.79 pg/mL TNF-α following 24 h.

5.4.5 Effect of *L. fermentum* NCIMB 2797 on colonic epithelial cell viability

The effect of the *L. fermentum* NCIMB 2797 formulations on Caco-2 colon epithelial cell viability when co-incubated was investigated. The colon epithelial cells demonstrated no significant loss ($p > 0.05$) in cell viability when exposed to *L. fermentum* NCIMB 2797 whole cell and cell-free supernatant probiotic extracts for 24 h (**Figure 5.6**).

5.4.6 Development and characterization of the co-culture model for inflammatory studies

To develop the co-culture colon epithelial model, Caco-2 cells were grown on semi-permeable inserts and the TER was monitored over 17 days, as a measurement of epithelial integrity (**Figure 5.7A**). The TER increased steadily over time, closely

following a second-order equation (R^2 = 0.9853). The desired TER of 1000 Ω/cm^2 was achieved by day 15 with an epithelial integrity of 982.5 ± 37.63 Ω/cm^2 and of 1049.5 ± 14.97 Ω/cm^2 on day 17.

Following establishment of the desired epithelium, co-culture with LPS-activated RAW 264.7 macrophages led to a decrease in epithelial integrity, as determined by TER (**Figure 5.7B**). Following 6 h of incubation with the RAW 264.7 macrophages, there was a significant reduction (p = 0.002) in epithelial integrity, with 93.3 ± 1.11% integrity. An even greater significant reduction (p < 0.0001) in epithelial integrity, with 58.11 ± 0.79% integrity, was demonstrated following 24 h of co-incubation.

5.4.7 Effect of *L. fermentum* NCIMB 2797 on colonic epithelial integrity

L. fermentum NCIMB 2797 was investigated for its ability to maintain the epithelial integrity, measured by TER, of Caco-2 cells co-cultured with LPS-activated macrophages (**Figure 5.8**). Treatment with the probiotic whole cell extract of *L. fermentum* NCIMB 2797 had no significant effect (p = 0.998) on the TER of the colonic epithelium, with an epithelial integrity of 73.53 ± 4.08%, similar to the untreated control epithelium with an integrity of 72.75 ± 4.86%. Conversely, treatment with *L. fermentum* NCIMB 2797 cell-free supernatant significantly increased (p < 0.0001) colonic epithelial integrity, with an integrity of 98.61 ± 7.58%.

5.5 Discussion

Chronic low-grade inflammation contributes to the pathogenesis of many of the leading causes of mortality in the United States, including cancer, diabetes, heart disease, Alzheimer's disease, stroke, inflammatory bowel syndrome and chronic lower respiratory

tract disease (2011; Bastard et al. 2006; Cao 2011; Ferrucci et al. 2010; Kundu and Surh 2008; Murphy et al. 2012; Singh and Newman 2011). Recent work demonstrates that probiotic bacteria have great immunomodulatory potential for the development of therapeutics to combat these diseases, although there is a need for a better understanding of the mechanisms involved (Corthesy et al. 2007). Probiotic bacteria have been proposed and investigated for a number of health disorders, discussed in detail in a recent review (Prakash et al. 2011c). Probiotic bacteria are naturally occurring, inexpensive, generally regarded as safe (GRAS) and are free of long-term negative side-effects (Branton et al. 2011). Specifically, *L. fermentum* has shown promising results: as an adjuvant for vaccines (Olivares et al. 2007), as an inflammatory bowel disease prevention strategy (Peran et al. 2007), as a cholesterol-lowering therapeutic (Bhathena et al. 2009), as capable of inhibiting oral disease progression (Saha et al. 2012a), as a non-alcoholic fatty liver disease treatment (Bhathena et al. 2013) and as a metabolic syndrome treatment option (Bhathena et al. 2012; Tomaro-Duchesneau et al. 2013a).

Interestingly, a number of *L. fermentum* probiotic strains have demonstrated intrinsic FAE activity, characterized by the production of FA (Abeijon Mukdsi et al. 2012; Bhathena et al. 2007; Bhathena et al. 2008; Hole et al. 2012; Tomaro-Duchesneau et al. 2012a; Tomaro-Duchesneau et al. 2012b). FA is a natural phenolic acid with potent antioxidant activity capable of neutralizing free radicals such as reactive oxygen species (ROS), implicated in DNA damage, accelerated cell aging and cancer (Lombard et al. 2005b; Rice-Evans et al. 1996). The oral delivery of free FA is limited due to its rapid absorption in the jejunum leading to its rapid excretion from the body (Spencer et al. 1999; Zhao et al. 2003). The delivery of FA-producing probiotic bacteria to the colon

enhances the FA bioavailability in the lower human digestive tract, giving rise to a constant and controlled release of the anti-inflammatory FA. The goal of this research was to investigate *L. fermentum* NCIMB 2797, a FA producing probiotic, for its anti-inflammatory activity using inflamed macrophage cells as well as an *in vitro* co-culture model of the human colon epithelium.

Using EFA, the simplest substrate of the FAE enzyme, we demonstrated that *L. fermentum* NCIMB 2797 produces 532.91 ± 13.14 μM FA following 24 h of incubation, similar to the levels produced in previous studies (Bhathena et al. 2007; Tomaro-Duchesneau et al. 2012a). Indeed, very low levels of the EFA substrate were remaining in solution following 24 h, suggesting almost complete hydrolysis by FAE activity. FA is a known antioxidant, a molecule that can inhibit oxidation and the production of damaging free radicals. Therefore, we investigated the total antioxidant capacity of *L. fermentum* NCIMB 2797 using a commercially available assay based on the reduction of Cu^{2+} to Cu^{+}. Indeed, following 24 h of growth, we observed a significant production of probiotic antioxidants, 595.71 ± 23.58 μM Trolox equivalents. Trolox is a water-soluble analog of vitamin E provided as a standard with the commercial antioxidant kit. As could be predicted, there was a close association between the FA production measured by HPLC and the antioxidant production measured by the commercial kit, confirming the antioxidant nature of the produced FA and, potentially other probiotic-produced antioxidant compounds.

Previous research groups have demonstrated that probiotic bacteria, including *L. fermentum* strains can produce NO by nitrate reduction (Xu and Verstraete 2001). Indeed, previous work, by Lamine *et al.,* demonstrated that *L. farciminis* produces NO which

improved chemically-induced rat colitis (Lamine et al. 2004). In the presented work, we investigated whether *L. fermentum* NCIMB 2797 can produce NO, and demonstrated that it produced significant levels of NO, 144.83 ± 7.95 μM when grown in MRS broth which contains small amounts of nitrate (Xu et al. 2000). The NO production profile was closely associated with the growth profile of the probiotic cells. NO has been shown to have anti-inflammatory properties due to a number of mechanisms including the modulation of leukocyte adherence (Kubes et al. 1991), decreasing the expression of endothelial adhesion molecules (De Caterina et al. 1995) and inhibiting leucocyte adhesion to mesenteric post-capillary venules of the intestine (Wallace et al. 1999). Jones *et al.*, in recent work, demonstrated that patches containing LAB that produce significant amounts of NO helped in the healing of ischemic and infected wounds (Jones et al. 2012c). Interestingly, NO, similar to FA, can also act as an antioxidant. During an inflammatory process, the free radicals, superoxide anion and NO, produced by macrophages, can combine to form peroxynitrite, a highly toxic free radical, present in an inflamed intestine (Miller et al. 1995). Earlier research demonstrated thatsuperoxide and NO must be present in equimolar concentrations for peroxynitrite production, as normally produced by macrophages. However, an excess production of either component leads to the inhibition of peroxynitrite production (Miles et al. 1996). Supplementing low levels of NO via probiotic production may, thus, allow for an antioxidant effect.

To further investigate the potential of *L. fermentum* NCIMB 2797 for reducing an inflammatory response, we investigated the effect of probiotic whole cells and cell-free supernatant on LPS-inflamed RAW 264.7 macrophages. We demonstrated that, as in similar studies, LPS stimulation led to significant increases in TNF-α and NO production

by the macrophages (Kiemer et al. 2002; Rodes et al. 2013). It is important to note that neither of the two *L. fermentum* extracts impaired the cell viability of the macrophage cells during their exposure. Treatment with *L. fermentum* NCIMB 2797 whole cells did not alter the inflammatory response of the LPS-stimulated macrophages, with respect to TNF-α and NO production. In contrast, treatment with cell-free probiotic supernatant led to a significant reduction in both TNF-α and NO production, suggesting anti-inflammatory activity by the secreted bacterial products. These results are in agreement with previous studies using different extracts of another strain of *L. fermentum* (Frick et al. 2007). In previous studies, pure FA, in a similar concentration found in the cell-free extract of *L. fermentum* NCIMB 2797, was indeed shown to decrease macrophage TNF-α secretion by similar levels observed in this study (Sakai et al. 1997). NO may also be responsible for the observed reduction in inflammation through antioxidant activity related to peroxynitrite, previously mentioned.

In humans, the intestinal epithelium is continuously in contact with the microbiota and luminal contents. It, therefore, has to protect itself from uncontrolled bacterial growth and from bacterial dissemination into the underlying tissues of the intestinal epithelium, by ensuring the integrity of the epithelium and the tight junctions between enterocytes. In disorders such as inflammatory bowel diseases and chronic-systemic inflammation, present in diabetes and obesity, the intestinal epithelium integrity, specifically the tight junctions between epithelial cells, are disrupted (Brun et al. 2007; Schulzke et al. 2009; Ulluwishewa et al. 2011). The increased intestinal epithelium permeability permits the diffusion of bacterial antigens, such as LPS, to the underlying tissues and blood stream, resulting in chronic systemic inflammation and, potentially, septic shock. It has been

suggested that probiotic bacteria can improve intestinal epithelial barrier function. To investigate if *L. fermentum* NCIMB 2797 has this beneficial property, we used an *in vitro* co-culture model of the colon epithelium using colon epithelial cells and macrophages, as described by Tanoue *et al.* (Tanoue et al. 2008). Following successful formation of a colon epithelium, we demonstrated that exposure of a colon epithelium to LPS-inflamed macrophages, significantly increased the intestinal permeability, as demonstrated by transepithelial electrical resistance readings. However, treatment with *L. fermentum* NCIMB 2797 cell-free supernatant, not the whole bacterial cells, countered the inflammatory processes and allowed for the maintenance of epithelial integrity. We hypothesize that the maintenance in epithelial integrity is correlated by the anti-inflammatory effects observed in the single-cell line macrophage model. In addition, research by Dai *et al.* previously demonstrated that the VSL#3 probiotic mixture, with NO production, increased tight junction protein expression, reducing colonic permeability in a rat model of irritable bowel syndrome, suggesting the potential role of *L. fermentum* NCIMB 2797 NO production in enhancing epithelial integrity *in vitro* (Dai et al. 2012).

This work supports the use of *L. fermentum* NCIMB 2797 in future studies as a probiotic with anti-inflammatory properties. This probiotic possesses antioxidant properties linked to FA and NO production. In addition, *L. fermentum* NCIMB 2797 secretory compounds significantly reduced inflammation in LPS-inflamed macrophages and helped to protect the colonic epithelial integrity, as demonstrated using an *in vitro* co-culture model of the colon epithelium.

Future studies should investigate further the exact probiotic mechanism(s) of action responsible for the observed anti-inflammatory effects. Other secreted probiotic compounds may yet be identified that play an important role. In addition, *in vivo* studies in animals are required to demonstrate the clinical impact of *L. fermentum* on epithelial integrity. As well, studies are needed to investigate the presence of the levels of probiotic-produced FA and NO *in vivo*. Other markers of inflammation, both pro- and anti-inflammatory, may also be investigated to provide a complete understanding of the inflammatory responses modulated by the secreted products of *L. fermentum*. Nonetheless, the presented work demonstrates the anti-inflammatory activity of *L. fermentum* NCIMB 2797 and its potential as a future biotherapeutic for the prevention and treatment of a number of health disorders.

5.6 Acknowledgments

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5.7 Conflict of interest

The authors declare that there is no financial conflict of interest related to this work.

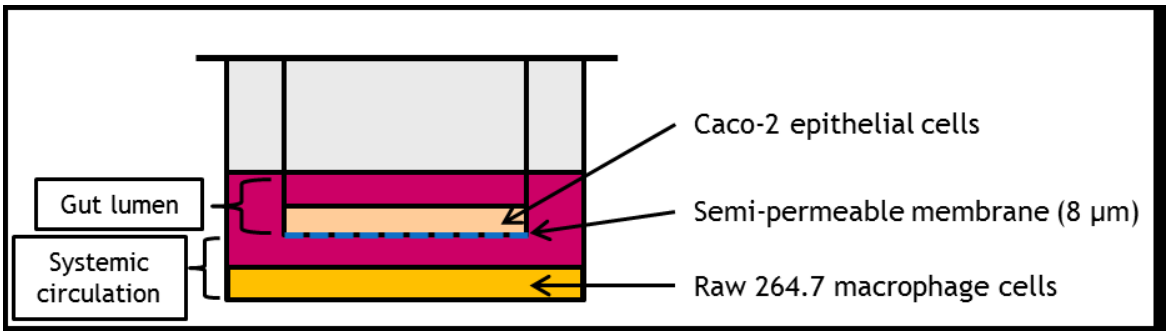

Figure 5.1: A colonic epithelium model was developed based on previous research by Tanoue *et al.* (Tanoue et al. 2008). Caco-2 cells (2 x 10^7 cells/mL) were seeded onto transwell inserts (0.3 cm^2, 8 µm pore size) and grown to represent the colon epithelium. RAW 264.7 macrophages were seeded on the basolateral side to represent the local and systemic inflammatory responses.

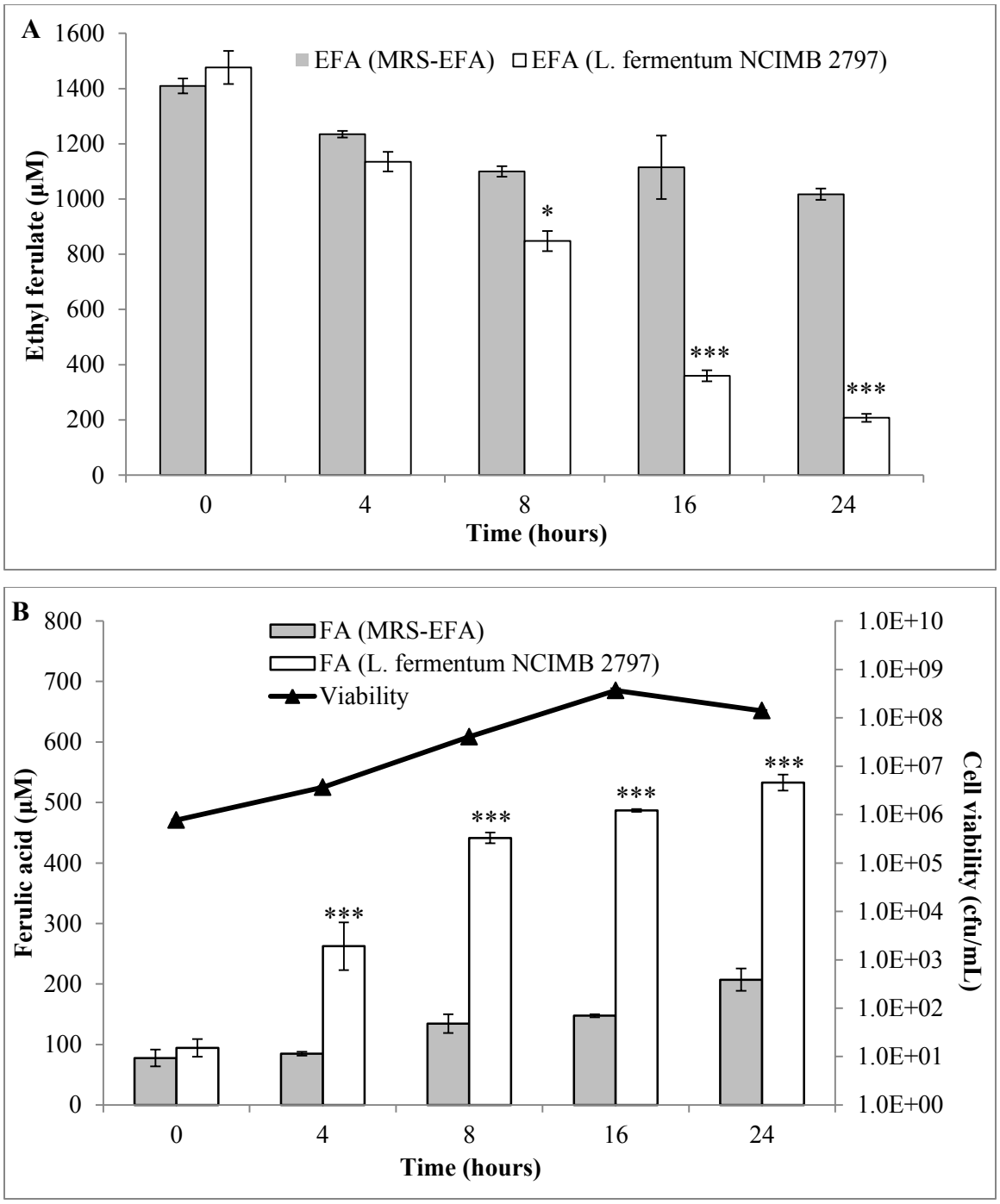

Figure 5.2: EFA hydrolysis (A) and FA production (B) by *L. fermentum* NCIMB 2797 were monitored by HPLC during its growth in MRS-EFA. Following 24 h of incubation, *L. fermentum* NCIMB 2797 demonstrated significant EFA hydrolysis with 207.51 ± 14.57 µM EFA remaining in solution and a highly significant FA production of 532.91 ± 13.14 µM. EFA hydrolysis and FA production were closely associated with the growth kinetics of *L. fermentum* NCIMB 2797. In addition, EFA hydrolysis and FA production were inversely correlated. Data is presented as mean ± SEM, n = 3 (*$p < 0.05$, ***$p < 0.0001$).

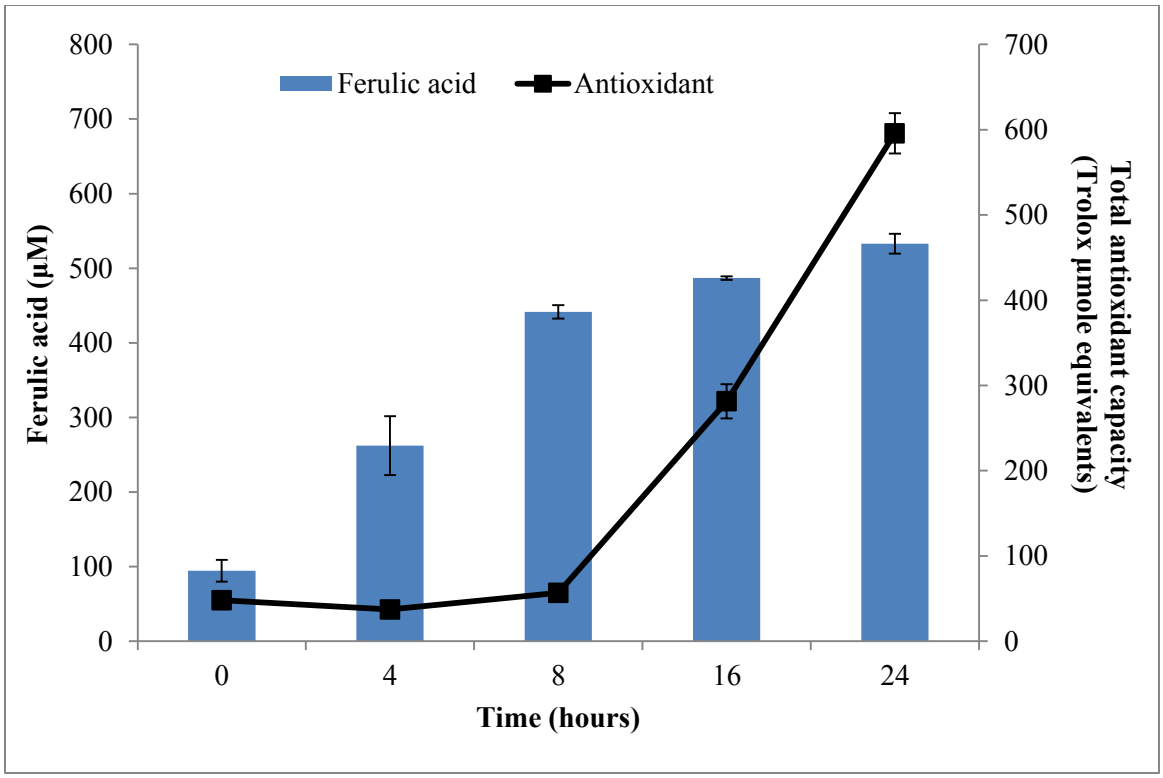

Figure 5.3: FA and antioxidant production by *L. fermentum* NCIMB 2797 were monitored during its growth in MRS-EFA. FA production showed a close association with antioxidant production (in Trolox equivalents). A FA production of 532.91 ± 13.14 µM demonstrated a total antioxidant activity of 595.71 ± 23.58 µM Trolox equivalents, following 24 h of incubation. Data is presented as mean ± SEM, n = 3.

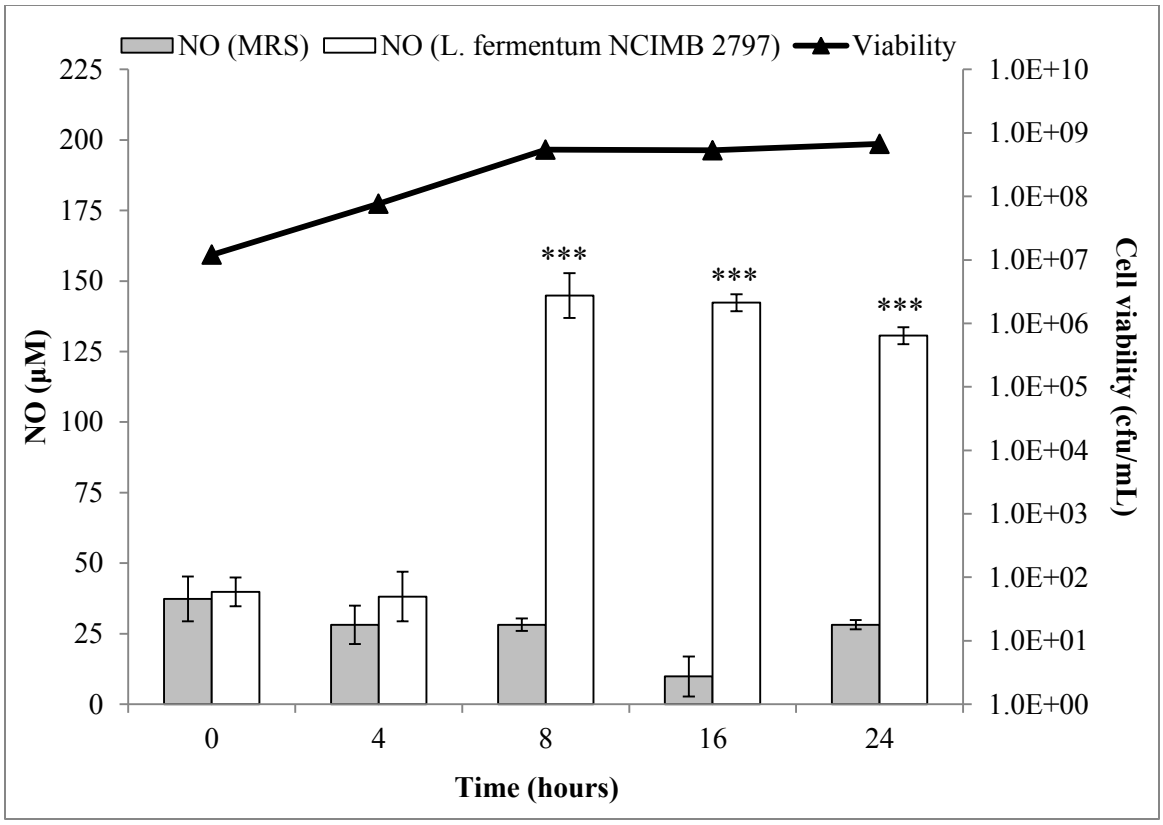

Figure 5.4: NO production by *L. fermentum* NCIMB 2797 grown in MRS broth. Following 8 h of growth, *L. fermentum* NCIMB 2797 had a NO production of 144.83 ± 7.95 µM which did not increase significantly following 16 h and 24 h of incubation. In addition, following 16 h and 24 h of incubation, the viability of *L. fermentum* NCIMB 2797 did not significantly increase as compared to the bacterial cell count following 8 h, suggesting a cell growth dependent NO production. Data is presented as mean ± SEM, n = 3 (***$p < 0.0001$).

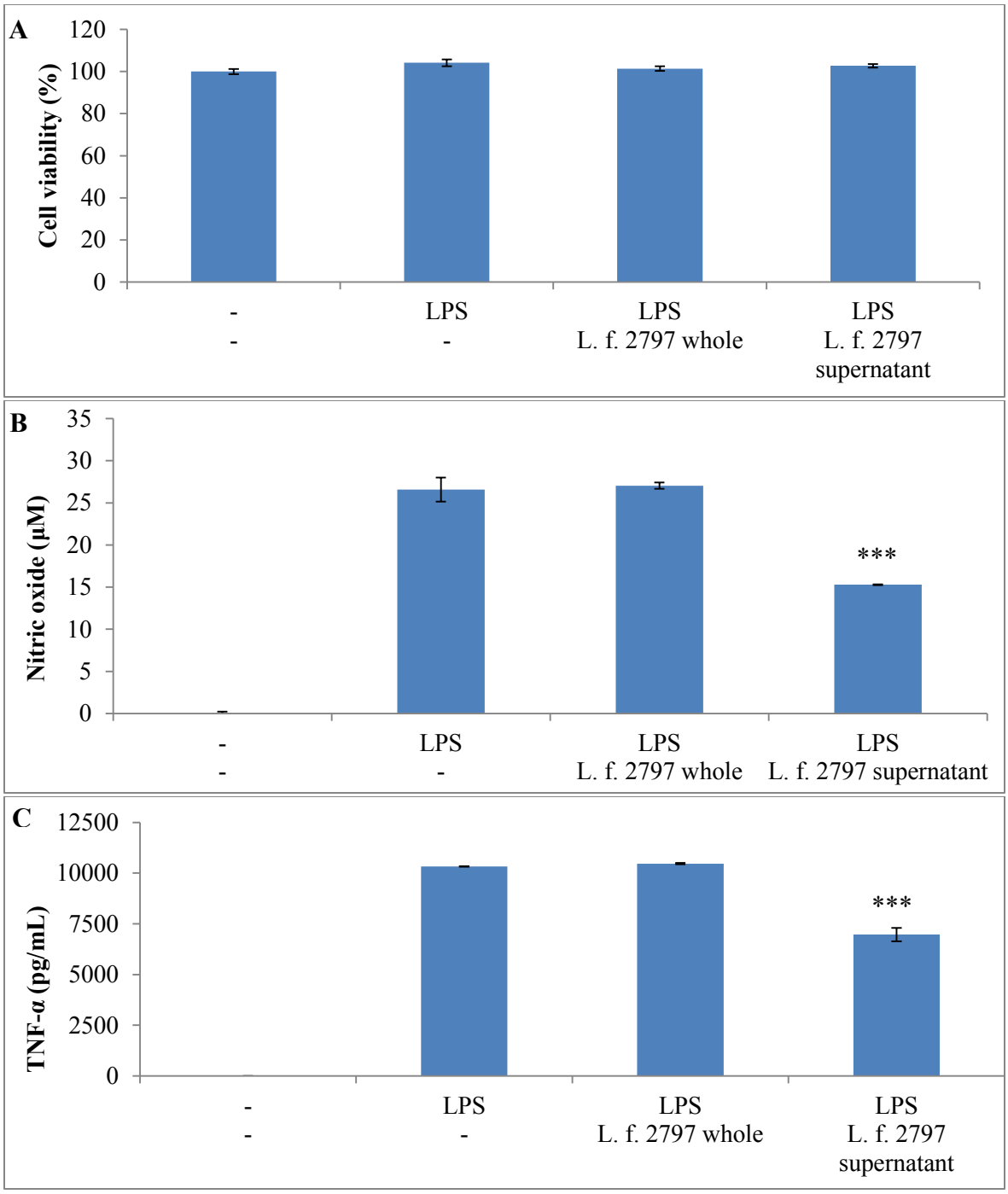

Figure 5.5: Effect of *L. fermentum* NCIMB 2797 whole cell and cell-free supernatant on LPS-stimulated macrophage viability (A), NO production (B) and TNF-α production (C) following 24 h of exposure. There was no significant loss in macrophage viability upon exposure to the probiotic whole cell and cell-free supernatant probiotic extracts. *L. fermentum* NCIMB 2797 whole cells had no significant effect on NO nor TNF-α production by LPS-activated macrophages. Conversely, treatment with *L. fermentum* NCIMB 2797 cell-free supernatant significantly decreased macrophage NO and TNF-α production. Data is presented as mean ± SEM, n = 3 (***$p < 0.0001$).

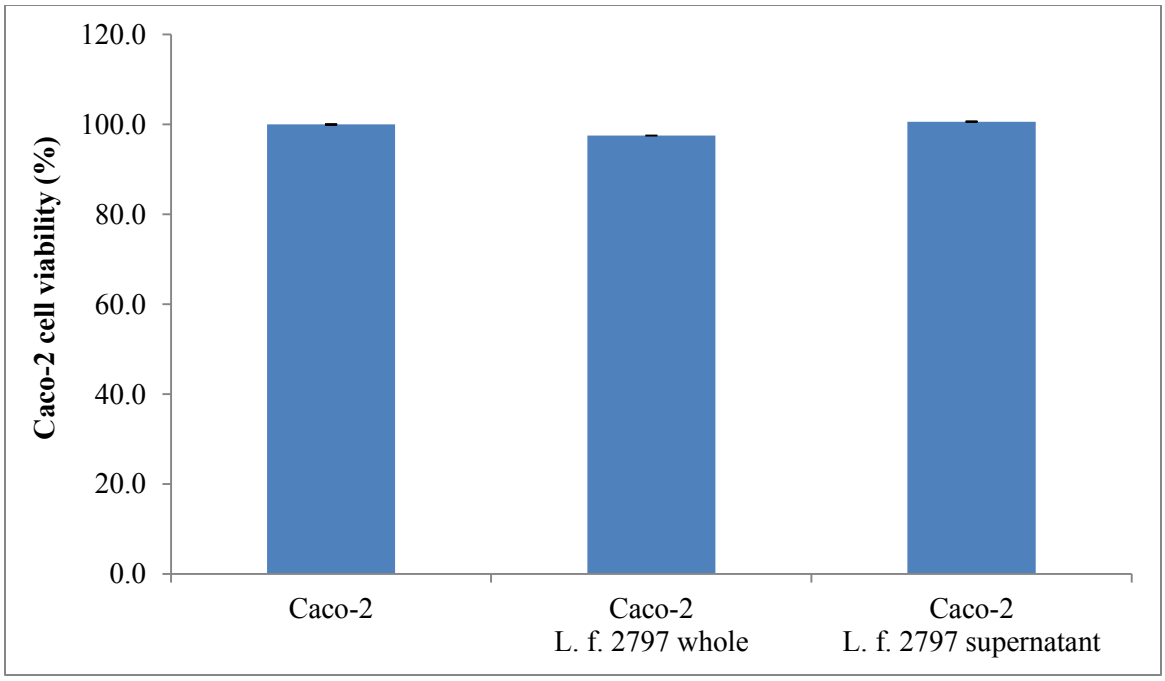

Figure 5.6: The effect of *L. fermentum* NCIMB 2797 whole cell and cell-free supernatant on Caco-2 colon epithelial cell viability when co-incubated for 24 h. The colon epithelial cells demonstrated no significant loss in cell viability when exposed to *L. fermentum* NCIMB 2797 whole cell and cell-free supernatant probiotic extracts. Data is presented as mean ± SEM, n = 3.

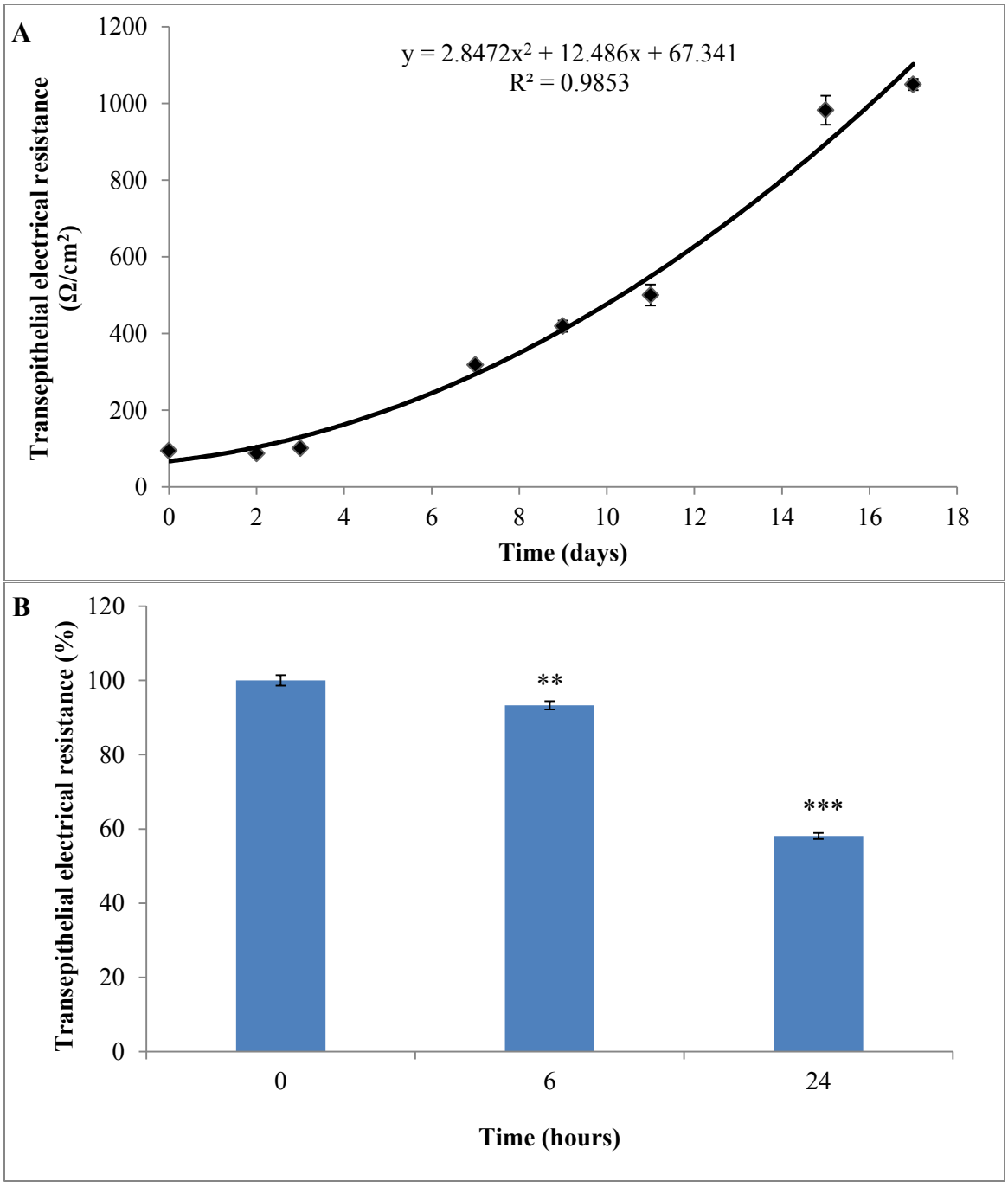

Figure 5.7: Establishment of co-culture colon epithelial model (A). Transepithelial electrical resistance (TER) was monitored over 17 days, as a measurement of epithelial integrity, and increased steadily over time, closely following a second-order equation. Impact of LPS-inflamed macrophages on TER (B) Following 6 h of incubation with the macrophages, there was a significant reduction in epithelial integrity, with 93.3 ± 1.11% integrity. An even greater significant reduction in epithelial integrity, with 58.11 ± 0.79% integrity, was demonstrated following 24 h of co-incubation. Data is presented as mean ± SEM, n = 3 (**$p < 0.01$, ***$p < 0.0001$).

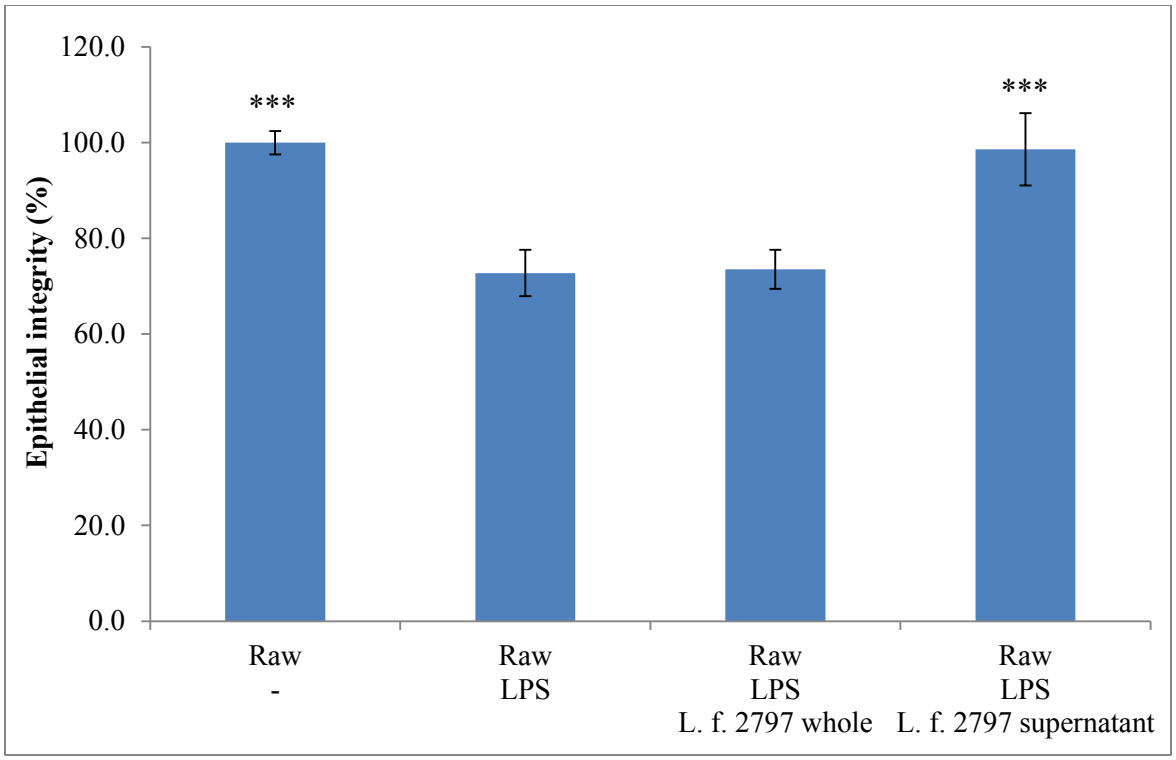

Figure 5.8: Investigation of *L. fermentum* NCIMB 2797's ability to maintain the epithelial integrity, measured by transepithelial electrical resistance (TER), of Caco-2 cells co-cultured with LPS-activated macrophages. Treatment with the probiotic whole cell extract had no significant effect on the TER of the colonic epithelium, with an epithelial integrity of 73.53 ± 4.08%, similar to the untreated control epithelium with an integrity of 72.75 ± 4.86%. Conversely, treatment with *L. fermentum* NCIMB 2797 cell-free supernatant significantly increased colonic epithelial integrity, with an integrity of 98.61 ± 7.58%. Data is presented as mean ± SEM, n = 3 (***$p < 0.0001$).

Original Research Article 4

CHAPTER 6: FERULIC ACID PRODUCING *L. FERMENTUM* AS CHOLESTEROL-LOWERING PROBIOTIC BIOTHERAPEUTICS

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Preface: MetS is also characterized by dyslipidemia. The goal of the research presented in this chapter was to investigate, *in vitro*, the cholesterol-lowering properties of *L. fermentum*. Our results indicate that *L. fermentum* can remove cholesterol from solution. The cell surface hydrophobicity of the probiotic was also determined by hydrocarbon adhesion. *L. fermentum* showed no significant impact on Caco-2, colon epithelial cell viability. In addition, colon epithelial cells pre-exposed to *L. fermentum* significantly decreased cholesterol uptake compared to the untreated cells. These results demonstrate that *L. fermentum* has important properties for the successful development of a cholesterol-lowering biotherapeutic and potentially a therapeutic for MetS.

Article submitted: **Bristish Journal of Nutrition**

6.1 Abstract

Cardiovascular disease and coronary artery disease risk, directly correlate with serum cholesterol levels and are significant health concerns of industrialized countries. Current cholesterol-lowering therapeutic modalities, including lifestyle and diet modifications, as well as statin drugs, have shortcomings, with many patients not attaining their cholesterol goals. Probiotic bacteria have great potential as biotherapeutics for a number of health disorders. *Lactobacillus fermentum* NCIMB 5221 and *L. fermentum* NCIMB 2797 have demonstrated some cholesterol-lowering potential in *in vivo* animal studies. The goal of this work is to investigate, *in vitro*, the cholesterol-lowering properties of these two probiotic strains, specifically: probiotic cholesterol assimilation, colon epithelial adhesion and inhibition of cholesterol uptake by colon epithelial (Caco-2) cells. Our results indicate that *L. fermentum* NCIMB 2797 ($p = 0.012$) and *L. fermentum* NCIMB 5221 ($p = 0.003$) assimilated cholesterol and the cell surface hydrophobicity, determined by hydrocarbon adhesion, was 70.30 ± 8.85% and 55.60 ± 2.59 % for the two strains, respectively. Both *L. fermentum* strains showed no significant impact ($p > 0.05$) on Caco-2 cell viability. Of most interest, Caco-2 pre-exposure to *L. fermentum* NCIMB 5221 significantly decreased ($p = 0.015$) cholesterol uptake, with 85.98 ± 2.07% uptake compared to the untreated cells. Similarly, *L. fermentum* NCIMB 2797 probiotic cells significantly decreased ($p = 0.019$) cholesterol uptake by Caco-2 cells, with 86.45 ± 1.71% uptake observed compared to the control cells. Additional studies are required to understand the mechanism(s) of action behind probiotic cholesterol assimilation and behind the inhibition of cholesterol uptake by colon epithelial strains. Nonetheless, these results demonstrate that *L. fermentum* NCIMB 5221

and *L. fermentum* NCIMB 2797 have important properties for the successful development of a cholesterol-lowering biotherapeutic and potentially a therapeutic for a number of other health disorders.

6.2 Introduction

The leading cause of worldwide mortality and morbidity is cardiovascular disease (CVD) (Tarride et al. 2009). According to the World Health Organization (WHO), the most common form of CVD, coronary artery disease (CAD), accounts for 7.25 million deaths, globally, per year (World Health Organization 2011). The statistics are staggering in the United States, with one in two healthy males and one in three healthy females projected to develop CAD in their lifetime (Durrington 2003). A clear link has been made between elevated serum cholesterol levels and CAD, over a range of cholesterol values and in the global population (1984; Muldoon et al. 1990; Ridker 1999). The first line of treatment for CAD involves dietary and lifestyle intervention, which has proven insufficient for the majority of individuals. There are a number of pharmacologic agents administered for the management of CAD, including statins, fibrates, niacins, cholesterol absorption inhibitors and bile acid sequestrants. Statins have a long history of safety and efficacy and, thus, have become the cornerstone of lipid-lowering therapy (Brautbar and Ballantyne 2011). Nonetheless, only 38% of patients undergoing dyslipidemia treatment, and only 18% of those with CAD, attained the goals established by the National Cholesterol Education Program (NCEP) (Pearson et al. 2000). In addition, non-compliance is an important issue with cholesterol-lowering medications, with a discontinuation rate reaching 30% following 5 years of use (Insull 1997). With the shortcomings associated with currently used cholesterol-lowering therapeutics, it is clear that additional treatment modalities should be explored.

In recent years, there has been an increasing interest in the field of probiotic research (Prakash et al. 2011c). Probiotic bacteria are defined by the WHO as “live

microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO and WHO 2001). Probiotic bacteria have shown great interest for a number of health disorders, including preventing metabolic syndrome (Bhathena et al. 2012; Tomaro-Duchesneau et al. 2013a), modulating non-alcoholic fatty liver disease (Bhathena et al. 2013), preventing and treating allergic disorders (Prakash et al. 2013), preventing periodontal diseases (Saha et al. 2012a; Saha et al. 2012b) and, of great interest, reducing lipids and cholesterol levels (Agerbaek et al. 1995; Agerholm-Larsen et al. 2000; Jones et al. 2012a; Jones et al. 2012b; Jones et al. 2013). In terms of probiotic hypocholesterolemic properties, there are several mechanisms that have been proposed as responsible for the observed effects. One mode of action involves the action of the probiotic enzyme bile salt hydrolase, described in a recent review (Jones et al. 2013). In addition, probiotic bacteria have been shown to assimilate cholesterol and incorporate cholesterol into their cellular membranes (Lye et al. 2010; Pereira and Gibson 2002), thereby lowering the levels of luminal cholesterol available for absorption. Another mechanism involves the production of ferulic acid (FA). Probiotic bacteria, including *Lactobacillus fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221 have been shown to produce large amounts of FA due to intrinsic ferulic acid esterase (FAE) activity (Tomaro-Duchesneau et al. 2012a). FA has been shown to have important cholesterol-lowering activity, suggested to act by competitively inhibiting the activity of hydroxymethylglutaryl CoA reductase in the liver and promoting the excretion of acidic sterol (Kim et al. 2003; Ou and Kwok 2004). FA-producing *L. fermentum*, in previous work, have indeed demonstrated cholesterol-lowering properties *in vivo*, although the mechanism(s) of action were not elucidated (Bhathena et al. 2009; Bhathena et al. 2013;

Tomaro-Duchesneau et al. 2013a). Indeed, i*n vitro* and *in vivo* research is required to determine the mechanism(s) by which the probiotic *L. fermentum* are decreasing cholesterol levels to allow for the formulation of a successful probiotic biotherapeutic for the prevention and treatment hypercholesterolemia and hypercholesterolemia-associated health disorders, including CVD and CAD.

The goal of the presented work is to investigate the cholesterol-lowering properties of two *L. fermentum* strains, *L. fermentum* NCIMB 5221 and *L. fermentum* NCIMB 2797. We investigate the capability of the probiotic strains to remove cholesterol from solution during bacterial growth, potentially leading to decreasing the cholesterol available for *in vivo* absorption. We then investigate the capability of the probiotic bacteria to adhere to the colon epithelium using cell surface hydrophobicity, viability and cell adhesion assays, which indicate the capability of probiotic bacteria to reside longer in the intestinal habitat. Finally, we investigate the effect of probiotic *L. fermentum* exposure on cholesterol uptake by colon epithelial cells, relevant to limiting cholesterol absorption by the intestinal epithelium *in vivo*.

6.3 Materials and methods

6.3.1 Bacterial and mammalian growth media and chemicals

De Man-Rogosa-Sharpe (MRS) broth was obtained from Fisher Scientific (Ottawa, ON, Canada). Water was purified with an EASYpure Reverse Osmosis System and a NANOpure Diamond Life Science (UV/UF) ultrapure water system from Barnstead (Dubuque, IA, USA). Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Life Technologies (Burlington, ON, Canada).

All other chemicals were of analytical or HPLC grade and purchased from commercial sources.

6.3.2 Bacterial strains and culture conditions

The probiotic bacterial strains *Lactobacillus fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221 were purchased from NCIMB (Aberdeen, Scotland, UK) and stored at -80°C in MRS broth containing 20% (v/v) glycerol. From the frozen stock, an MRS-agar plate was streaked, for isolation and to ensure purity, and incubated at 37°C with 5% CO_2 for 24 h. Following incubation, one colony from the agar plate was inoculated into 5 mL MRS broth and incubated at 37°C for 24 h. A 1% (v/v) bacterial inoculum was then used for sub-culturing and incubated at 37°C for 24 h immediately prior to use. The bacterial viabilities of *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221 were determined using standard colony counting methods. Briefly, overnight cultures were diluted in 0.85% (w/v) NaCl using 10-fold dilutions which were plated on MRS-agar incubated at 37°C and 5% CO_2 for 48 h, followed by counting colony forming units (cfu). All viability tests were performed in triplicate to ensure accuracy and reproducibility.

6.3.3 Determining probiotic cholesterol assimilation

To determine cholesterol assimilation by *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221, bacteria were incubated with cholesterol and cholesterol remaining was measured following incubation. A 1% (v/v) inoculum of overnight probiotic culture was added to MRS-broth containing at a final concentration of 100 µg/mL of water-soluble cholesterol, cholesterol-polyethylene glycol (PEG) 600 from Sigma-Aldrich (Oakville, ON, Canada). The probiotic cultures were then incubated at

37°C for 24 h. The bacterial cell viability of each probiotic was measured using standard colony counting methods. The bacterial supernatant was collected by centrifuging the probiotic cultures at 4000 rpm for 10 min at 4°C using a Napco 2028R centrifuge (Fisher Scientific, Ottawa, ON, Canada). Cholesterol concentrations were determined using a protocol modified from Rudel and Morris (Rudel and Morris 1973). Briefly, 500 µL of 33% (w/v) KOH and 1 mL absolute ethanol were added to 500 µL of the samples / standards. This solution was then vortexed for 1 min and heated at 37°C for 15 minutes, followed by cooling to room temperature. 1 mL of distilled H_2O and 1.5 mL of hexanes were added to the solution which was then vortexed for 1 min. The phases were allowed to separate. Following phase separation, 0.5 mL of the hexane layer was transferred into a glass tube. The solvent was then evaporated under nitrogen gas, after which 1 mL of 50 mg/dL o-phthalaldehyde reagent (Sigma-Aldrich, Oakville, ON, Canada) in acetic acid was added, followed by thorough mixing. Following mixing, 250 µL of concentrated H_2SO_4 was added and the solution was vortexed for 1 min. The solution was allowed to sit for 10 min and absorbance was read at 550 nm using a UV spectrophotometer Victor^{3}V 1420 Multilabel Counter (Perkin Elmer, Boston, MA). A standard curve of absorbance vs. cholesterol concentrations was generated using the concentrations: 0, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250 and 500 µg/mL cholesterol (R^2 = 0.965). The cholesterol assimilated by *L. fermentum* NCIMB 5221 and *L. fermentum* NCIMB 2797 was calculated using the following equation:

$$\textit{Cholesterol assimilated (µg/mL)} = \textit{Cholesterol (µg/mL)}_{0\,h} - \textit{Cholesterol (µg/mL)}_{24\,h}$$

In addition, the amount of cholesterol assimilated by each cfu of the *L. fermentum* probiotic strains following 24 h of incubation in the MRS-cholesterol was determined according to the following equation:

µg cholesterol assimilated / cfu = cholesterol assimilated (µg/mL) / cell viability (cfu/mL)

Samples and standards were tested with n=6 to ensure accuracy and reproducibility.

6.3.4 Measuring probiotic cell surface hydrophobicity

The probiotic cell surface hydrophobicity of *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221 were determined using a protocol modified from Rosenberg *et al.* (Rosenberg et al. 1983). PUM buffer was prepared with 22 g/L dipotassium phosphate, 7.26 g/L potassium dihydrogen phosphate, 1.8 g/L urea and 0.2 g/L magnesium sulfate. 10 mL of the overnight cultures of the two probiotic *L. fermentum* grown in MRS broth were centrifuged at 4000 rpm for 5 min at 4°C. The bacterial supernatants were discarded and the cells were washed with 10 mL of PUM buffer and centrifuged again. The wash step with PUM buffer was repeated and the bacterial pellet was resuspended in 5 mL PUM buffer. The absorbance of the cell resuspension was measured at 620 nm using a UV spectrophotometer Victor^{3}V 1420 Multilabel Counter, and was adjusted to approximately 0.70 (A_0). 5 mL of the adjusted solution was added to 1 mL toluene and the mixture was vortexed for 2 min. The phases were then allowed to settle for 1 h, following which the aqueous phase was collected and its absorbance was read at 620 nm (A_1). The percentage cell surface hydrophobicity (H %) was determined according to the following equation:

$$H\ \% = (1 - A_1/A_0) \times 100$$

The experiment was performed in triplicate and with triplicate measurements to ensure the reproducibility and precision of the results.

6.3.5 Investigating the effect of *L. fermentum* on colon epithelial cell viability

The effect of *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221 on colon epithelial cell viability was investigated. Human colon epithelial cells, Caco-2, were purchased from Cedarlane Laboratories (Burlington, ON, Canada) and maintained in DMEM with 10% (v/v) FBS, with no antibiotics, and incubated at 37°C and 5% CO_2 with passages every 5-7 days. The cells were sub-cultured into 12-well plates, at a cell concentration of 1 x 10^6 cells/well, determined by cell counting using a haemocytometer and trypan blue. The Caco-2 cells were allowed to attach for 48 h prior to the assay. The *L. fermentum* probiotic overnight cultures were centrifuged at 4000 rpm for 5 min at 4°C. The probiotic bacterial pellets were washed with phosphate buffered saline (PBS) and resuspended in DMEM, obtaining 1.40 x 10^8 cfu/mL of *L. fermentum* NCIMB 5221 and 3.27 x 10^7 cfu/mL of *L. fermentum* NCIMB 2797. 500 µL of the probiotic suspensions were added to the wells containing the Caco-2 cells which were incubated for 24 h at 37°C with 5% CO_2. DMEM with no probiotic was used as a control treatment. Viability of the Caco-2 cells was determined using a CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) from Promega (Madison, WI, USA). The manufacturer's instructions were followed and the absorbance of formazan was read at 490 nm using a UV spectrophotometer Victor^{3}V 1420 Multilabel Counter. The assay was performed in triplicate to ensure accuracy and reproducibility.

6.3.6 Determining probiotic cell attachment to the colon epithelium

The probiotic *L. fermentum* attachment to the colon epithelium was investigated using Caco-2 cells. The human colon epithelial cells were sub-cultured into 12-well plates at a cell concentration of 1 x 10^6 cells/well and incubated for 48 h at 37°C and 5% CO_2 to allow cell attachment. The *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221 overnight cultures were centrifuged at 4000 rpm for 5 min at 4°C. The bacterial pellets were washed with PBS and resuspended in DMEM and 500 µL of each treatment were added to each well, with DMEM alone as a control. The probiotic-Caco-2 cells were incubated at 37°C and 5% CO_2 with probiotic attachment measured following 0, 3 and 6 h of co-incubation. To measure probiotic attachment, the Caco-2 cells containing the probiotic cells were washed 3 times with PBS. 500 µL of 0.1% (v/v) Triton X-100 were added to each well and the cells were shaken at 150 rpm for 15 min using a rotary shaker (LW Scientific, Lawrenceville, GA, USA). The resulting solutions were serially diluted and plated on MRS agar plates which were then incubated at 37°C and 5% CO_2 for 48 h followed by colony counting to determine the percent and number of probiotic cells attached to the colon epithelial cells, using triplicate experiments.

6.3.7 Preparation of cholesterol micelles

Cholesterol micelles were prepared for the *in vitro* studies investigating cholesterol uptake by Caco-2 cells. The constituents were purchased from Sigma-Aldrich (Oakville, ON, Canada) and consisted of 1.65 mM sodium taurocholate (stock in ethanol), 0.5 mM cholesterol (stock in chloroform), 0.25 mM oleic acid (stock in ethanol) and 0.125 mM monoolein (stock in chloroform), listed in **Table 6.1**. The components were mixed and the solvents were evaporated under nitrogen gas. The resulting deposit

was resuspended in DMEM and sonicated for 1 h at 60 pulses/min (Branson Ultrasonics, CT, USA). Cholesterol in solution was measured by combining 100 µL of sample with 2 mL of 50 mg/dL o-phthalaldehyde reagent and 1 mL of concentrated H_2SO_4. The solutions were thoroughly mixed and were incubated at room temperature for 10 min, and the absorbance was read at 570 nm using a UV spectrophotometer Victor^{3}V 1420 Multilabel Counter. The cholesterol micellar solution was serially diluted to generate a standard curve for quantification with the cholesterol concentrations of 0, 0.0313, 0.0625, 0.125, 0.250, 0.500 mM (R^2= 0.999).

6.3.8 Investigating *L. fermentum* inhibition of cholesterol uptake by colon epithelial cells

The effect of the *L. fermentum* probiotic bacteria on cholesterol uptake by Caco-2 colon epithelial cells was investigated. Caco-2 cells were sub-cultured into 24-well plates at a cell concentration of 5 x 10^5 cells/well and were incubated for 48 h at 37°C and 5% CO_2 to permit cell attachment. 1 mL of the overnight cultures of *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221 were centrifuged at 4000 rpm for 5 min at 4°C and the obtained bacterial pellets were washed with PBS and then resuspended in 10 mL DMEM and diluted 10-fold. The bacterial concentrations were 1.0 x 10^7 ± 2.52 x 10^6 cfu/mL for *L. fermentum* NCIMB 5221 and 5.4 x 10^7 ± 3.51 x 10^6 cfu/mL for *L. fermentum* NCIMB 2797, as determined by standard colony counting methods, of which 500 µL were added to each well containing attached Caco-2 cells. DMEM with no probiotic was added as a control. The cells were incubated for 24 h at 37°C and 5% CO_2. Following probiotic incubation, the cells were washed 3 times with PBS. The cholesterol (0.5 mM) micelles were then added to the Caco-2 cells and incubated for 4 h at 37°C and

5% CO_2. Following incubation, the cell-supernatant containing the cholesterol micelles was collected and the cholesterol remaining was quantified according to the method aforementioned. The experiment was performed with n=12 to ensure accuracy and reproducibility.

6.3.9 Statistical analysis

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, USA). Linear regression was performed for generating standard curves. Statistical comparisons were carried out using the general linear model, followed by multiple comparisons of the means using Tukey's post-hoc analysis. Statistical significance was set at $p < 0.05$ and p-values less than 0.01 were considered highly significant.

6.4 Results

6.4.1 *L. fermentum* cholesterol assimilation

The capability of the probiotic *L. fermentum* to assimilate cholesterol in media during growth was determined. The viability of both *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221 were not significantly ($p > 0.05$) affected by the presence of cholesterol in the growth media, as observed in **Figure 6.1A**. *L. fermentum* NCIMB 5221 had a viability of $8.33 \times 10^8 \pm 1.00 \times 10^9$ cfu/mL with cholesterol and $1.00 \times 10^9 \pm 2.52 \times 10^8$ cfu/mL without cholesterol. Similarly, *L. fermentum* NCIMB 2797 had a viability of $5.13 \times 10^9 \pm 5.33 \times 10^8$ cfu/mL with cholesterol and $5.40 \times 10^9 \pm 3.51 \times 10^8$ cfu/mL

without cholesterol. Both *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221 were successful at assimilating cholesterol during 24 h of incubation in cholesterol-containing MRS, as seen in **Figure 6.1B**. *L. fermentum* NCIMB 5221 assimilated 327.8 ± 62.1 µg of cholesterol (p = 0.003) and *L. fermentum* NCIMB 2797 assimilated 277.8 ± 62.1 µg of cholesterol (p = 0.012). In addition, *L. fermentum* NCIMB 5221 (0.0393 ± 0.0048 pg cholesterol/cfu) had significantly better cholesterol assimilation ($p < 0.001$) than *L. fermentum* NCIMB 2797 (0.0054 ± 0.0014 pg cholesterol/cfu) when considering the probiotic cell counts, as shown in **Table 6.2**.

6.4.2 Probiotic *L. fermentum* cell surface hydrophobicity

Probiotic cell surface hydrophobicity as an indicator of probiotic cell attachment was determined for *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221, using phase separation. The cell surface hydrophobicity of *L. fermentum* NCIMB 5221 was determined as 55.60 ± 2.59 % and the cell surface hydrophobicity of probiotic *L. fermentum* NCIMB 2797 was determined as 70.30 ± 8.85%, as indicated in **Figure 6.2**.

6.4.3 Effect of *L. fermentum* on colon epithelial cell viability

Upon co-incubation of the probiotic *L. fermentum* strains with human colon epithelial cells, the effect on the viability of the mammalian cells was determined. Both *L. fermentum* NCIMB 5221 and *L. fermentum* NCIMB 2797 did not significantly ($p > 0.05$) impact the Caco-2 cell viability following 24 h of co-incubation, as observed in **Figure 6.3**. *L. fermentum* NCIMB 5221 treated Caco-2 cells had a viability of 96.85 ± 1.54% and *L. fermentum* NCIMB 2797 cells had a viability of 95.68 ± 1.55% as compared to control untreated Caco-2 cells.

6.4.4 Attachment of *L. fermentum* cells to colon epithelial cells

The probiotic attachment to human colon epithelial cells was determined for *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221 upon probiotic co-incubation with the Caco-2 cells, with the results presented in **Figure 6.4**. *L. fermentum* NCIMB 5221 was attached following 3 h of incubation, with 3.87 x 10^6 ± 5.83 x10^5 cfu/well attached, representing 2.77 ± 0.42% of the probiotic cells added to the colon epithelial cells. Following 6 h of incubation, *L. fermentum* NCIMB 5221 had 5.30 x 10^6 ± 6.45 x10^5 cfu/well attached to the colon epithelial cells, representing 3.79 ± 0.46% of the added probiotic cells. Similarly, *L. fermentum* NCIMB 2797 cells were attached following 3 h of co-incubation with 1.32 x 10^6 ± 6.67 x10^4 cfu/well attached (~5.5 bacteria per Caco-2 cell), or 4.03 ± 0.20% of the probiotic cells added to the colon epithelial cells. Following 6 h of incubation, *L. fermentum* NCIMB 2797 had 1.42 x 10^6 ± 1.33 x10^5 cfu/well attached (~1.6 bacteria per Caco-2 cell) to the colon epithelial cells, representing 4.34 ± 0.41% of the added bacterial cells. A greater percentage of probiotic *L. fermentum* NCIMB 2797 cells were attached to the Caco-2 cells, compared to *L. fermentum* NCIMB 5221. However, the *L. fermentum* NCIMB 5221 cells attached to the Caco-2 cells in greater cell counts than *L. fermentum* NCIMB 2797.

6.4.5 *L. fermentum* inhibition of colon epithelium cholesterol uptake

The effect of *L. fermentum* on the uptake of cholesterol by colon epithelial cells was investigated. Caco-2 cells with no exposure to probiotic bacteria absorbed 29.97 ± 1.16 µg of cholesterol following 4 h of incubation with the cholesterol micelles. Pre-exposure by the probiotic *L. fermentum* strains had a significant impact on cholesterol uptake, as presented in **Figure 6.5**. Following 4 h of incubation with the cholesterol

micelles, subsequent to exposure by *L. fermentum* NCIMB 5221, the colon epithelial cells had significantly lower (p = 0.015) cholesterol uptake of 85.98 ± 2.07% compared to that uptaken by the untreated control cells. Similarly, pre-exposure to *L. fermentum* NCIMB 2797 significantly decreased (p = 0.019) colon epithelium cholesterol uptake, with 86.45 ± 1.71% of the control cells' uptake.

6.5 Discussion

Elevated serum cholesterol levels are directly associated with the risk of developing CAD. Recent studies have turned to probiotic bacteria as these have demonstrated important hypocholesterolemic properties (Bhathena et al. 2013; du Toit et al. 1998; Huang and Zheng 2010; Jones et al. 2013; Yoon et al. 2011). Probiotic bacteria are naturally occurring in foods such as milk and yoghurt, are inexpensive, are generally regarded as safe (GRAS) and are free of long-term negative side-effects (Branton et al. 2011). The probiotic bacteria *L. fermentum* have shown promising results for a number of applications, including as adjuvants for vaccines (Olivares et al. 2007), as inflammatory bowel disease therapeutics (Peran et al. 2007), as metabolic syndrome treatment options (Bhathena et al. 2012; Tomaro-Duchesneau et al. 2013a), as capable of inhibiting dental caries and periodontitis progression (Saha et al. 2012a) and as non-alcoholic fatty liver disease treatments (Bhathena et al. 2013). A number of *L. fermentum* probiotic strains have demonstrated intrinsic FAE activity, characterized by the production of FA (Abeijon Mukdsi et al. 2012; Bhathena et al. 2007; Bhathena et al. 2008; Hole et al. 2012; Tomaro-Duchesneau et al. 2012a; Tomaro-Duchesneau et al. 2012b), which has been shown to have important cholesterol-lowering effects (Kim et al. 2003). Since the oral delivery of

free FA is limited due to its rapid absorption in the jejunum leading to its rapid excretion from the body (Spencer et al. 1999; Zhao et al. 2003), the delivery of FA-producing probiotic bacteria enhances the gastrointestinal bioavailability of FA, giving rise to cholesterol-lowering effects. The aim of this study was to investigate the properties and mechanisms of action of two *L. fermentum* probiotic strains, *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221, demonstrated to have cholesterol-lowering capabilities in previously published *in vivo* studies (Bhathena et al. 2013; Tomaro-Duchesneau et al. 2013a).

Previous groups have demonstrated that certain probiotic bacteria can assimilate cholesterol when grown in its presence, contributing to cholesterol-lowering effects *in vivo* (Lin and Chen 2000; Tahri et al. 1996). Based on this principle, we investigated the two *L. fermentum* strains for cholesterol assimilation activities when grown in MRS broth containing water-soluble cholesterol. Both *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221 successfully assimilated significant quantities of cholesterol following 24 h of incubation, indicated by decreases in the cholesterol remaining in the culture supernatant. This could be due to a number of mechanisms, including probiotic-cholesterol co-precipitation, however this is normally observed when oxgall or bile is supplemented in the growth media (Gilliland et al. 1985). It is suggested that the observed probiotic assimilation *in vitro* would allow for a decreased availability of cholesterol for absorption into the blood when probiotic bacteria are present in the gastrointestinal tract, providing a hypocholesterolemic effect.

In terms of probiotic properties required for efficient cholesterol-lowering, adhesion to human intestinal epithelial cells is critical. Adhesion to intestinal epithelial

cells ensures an extended probiotic transit time in the gastrointestinal tract, upon oral administration, promoting efficient probiotic cholesterol-lowering effects *in vivo*. Adhesion to epithelial cells involves a number of processes, some specific and non-specific, including hydrophobic processes, van der Waals and electrostatic forces. In general, strains that adhere well to hydrocarbons are considered hydrophobic and those adhering poorly are hydrophilic (Duary et al. 2011). As such, we investigated the cell surface hydrophobicity of *L. fermentum* NCIMB 5221 and *L. fermentum* NCIMB 2797 using a method based on microbial adhesion to hydrocarbons, hexanes in this study. Both of the *L. fermentum* strains were demonstrated to have significant cell surface hydrophobicity, of 55.60 ± 2.59 % for *L. fermentum* NCIMB 5221 and 70.30 ± 8.85% for *L. fermentum* NCIMB 2797. Compared to previously studied probiotic bacteria, both of the *L. fermentum* strains have relatively high cell surface hydrophobicity based on the assay used, suggesting great potential for cellular adhesion (Duary et al. 2011; Ocana et al. 1999). It is important to note that other cell surface hydrophobicity assays could be performed using different hydrocarbons, such as xylene and toluene, as the surface hydrophobicity values differ depending on the solvent used (Duary et al. 2011; Ocana et al. 1999). However, the use of hexanes as a hydrocarbon source provides an initial indication of the potential attachment of the probiotic bacteria to the colon epithelium.

For a better indication of the potential for intestinal epithelial adhesion of the two probiotic *L. fermentum* strains following oral administration, we co-incubated the probiotic cells with human colon epithelial cells. Upon co-incubation of the bacterial and mammalian cells there was no significant difference in colon epithelial cell viability, demonstrating that *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221 were not

toxic to the cell monolayers. In terms of adhesion, following 3 h of co-incubation of the probiotic *L. fermentum* strains with the Caco-2 cells, there was significant probiotic adhesion to the monolayers, with only a slight increase by 6 h. Candela *et al.* defined bacterial cells as adhesive when 5-40 bacterial cells adhere to one Caco-2 cell, with less than 5 bacterial cells adhering to one Caco-2 cell considered as non-adhesive (Candela et al. 2005). By this definition, *L. fermentum* NCIMB 5221 (5.5 cfu/Caco-2 cell) is considered as an adhesive probiotic strain, but *L. fermentum* NCIMB 2797 (1.55 cfu/Caco-2 cell) is considered as non-adhesive. Interestingly, *L. fermentum* NCIMB 2797, as aforementioned, had a greater cell surface hydrophobicity, suggesting that *L. fermentum* NCIMB 5221 adherence with Caco-2 cells may be based more on specific binding rather than through non-specific electrostatic interactions.

Recent work has demonstrated that certain probiotic Lactobacilli can decrease cholesterol uptake by Caco-2 colon epithelial cells (Yoon et al. 2013). Both *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221 significantly decreased cholesterol uptake by Caco-2 cells, when the latter cells were exposed to the probiotic prior to cholesterol micelle addition. A cholesterol uptake inhibition of a moderate 14% was observed for both of the probiotic strains, as previous studies by Yoon *et al.* observed cholesterol uptake inhibition up to 40-45% for certain probiotic strains (Yoon et al. 2013). This higher inhibition of cholesterol uptake may be attributed to the presence of bile salt hydrolase activity in the probiotic strains investigated (Jones et al. 2013). The activity of the BSH-active strains on the inhibition of cholesterol uptake, as proposed by Yoon *et al.*, may be related to the downregulation of Niemann-Pick C1-like (NPC1L1) (Yoon et al. 2013) and the upregulation of ATP-binding cassette sub-family G members 5 and 8

(ABCG5/G8) and Liver X receptor (LXR) (Yoon et al. 2011). Further investigations are required into the mechanisms behind the observed inhibition of Caco-2 cholesterol-uptake by pre-exposure to *L. fermentum* NCIMB 5221 and *L. fermentum* NCIMB 2797.

The results presented here demonstrated that *L. fermentum* NCIMB 5221 and *L. fermentum* NCIMB 2797 have important properties necessary, on top of the previously demonstrated FA production, for the successful development of a cholesterol-lowering biotherapeutic. Both probiotic strains were capable of assimilating cholesterol in solution, suggesting rapid cholesterol excretion from the gastrointestinal tract upon *in vivo* probiotic oral administration. In addition, both *L. fermentum* had some degree of colon epithelial adhesion, suggesting a longer residence time in the gastrointestinal tract. Of greater importance is the fact that *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221 both decreased cholesterol uptake by human colon epithelial cells, which would lead to decreased systemic cholesterol levels *in vivo.* Additional *in vitro* studies are required to better understand the mechanism(s) of action behind probiotic cholesterol assimilation and behind the inhibition of cholesterol uptake by colon epithelial strains. However, both of the *L. fermentum* strains investigated in this work have great potential for the future development of a successful probiotic biotherapeutic for the prevention and treatment of hypercholesterolemia and a number of other associated health disorders.

6.6 Acknowledgments

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6.7 Conflict of interest

The authors declare that there is no financial conflict of interest related to this work.

Table 6.1: Constituents of the cholesterol micelles.

Component	Role	Concentration in micellar solution (mM)
Sodium taurocholate	Bile salt	1.650
Oleic acid	Fatty acid	0.250
Monoolein	Fatty acid	0.125
Cholesterol	Cholesterol	0.500

Table 6.2: Cholesterol assimilation by *L. fermentum* NCIMB 5221 and *L. fermentum* NCIMB 2797 incubated in MRS containing 100 μg/mL cholesterol for 24 h.

	L. fermentum **NCIMB 5221**	***L. fermentum*** **NCIMB 2797**
Viability (cfu/mL)	$8.33 \times 10^8 \pm 2.03 \times 10^8$	$5.13 \times 10^9 \pm 0.53 \times 10^9$
Cholesterol assimilated (μg)	327.8 ± 62.1	277.8 ± 62.1
pg of cholesterol assimilated per cfu	$0.0393 \pm 0.0048^{***}$	0.0054 ± 0.0014

Data is presented as mean ± SEM, n = 6, ***$p < 0.001$ vs. *L. fermentum* NCIMB 2797.

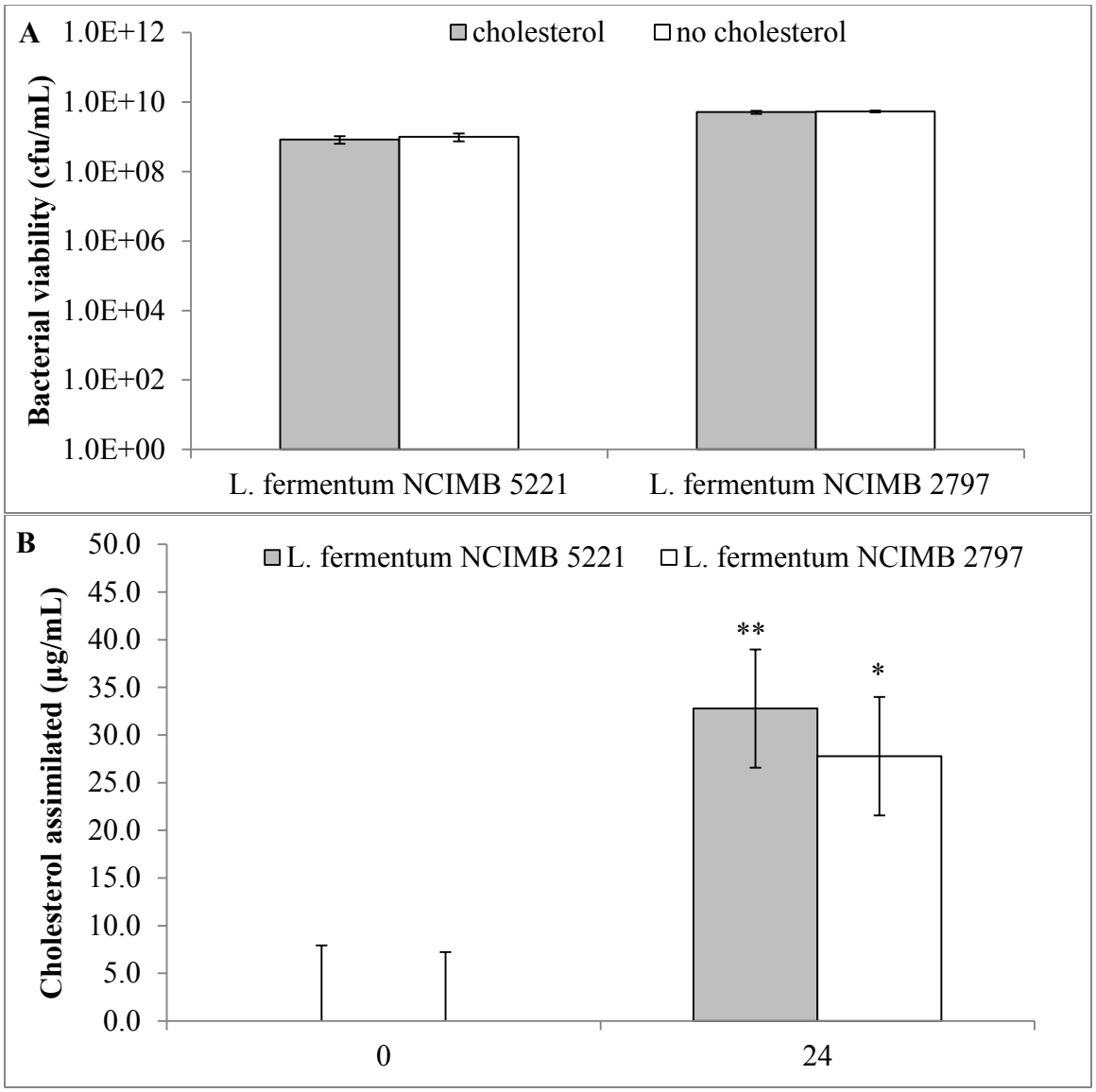

Figure 6.1: Cholesterol assimilation by *L. fermentum* NCIMB 5221 and *L. fermentum* NCIMB 2797. (A) The viability of both *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221 were not significantly ($p > 0.05$) affected by the presence of cholesterol in the growth media. (B) Both *L. fermentum* NCIMB 2797 ($p = 0.012$) and *L. fermentum* NCIMB 5221 ($p = 0.003$) were successful at assimilating cholesterol during 24 h of incubation in cholesterol-containing MRS. Data is expressed as means ± SEM, n = 6.

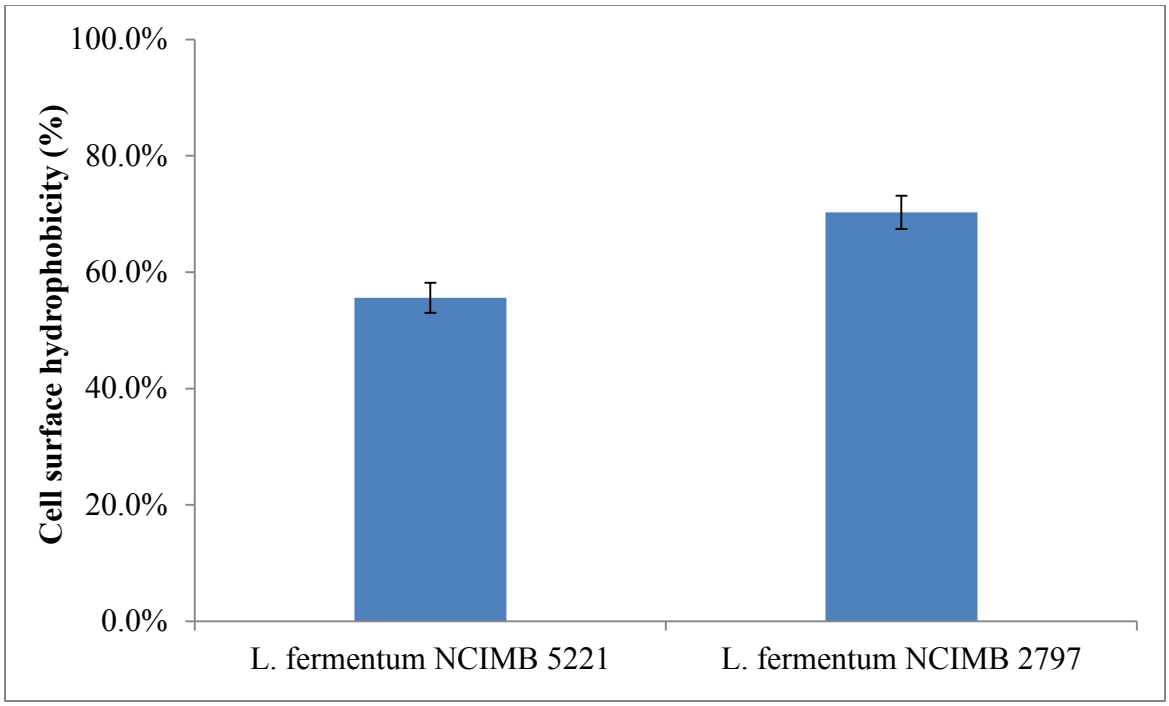

Figure 6.2: Probiotic cell surface hydrophobicity was determined for *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221, by a phase separation method. The cell surface hydrophobicity of *L. fermentum* NCIMB 5221 was 55.60 ± 2.59 % and *L. fermentum* NCIMB 2797 was 70.30 ± 8.85%. Data is presented as mean ± SEM, n = 3.

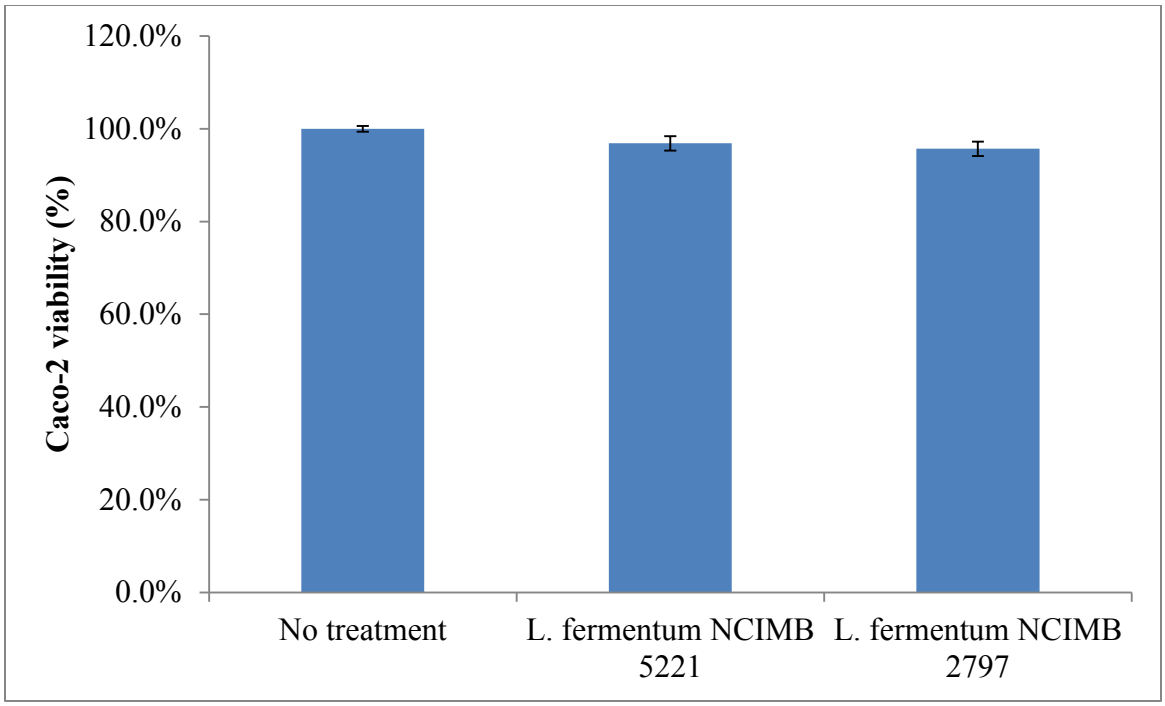

Figure 6.3: Effect of probiotic *L. fermentum* NCIMB 5221 and *L. fermentum* NCIMB 2797 on Caco-2 epithelial cell viability. Both *L. fermentum* NCIMB 5221 (96.85 ± 1.54%) and *L. fermentum* NCIMB 2797 (95.68 ± 1.55%) did not significantly ($p > 0.05$) impact the Caco-2 cell viability following 24 h of co-incubation as compared to the untreated control cells. Data is presented as mean ± SEM, n = 3.

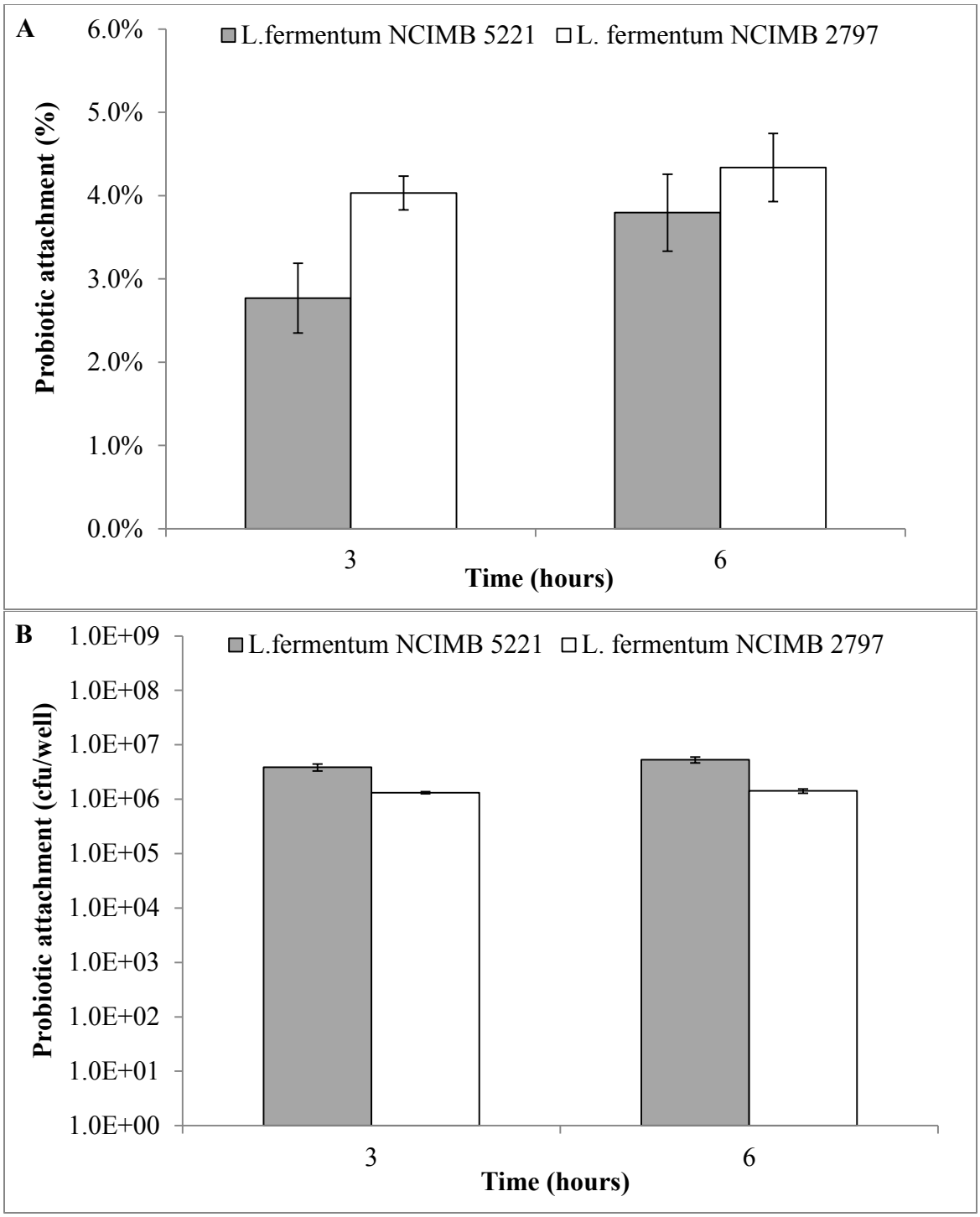

Figure 6.4: Probiotic attachment to Caco-2 cells. (A) A greater percentage of probiotic *L. fermentum* NCIMB 2797 (4.34 ± 0.41%) cells were attached to the Caco-2 cells, following 6 h of co-incubation, as compared to *L. fermentum* NCIMB 5221 (3.79 ± 0.46%). (B) In terms of cell counts, following 6 h of co-incubation the *L. fermentum* NCIMB 5221 ($5.30 \times 10^6 \pm 6.45 \times 10^5$ cfu/well) cells attached to the Caco-2 cells in greater cell counts than *L. fermentum* NCIMB 2797 ($1.42 \times 10^6 \pm 1.33 \times 10^5$ cfu/well). Data is presented as mean ± SEM, n = 3.

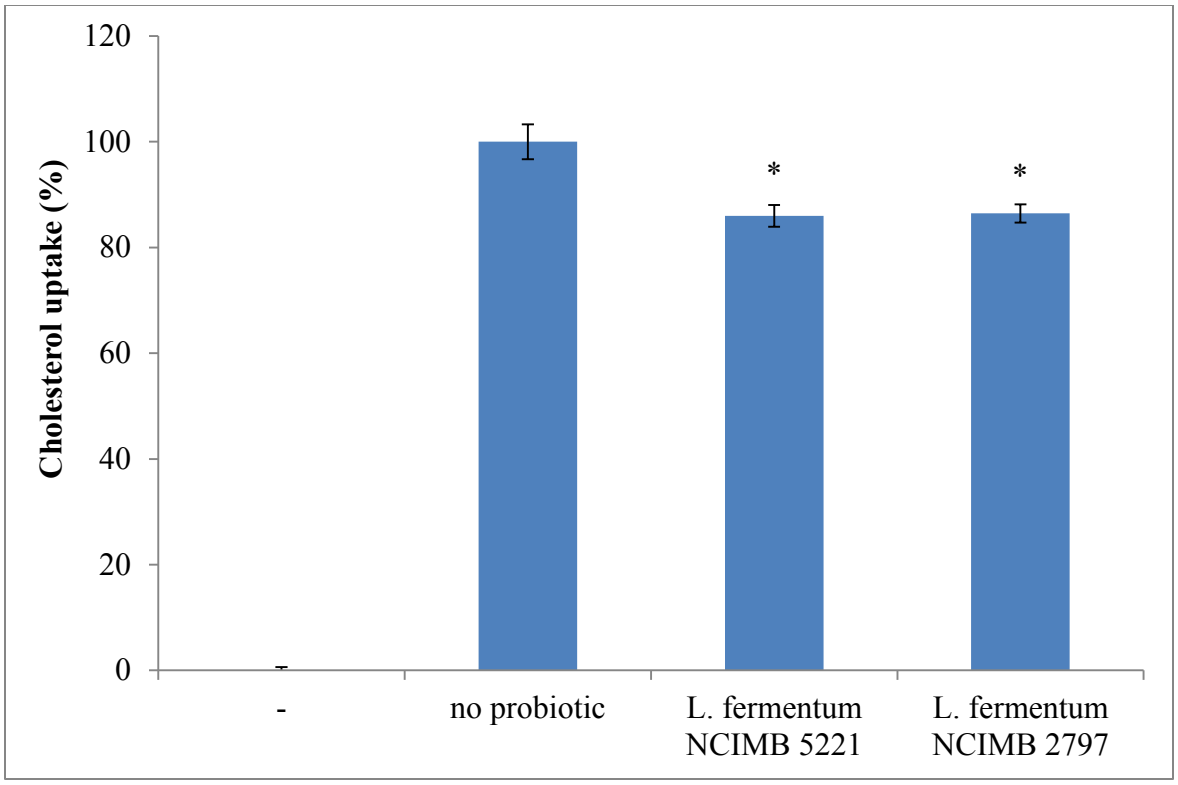

Figure 6.5: Probiotic inhibition of Caco-2 colon epithelial cholesterol uptake. Caco-2 pre-exposure for 24 h to *L. fermentum* NCIMB 5221 significantly decreased ($p = 0.015$) cholesterol uptake over 4 h, with 85.98 ± 2.07% of the untreated cells' uptake. Similarly, *L. fermentum* NCIMB 2797 probiotic cells significantly decreased ($p = 0.019$) cholesterol uptake, with 86.45 ± 1.71% of the uptake observed with the control cells. Data is expressed as means ± SEM, n = 12.

Original Research Article 5

CHAPTER 7: EFFECT OF ORALLY ADMINISTERED MICROENCAPSULATED FA-PRODUCING *L. FERMENTUM* ON MARKERS OF METABOLIC SYNDROME: AN *IN VIVO* ANALYSIS

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Preface: Following characterization, *in vitro*, of the probiotic formulation for its potential as a MetS therapeutic, the aim of this chapter was to investigate the role of alginate-polylysine alginate microencapsulated *L. fermentum* to modulate markers of MetS *in vivo*, using a high-fat fed $BioF_1B$ Golden Syrian hamster model. We investigated the effects on the adiposity index, serum insulin, insulin resistance, glycosylated albumin, serum leptin, serum uric acid, serum total cholesterol, serum esterified cholesterol and free fatty acid levels in the treated animals. This research indicates that the probiotic *L. fermentum* formulation may significantly delay the onset of insulin resistance, hyperglycemia, hyperinsulinemia, dyslipidemia and obesity, indicating a lower risk of diabetes and cardiovascular disease.

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7.1 Abstract

Ferulic acid (FA) is a natural phenolic acid produced by a number of lactic acid bacteria. FA has a number of beneficial properties, including: antioxidant activity, anti-tumorigenic properties and cholesterol-lowering capabilities. Our group has previously screened Lactobacilli for FA production, and selected *L. fermentum* ATCC 11976 (*L.f.* 11976) as one of the best producers. Alginate-polylysine-alginate (APA) microencapsulation has proven successful for the oral delivery of this strain to the colon, where production of FA is greatest. The aim of this study was to investigate the role of APA microencapsulated *L.f.* 11976 to modulate markers of metabolic syndrome. The antioxidant activity, as a potential mechanism of action to treat/prevent metabolic syndrome, of free and microencapsulated *L.f.* 11976 was quantified. A high-fat fed $BioF_1B$ Golden Syrian hamster model was used to investigate the effects of orally administered microencapsulated *L.f.* 11976 on markers of metabolic syndrome. Results demonstrate that the microencapsulated *L.f.* 11976 formulation reduced the adiposity index (p = 0.0014), serum insulin (p = 0.0042), insulin resistance (p = 0.0096), glycosylated albumin (p = 0.00013), serum leptin (p = 0.048), serum uric acid (p = 0.025) serum total cholesterol (p = 0.024), serum esterified cholesterol (p = 0.0328) and free fatty acid (p = 0.029) levels in the treated animals. This research indicates that the probiotic *L.f.* 11976 microencapsulated formulation may significantly delay the onset of insulin resistance, hyperglycemia, hyperinsulinemia, dyslipidemia and obesity, indicating a lower risk of diabetes and cardiovascular disease. We propose and discuss the potential mechanism(s) of action by which FA is acting. With these in mind, further *in vivo* studies

are required to validate the therapeutic effects of the formulation and to investigate the mechanism(s) of action by which the probiotic formulation is acting.

7.2 Introduction

Ferulic acid [*trans*-4-hydroxy-3-methoxycinnamic acid] (FA) is a naturally found phenolic acid that is abundantly bound to most of the foods consumed by humans (bran, whole grains, fruits, vegetables, tea, coffee). This molecule is a potent antioxidant able to neutralize free radicals, such as Reactive Oxygen Species (ROS) (Rice-Evans et al. 1996) which have been implicated in DNA damage (Lombard et al. 2005a), cancer (Hu et al. 2011), accelerated cell aging (Ishii et al. 1998), obesity (Sonta et al. 2004) and type 2 diabetes mellitus (T2DM) (Donath and Shoelson 2011; Sonta et al. 2004). A number of gastrointestinal tract (GIT) bacterial strains have been shown to produce feruloyl esterase (FAE) (Bhathena et al. 2007; Bhathena et al. 2008; Bhathena et al. 2009; Lai et al. 2009a; Tomaro-Duchesneau et al. 2012a; Tomaro-Duchesneau et al. 2012b), an enzyme that hydrolyses and releases free FA from its bound state. Orally ingested FA-producing lactic acid bacteria should demonstrate an increased bioavailability of FA due to an increased FAE activity in the intestine. Live microorganisms have been ingested and fermented in foods for centuries, with Metchnikoff establishing the concept of probiotics in the early 1900s (Metchnikoff 1907). Unfortunately, probiotic survival incorporated in foods is usually low due to the harsh environment (low pH, digestive enzymes, bile) of the GIT. Microencapsulation in polymer membranes, such as alginate-polylysine-alginate (APA) has been shown to successfully protect live bacterial cells for oral delivery (Bhathena et al. 2008; Prakash et al. 2011b; Tomaro-Duchesneau et al. 2012b). Specifically, the microencapsulation of FAE bacteria has demonstrated a higher viable cell count delivery through the GIT, allowing for a higher FA bioavailability (Bhathena et al. 2008; Bhathena et al. 2009; Tomaro-Duchesneau et al. 2012b). Previous research by our group

has demonstrated that microencapsulated *Lactobacillus fermentum* ATCC 11976 (*L.f.* 11976) can be used as a probiotic formulation in the context of cholesterol management in a Bio F_1B hypercholesterolemic Golden Syrian hamster model (Bhathena et al. 2009).

The increased bioavailability of FA in the GIT may also induce additional metabolic changes which should prove beneficial for the treatment of metabolic syndrome, specifically T2DM, obesity and cardiovascular diseases. T2DM and obesity are two major public health concerns in industrialized countries. The prevalence of diabetes in the United States in 2005 was estimated at 16.2 million individuals and is projected to rise to 48.3 million individuals by 2050 (Narayan et al. 2006b). Obesity, also related to T2DM, is an epidemic, not only because of its increasing prevalence but, also because of its frequent association with major atherosclerotic and cardiovascular risk factors (Lavie and Milani 2003). As a potential natural therapeutic, FA has been shown to regulate blood glucose levels through a variety of mechanisms including the modulation of insulin secretion, the promotion of pancreatic beta-cell survival and the reduction of inflammatory markers linked to antioxidant activity (Adisakwattana et al. 2008). With these properties in mind, FA could provide a way to modulate hyperglycemia, hyperinsulinema, adiposity and other markers involved in a number of metabolic diseases, including T2DM and obesity.

The presented research attempts to demonstrate the antioxidant properties of a commercially available FA, to subsequently investigate the antioxidant potential of *L.f.* 11976, associated with its growth in ethyl ferulate (EFA), a natural substrate of FAE. Furthermore, we investigate, *in vivo*, the use of the FA-producing *L.f.* 11976 microencapsulated formulation to modulate markers involved in the pathogenesis of

metabolic disorders such as T2DM and obesity using a diet-induced hypercholesterolemic hamster model.

7.3 Materials and methods

7.3.1 Bacterial growth media and chemicals

EFA (ethyl 4-hydroxy-3-methoxycinnamate), FA, poly-l-lysine, calcium chloride and low viscosity sodium alginate were purchased from Sigma-Aldrich (Oakville, ON, Canada). De Man-Rogosa-Sharpe (MRS) broth was obtained from Fisher Scientific (Ottawa, ON, Canada). Water was purified with an EASYpure Reverse Osmosis System and a NANOpure Diamond Life Science (UV/UF) ultrapure water system from Barnstead/Thermoline (Dubuque, IA, U.S.A.). All other chemicals were of analytical or high-performance liquid chromatrography (HPLC) grade and purchased from commercial sources.

7.3.2 Bacterial strain and culture conditions

L.f. 11976 was purchased from Cedarlane Laboratories (Burlington, ON, Canada). The bacterial strain was stored at -80°C in MRS containing 20% (v/v) glycerol. An MRS-agar plate was streaked for isolation from the frozen stock and incubated at 37°C with 5% CO_2 for 24 h to ensure purity. One colony from the MRS-agar plate was inoculated into 5 mL of MRS broth and incubated at 37°C for 24 h. A 1% (v/v) inoculum was then used for subculturing and incubated at 37°C for 24 h immediately before use. Viability on microcapsules was performed by exposure to 0.1 M sodium citrate until complete disruption of the microcapsules was observed. Ten-fold serial dilutions in physiological

saline followed by plating on MRS-agar plates were then performed to determine the colony forming units.

7.3.3 APA microencapsulation of L.f. 11976

The microencapsulation of *L.f.* 11976, using APA microencapsulation, was performed according to the standard protocol, described in our previous studies (Bhathena et al. 2008; Tomaro-Duchesneau et al. 2012b). The microencapsulation procedure was performed using an Inotech encapsulator (Inotech Biosystems International, MD' U.S.A.) and optimized flow rate, vibration frequency and voltage. The semi-permeable microcapsules obtained have a Molecular weight cut-off of 60-70 kDa, as previously demonstrated (Bhathena et al. 2008). Microcapsules termed "empty," which do not contain bacteria were also prepared as controls. Microencapsulated lactobacilli were stored at 4°C, in minimal media consisting of 10% MRS until further use. Previous work demonstrated no significant loss of cell viability or changes in microcapsule stability using this storage method (Bhathena et al. 2007).

7.3.4 Total antioxidant capacity of L.f. 11976

The total antioxidant production of *L.f.* 11976 was measured using a QuantiChrom^{TM} Antioxidant Assay Kit (BioAssay systems, CA, U.S.A.), a spectrophotometric assay based on the reduction of Cu^{2+} to Cu^{+} measured using a UV spectrophotometer Victor^{3}V 1420 Multilabel Counter (Perkin Elmer, MA, U.S.A.). The protocol provided with the assay kit was followed. A standard curve was generated for Trolox, a standard provided with the kit, at concentrations of 0, 300, 600 and 1000 μM plotted against absorbance at 570 nm (R^2=0.9970). Antioxidant activity of commercially available FA at concentrations of 0.3, 0.6, 0.9, 1.2 and 1.5 mM was plotted against

Trolox concentrations at an absorbance of 570 nm. Unencapsulated *L.f.* 11976 was subcultured from MRS broth at 1% (v/v) and microencapsulated *L.f.* 11976 at a concentration of 0.05 g/mL was added to MRS-EFA broth at an EFA concentration of 1.5 mM. Uninoculated MRS-EFA broth was used as a negative control. At each time point, the viability of the microcapsules and free cells was determined using ten-fold serial dilutions in physiological saline followed by plating on MRS-agar plates. Bacterial supernatant was obtained from each sample by centrifuging at 4000 rpm at 4°C for 15 minutes. The supernatant was stored at 4°C until the assay was performed. All samples and controls were treated in triplicate and incubated at 37°C during the course of the experiment.

7.3.5 Animals

Male Bio F_1B Golden Syrian hamsters, 8 weeks old (BioBreeders, MA, U.S.A.) with an average body weight of 90 g were used in this study. The animals were housed three per cage in a climate controlled space with inversed, alternating light and dark cycles (12:12-hr light– dark cycle; lights on at 19:00). The inversed light cycle and strict timing of glucose sampling were ensured to minimize any daily variations in measurements. All the principles of laboratory animal care were followed and all of the experimental protocols complied with the Animal Care Committee of McGill University (Montréal, Canada) and the Canadian Council on Animal Care guidelines.

7.3.6 Animal diet and experimental protocol

Following their arrival, animals were allowed free access to a commercial rodent ration (LabDiet® Rodent Laboratory Chow 5001, Purina Laboratories, St. Louis, MO, U.S.A.) and water for 2 weeks during their acclimatization period. Baseline values of

serum total cholesterol (TC) were determined, as described below, at the end of the acclimatization from hamsters fasted over a 14-hour period. These basal serum TC values were used to randomize the hamsters into the two treatment groups.

Following randomization, the hamsters of both groups were fed a semi-purified hyperlipidemic hypercholesterolemic diet *ad libitum* (Modified LabDiet® Laboratory Rodent Diet 5001 with 10.0% Wheat Bran, 0.05% Cholesterol, 6.0% Saturated Fat and 10.0% Total Fat, Purina Laboratories, St. Louis, MO, U.S.A.). To investigate the therapeutic efficacy of the formulation, animals in the treatment group (n = 12) received the microcapsule formulation containing 10^{11} cfu of *L.f.* 11976 twice daily, a dose determined from previous work by our group (Bhathena et al. 2009). The control group (n = 12) received empty microcapsules as treatment vehicle-control. The treatment was administered during 10 weeks, with the hamsters orally fed the formulation using an 18G/50 mm with 2.25 mm ball diameter stainless steel gavage needle. During the course of the treatment, food consumption and body weight were measured biweekly. Blood samples were collected once every 14 days from 14-hour food-deprived hamsters via the saphenous vein. At the end of the experimental period, the hamsters were euthanized by carbon dioxide asphyxiation and blood was withdrawn by cardiac puncture.

7.3.7 Biological samples

Blood was collected either in Microtainer® serum separator tubes or in Vacutainer® plasma tubes with spray-coated lithium heparin from Fisher Scientific (Ottawa, ON, Canada). For serum, the blood was allowed to clot at 23°C for 30 minutes and subsequently placed on ice until centrifugation. Serum was separated by low-speed centrifugation at 2000 x*g* for 20 min at 4°C temperature and stored at –80°C until used.

To calculate the adiposity index, the weights of the epididymal, visceral and retroperitoneal fat were taken following their careful dissection. The adiposity index was calculated using the formula [Visceral fat / (body weight-visceral fat)] *100, and expressed as adiposity percentage (Taylor and Phillips 1996).

7.3.8 Clinical chemistry analysis

Serum lipids (TC, triglycerides), glucose, uric acid, glycosylated hemoglobin (HbA1c) and C-reactive protein (CRP) were assayed by conventional enzymatic methods on a Hitachi 911 automated clinical chemistry autoanalyzer (Roche Diagnostics, U.S.A.) using reagent kits supplied by Roche Diagnostics (Laval, QC, Canada). Serum free cholesterol (FC) and non-esterified fatty acids (NEFA) were similarly estimated using reagent kits from Wako Chemicals Inc. (Richmond, VA, U.S.A). Serum insulin and leptin were measured by an Enzyme Immunoassay kit from SPI-BIO (Massy, France). Glycosylated albumin (GA) was estimated using a Glycaben ELISA kit from Exocell Inc. (Philadelphia, PA, U.S.A.). Fasting serum insulin and glucose were used to calculate insulin resistance from the homeostasis model assessment for insulin resistance (HOMA-IR) [(fasting glucose * fasting insulin)/22.5] (de Roos et al. 2005). Serum cholesteryl ester (EC) concentrations were calculated as the difference between the TC and the FC concentrations.

7.3.9 Statistical analysis

Experimental results are expressed as mean ± SEM. Statistical analysis was carried out using SPSS Version 17.0 (Statistical Product and Service Solutions, IBM Corporation, New York, NY, USA). Linear regression was performed to correlate FA and Trolox concentrations. Statistical comparisons were carried out using the Independent

Samples t-test to compare the means of the treatment and control groups. Statistical significance was set at $p < 0.05$ and p-values less than 0.01 were considered highly significant.

7.4 Results

7.4.1 Microencapsulation of L.f. 11976

L.f. 11976 microencapsulation was optimized—by controlling the flow rate, stirring time, stirring speed, coating time, vibration frequency, and voltage—to obtain spherical and monodispersed microcapsules. The obtained *L.f.* 11976 microcapsules were observed under light microscopy at magnifications of 100X and 200X. The APA microcapsules were monodispersed with an approximate size of 602±30 μm in diameter. **Figure 7.1A** demonstrates the spherical shape of the microcapsule and Figure **7.1B** allows for the visualization of the outer coat of the APA microcapsule with the bacterial cells evenly distributed within the polymeric membrane.

7.4.2 Total antioxidant capacity of free and microencapsulated L.f. 11976

To investigate the antioxidative potential of the FA produced by *L.f.* 11976 and to ensure that the microcapsule does not inhibit this activity, a QuantiChrom^{TM} Antioxidant Assay Kit was used. Commercially purchased FA was shown to have significant antioxidant activity, with a linear correlation demonstrated using Trolox as the provided standard ($R^2 = 0.9881$, $p < 0.0001$) (**Figure 7.2A**). To quantify the bacterial antioxidant activity, the probiotic strain was incubated in MRS-EFA at an initial EFA concentration of 1.5 mM. Antioxidant activity and bacterial viability were measured at various time points for both free (**Figure 7.2B, 7.2C**) and microencapsulated *L.f.* 11976 (**Figure 7.2D,**

7.2E). Following 48 hours of incubation, the antioxidant activity of *L.f.* 11976 was 713.81 ± 3.72 μM Trolox equivalents for the free cells and 699.52 ± 15.26 μM Trolox equivalents for the microencapsulated cells. No significant difference in antioxidant production was detected between the free and microencapsulated cells ($p = 0.450$). Using the curve generated for FA the activity correlates to 1931.83 ± 10.07 μM for the free cells and 1893.15 ± 41.30 μM FA.

7.4.3 Effect of microencapsulated L.f. 11976 on body weight and adiposity

Dietary intake, food consumption and adiposity were monitored following the change to a hyperlipidemic hypercholesterolemic diet. The animals, of both groups, gained significant amounts of body weight over the course of the 10 week study (**Figure 7.3A**). Following the 10 weeks of treatment, there was no significant difference in body mass between the control (157.20 ± 2.25 g) and the treatment (151.42 ± 3.17 g) animals ($p = 0.178$). In addition, no significant difference in food consumption between the groups was observed (data not shown). As for the abdominal visceral fat weights, these were significantly lower in the animals treated with microencapsulated *L.f.* 11976. The adiposity index of the treated group of animals, at the end of the 10 weeks of treatment with microencapsulated *L.f.* 11976 (6.22 ± 0.25) proved to be significantly lower than that of the control animals (7.94 ± 0.35) ($p = 0.0014$) (**Figure 7.3B**).

7.4.4 Effect of microencapsulated L.f. 11976 on serum glucose and insulin

Fasted serum glycemia was monitored throughout the treatment period and consumption of the hyperlipidemic hypercholesterolemic diet. Hamsters administered *L.f.* 11976 (15.41 ± 2.26 mmol/L) showed no significant decrease in fasting serum glucose level following 10 weeks of treatment, when compared to the untreated control hamsters

(16.37 ± 1.20 mmol/L) (p=0.629) (**Figure 7.4A**). On the other hand, serum fasting insulin concentrations were significantly different between animals administered the microencapsulated bacterial formulation (0.59 ± 0.096 ng/mL) and the controls (1.40 ± 0.217 ng/mL) (p = 0.0042) (**Figure 7.4B**). Moreover, intervention with the microcapsule formulation showed a significant reduction in insulin resistance (HOMA-IR) in treated animals (66.51 ± 10.78) as compared with the controls (153.86 ± 28.24) (p = 0.0096) (**Figure 7.4C**).

7.4.5 Effect of microencapsulated L.f. 11976 on glycemic control and inflammation

Serum HbA1c levels were measured following 10 weeks of treatment with microencapsulated *L.f.* 11976. HbA1c was non-significantly reduced in the treated group (8.41 ± 1.40 %) when compared to the control group (12.85 ± 1.59 %) (p = 0.058) (**Figure 7.5A**). On the other hand, a highly significant reduction in GA levels of the hamsters treated with microencapsulated *L.f.* 11976 (0.86 ± 0.12 %) when compared with the control animals (2.38 ± 0.30 %) was noted (p = 0.00013) (**Figure 7.5B**).

Systemic inflammation, as measured by serum CRP levels, was not found to be significantly different between the treatment (12.04 ± 4.05 nmol/L) and control (16.27 ± 4.62 nmol/L) animals (p = 0.61) (**Figure 7.5C**). On the other hand, the serum concentration levels of uric acid, following 10 weeks of treatment, were found to be significantly higher in the control group (247.07 ± 23.02 μmol/L) when compared to the levels of the group administered microencapsulated *L.f.* 11976 (178.07 ± 15.32 μmol/L) (p = 0.025) (**Figure 7.5D**). A statistically significant difference in the serum leptin levels

was also noted between the treated (143.54 ± 19.46 pg/mL) and the control (211.75 ± 24.33 pg/mL) animals (p = 0.048) (**Figure 7.5E**).

7.4.6 Effect of microencapsulated L.f. 11976 on serum lipids

Figure 7.6A demonstrates that, following 10 weeks of treatment with microencapsulated *L.f.* 11976, there was a significant decrease (p = 0.024) in serum TC concentrations in the treated (14.33 ± 2.38 mmol/L) as compared to the control (23.18 ± 2.52 mmol/L) animals (p = 0.024). At the same time point, serum FC levels, did not show any difference between the treated (1.71 ± 0.31 mmol/L) and the control (1.55 ± 0.33 mmol/L) hamsters (p = 0.758). However, EC concentrations were significantly reduced in the treated animals (12.91 ± 2.51 mmol/L) when compared to the control animals (21.94 ± 2.83 mmol/L) (p = 0.0328). **Figure 7.6B** demonstrates that serum triglyceride (TG) concentrations were not significantly different between the treated (6.28 ± 0.97 mmol/L) and the control (8.36 ± 0.51) groups (p = 0.0948). On the other hand, there was a significant reduction in serum NEFA concentrations in the treated animals (2.65 ± 0.12 mmol/L) as compared to the control animals (3.33 ± 0.26 mmol/L) (p = 0.029).

7.5 Discussion

Probiotics are dietary supplements containing bacteria which, when administered in adequate amounts, confer a health benefit on the host (FAO and WHO 2001; Prakash et al. 2011b). Probiotics, as natural compounds, are generally considered safe, but can also be tested for set-out safety parameters (Branton et al. 2011). A number of studies have investigated bacterial strains for a range of conditions, including infections, allergies and metabolic disorders such as ulcerative colitis and Crohn's disease (Prakash et al.

2011b). Promising research focuses on the microbial secretion and production of beneficial biologically active enzymes and proteins, including: ornithine decarboxylase for the treatment of autoimmune diseases and accelerated cell apoptosis (Mates et al. 2002), bile salt hydrolase for hypercholesterolemia (Martoni et al. 2008; Tanaka et al. 1999a), and bile transport and tolerance proteins for the efficient delivery of probiotics (Pfeiler and Klaenhammer 2009). In recent studies, the products of another microbial protein, cinnamoyl esterase, have shown significant levels of antioxidant activity (Srinivasan et al. 2007; Tomaro-Duchesneau et al. 2012a) and other effects, including stimulation of insulin secretion (Adisakwattana et al. 2008; Sri Balasubashini et al. 2003), prevention of oxidative stress (Adisakwattana et al. 2008), lipid peroxidation (Balasubashini et al. 2004), cholesterol-lowering capabilities (Bhathena et al. 2009) and inhibition of diabetic nephropathy progression (Atsuyo et al. 2008). A product of hydrolysis activity of FAE, FA, is a well-characterised antioxidant (Tomaro-Duchesneau et al. 2012a).

FA is a naturally found phenolic acid that has numerous beneficial properties. Recent studies suggest that FA has antitumor activity against breast cancer (Chang et al. 2006; Kampa et al. 2003), liver cancer (Lee 2005; Taniguchi et al. 1999) and is effective at preventing cancer induced by the exposure to carcinogenic compounds such as benzopyrene (Lesca 1983) and 4-nitroquinoline 1-oxide (Tanaka et al. 1993). FA has also been demonstrated to have important antioxidant properties (Rice-Evans et al. 1996) while also reducing cell aging (Lombard et al. 2005a). Keeping this in mind, we propose that FA can be used to modulate a number of biomarkers related to metabolic syndrome, specifically cardiovascular diseases, T2DM and obesity (**Figure 7.7**). Unfortunately, the

oral delivery of free FA is hampered by its quick absorption in the jejunum, followed by its rapid excretion. It has been demonstrated in a number of recent studies that some GIT bacterial strains produce FAE, an enzyme that can produce FA from natural substrates. The oral delivery of these bacteria, however, is impeded by the harsh conditions of the upper GIT, specifically the presence of bile and an acidic pH. Microencapsulation, specifically APA microencapsulation, has been successfully used to overcome the challenge of delivering bacterial cells through the GIT (Tomaro-Duchesneau et al. 2012b). APA microencapsulation relies on a polyelectrolyte complexation mechanism for the association of alginate and PLL. Alginate is a natural biocompatible polymer, extracted from brown algae, that is increasingly being used in the biotechnology industry (Prakash et al. 2011b). Alginate is an unbranched polysaccharide containing 1,4′-linked *β*-D-mannuronic acid and *α*-L-guluronic acid blocks interdispersed with regions of the alternating structure, *β*-L-mannuronic acid-*α*-L-guluronic acid blocks. PLL is a polypeptide made up of the amino acid L-lysine that is available in a variable number of chain lengths. The addition of this polymer leads to the formation of a capsule membrane that provides selective permeability and immunoprotection. The alginate bead could not withstand the harsh conditions of the GIT in the absence of PLL, which provides it with an increased mechanical stability. Previous research, by our group, investigated APA microencapsulation as a method to protect bacterial viability through a GIT transit, and demonstrated a significant 2.5 log difference in viability between free and APA microencapsulated *L. fermentum* following transit (Tomaro-Duchesneau et al. 2012b).

Previous research by our group has screened for FA production by probiotic bacteria (Bhathena et al. 2007; Tomaro-Duchesneau et al. 2012a). For this research, *L.f.*

11976 was selected for its FAE activity. *In vitro* studies into the antioxidant properties of this strain were performed with relation to FA production. Following 48 hours of incubation, the antioxidant activity of *L.f.* 11976 was determined and correlated with a standard curve generated for FA. Using the curve generated for FA the activity correlated to 1931.83 ± 10.07 μM for the free cells, greater than that quantified by previous HPLC assays (Bhathena et al. 2007; Tomaro-Duchesneau et al. 2012a). These results suggest that the antioxidant activity of *L.f.* 11976 may not be explained solely by its FA production, but by potential other molecules produced by this strain, which require further investigations. The demonstrated antioxidant activity as well as its previously demonstrated effects on hypercholesterolemia make it an ideal candidate for this study (Bhathena et al. 2007).

Feeding of a high lipid diet provides a dietary model of metabolic syndrome—cardiovascular diseases, T2DM and obesity. A number of risk factors are involved in metabolic syndrome, including insulin resistance, hyperglycemia, hyperinsulinemia, chronic systemic inflammation and hypertriglyceridemia, to name a few. An overload of fat to the liver leads to the disruption of metabolic and glucose uptake pathways, potentially leading to an enhanced rate of *de novo* lipogenesis and triglyceride synthesis, ultimately inducing insulin resistance. The presented study uses a hamster model fed a hyperlipidemic hypercholesterolemic diet, leading to the induction of moderate insulin resistance and elevation of serum glucose. Future research may also want to investigate to what extent markers of metabolic syndrome are restored following probiotic treatment, using a comparative group fed a standard diet. One could also investigate the potential effect(s) of treatment on animals consuming a standard diet.

Following 10 weeks of treatment with APA microencapsulated *L.f.* 11976 the serum glucose levels were only numerically disparate in the treated animals when compared to the control group. Furthermore, no significant difference in animal body mass was noted, suggesting that a longer treatment period may be necessary to significantly alter hyperglycemia and obesity. On the other hand, insulin levels were significantly lower in the treatment group than the control, suggesting a control of hyperinsulinemia by the probiotic formulation. In addition, insulin resistance, as determined using HOMA-IR, was alleviated significantly ($p = 0.0096$), demonstrating the ability of microencapsulated *L.f.* 11976 to improve the sensitivity of the peripheral tissues to insulin.

The United Kingdom Prospective Diabetes Study demonstrated that a 1% reduction of HbA1c is associated with a 35% reduction in macrovascular endpoints, an 18% reduction in myocardial infarction, and a 17% reduction in all-cause mortality (UK Prospective Diabetes Study Group 1998). In our study, the HbA1c levels, following 10 weeks of treatment, demonstrated only a non-significant difference (8.41 ± 1.40 %) for the treated and (12.85 ± 1.59%) for the control animals ($p = 0.058$). One must keep in mind, however, that total HbA1c reveals the percentage of circulating hemoglobin that has chemically reacted with glucose, reflecting the blood glucose levels over the 120 days preceding the test (Gabbay et al. 1977), and is, therefore, unaffected by short term fluctuations in blood glucose levels present in a short (70 day) study like this one. Furthermore, the non-significant decrease in HbA1c levels observed in our study may reflect the need for the longer administration or a higher dose of probiotic to demonstrate an adequate and long-term reduction of hyperglycemia.

Serum GA levels have been shown to be a better alternative marker of glycemic control in patients with T2DM (Inaba et al. 2007) since it is free of interference from endogenous glycated amino acids and is unaffected by changes in albumin concentration. In the presented study, hamsters treated with microencapsulated *L.f.* 11976 demonstrated significantly lower GA concentrations than the control group ($p = 0.00013$). Furthermore, control animals had significantly increased TC, TG, and NEFA levels. Dyslipidemia is often one of the first risk factors identified and observed in pre-diabetic patients (Dagogo-Jack 2005), suggesting a link between dyslipidemia and T2DM initiation. For example, it has been reported that GA contributes to dyslipidemia in diabetes (and renal insufficiency) (Bucala et al. 1994) and GA modification impairs the low-density lipoprotein (LDL) receptor-clearance mechanism (Aronson and Rayfield 2002). Hence, even a small decrease in GA (such as that observed in this study) and TC, TG and NEFA levels may be sufficient to stop the triggering of pathogenic mechanisms underlying accelerated atherosclerosis, nephropathy and retinopathy (Schalkwijk and Miyata 2012). Future studies should take into consideration that GA measurements reflect glycemic control for only two weeks preceding the assay, and so should be taken more frequently throughout the study.

Our results demonstrate a significant reduction in serum NEFA levels in the animal group treated with microencapsulated *L.f.* 11976, following 10 weeks of treatment ($p = 0.029$). Recent studies support the idea of a correlation between elevated NEFA and peripheral insulin resistance (Barazzoni et al. 2012). In obese individuals, serum NEFA levels are chronically elevated, contributing to insulin resistance at the skeletal muscle level. Furthermore, these may have additional actions in the liver and pancreas,

contributing to the development of T2DM (Tushuizen et al. 2007). Hence, NEFA consist of an important therapeutic target that our probiotic formulation successfully modulated.

The administration of microencapsulated *L.f.* 11976 also reduced the accumulated levels of advanced glycosylation end-products, produced by non-enzymatic glycosylation of proteins (Maillard reaction) (Maillard 1912), as indicated by the decrease in total HbA1c and albumin. Glycosylated end-products are present in high levels in T2DM patients, stimulating macrophages to secrete inflammatory cytokines and growth factors, impairing normal macrophage function, hence, increasing susceptibility to infection (Liu et al. 1999). Similarly, inflammatory markers, particularly CRP, play an important role in cardiovascular disease as they are linked with an increased risk of atherosclerosis, myocardial infarction and diabetes (King et al. 2003; Ridker et al. 1998). These findings suggest an association between glycemic control and systemic inflammation in people with established diabetes, specifically with HbA1c levels (King et al. 2003). In the presented study, administration of microencapsulated *L.f.* 11976 decreased slightly, although not significantly, serum CRP levels ($p = 0.61$), which may be explained by the high levels of HbA1c observed.

Hyperuricemia also demonstrates a strong correlation with insulin resistance, and cardiovascular disease, but not with blood glucose levels (Lin et al. 2007). The link between insulin resistance and hyperuricemia, elucidated by Facchini et al, demonstrates that urinary uric acid clearance decreases in proportion to increases in insulin resistance, manifesting as an increase in serum uric acid concentrations (Facchini et al. 1991). Our results demonstrate simultaneous reduction of serum uric acid and insulin resistance in hamsters receiving microencapsulated *L.f.* 11976 when compared to control animals.

Previous research has also noted that elevated serum leptin levels, a neuroregulatory peptide that regulates lipid, glucose and insulin, contribute to increased serum uric acid levels (Matsubara et al. 2002). A majority of patients with T2DM have elevated leptin levels, associated with that seen in obese subjects (Considine et al. 1996). In our study, there was no significant difference in body weight ($p = 0.178$) between the treated and control animals, even though the serum leptin levels were significantly lower in the treated animals ($p = 0.0476$). Previous research has demonstrated a strong positive correlation between serum leptin concentration and the percentage of body fat, as we also demonstrate with a decreased adiposity index (Considine et al. 1996). As aforementioned, despite the difference in serum leptin levels, no significant difference in food consumption nor body mass was noted between the two groups. Previous research has demonstrated that obese subjects may have decreased sensitivity to leptin and require larger doses of leptin to induce weight loss, explaining the phenomena seen in the presented work (Considine et al. 1996).

The consumption of functional foods containing probiotics has been proposed as one of the best approaches to treat and/or inhibit the prevalence of T2DM, obesity and cardiovascular diseases. It has previously been demonstrated that probiotic lactic acid bacteria can control glucose levels and significantly delay the onset of hyperglycemia, hyperinsulinemia, dyslipidemia, glucose intolerance and oxidative stress (Yadav et al. 2007; Yadav et al. 2008). The supplementation of probiotic *L. acidophilus* and *L. casei* with dahi cultures, increased the efficacy of dahi in suppressing streptozotocin-induced diabetes in rats by inhibiting insulin depletion, diabetic dyslipidemia, lipid peroxidation and nitrite formation (Yadav et al. 2008). FA-producing microencapsulated lactic acid

bacteria have been shown to liberate FA in the gut (Bhathena et al. 2008; Tomaro-Duchesneau et al. 2012b). Currently, it is not clear exactly how FA is impacting metabolic syndrome, as presented in this study. However, as shown in Figure 7, the mechanism(s) of action by which FA exerts its beneficial effects has been hypothesized, with a number of biomarkers involved that play important roles in metabolic syndrome. Future research should focus on the potential mechanisms of action and further explore the role of FA in all of the markers presented. The exact role of FA in modulating the markers described in this research will be investigated further using both *in vitro* and *in vivo* studies, focusing on both the metabolic and anti-inflammatory effects of this molecule. The objective of this research was to demonstrate the use of a FA-producing probiotic on the modulation of metabolic markers, with mechanistic studies to follow. Future animal work should also be undertaken using other models of metabolic syndrome and T2DM.

7.6 Conclusion

The presented study investigated the effects of feeding microencapsulated live probiotic *L.f.* 11976 on biomarkers of metabolic syndrome, entailing cardiovascular diseases, obesity and T2DM. The evidence for a possible role of dietary FA for the treatment and prevention of these disorders is presented and discussed. Keeping in view the gaps in these studies, the various mechanisms by which FA may improve the control of glucose tolerance, lipid metabolism, body mass and other markers presented here, need to be further investigated.

7.7 Acknowledgments/Disclosures

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SP and JB have a conflict of interest with Micropharma Ltd., Montreal, Canada; the technology mentioned in this research is optioned to Micropharma Ltd., for commercialization. None of the other authors have any personal or financial conflict of interest. The design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation of the manuscript was independent of the funding organizations or sponsors. The manuscript was reviewed and approved by the sponsor.

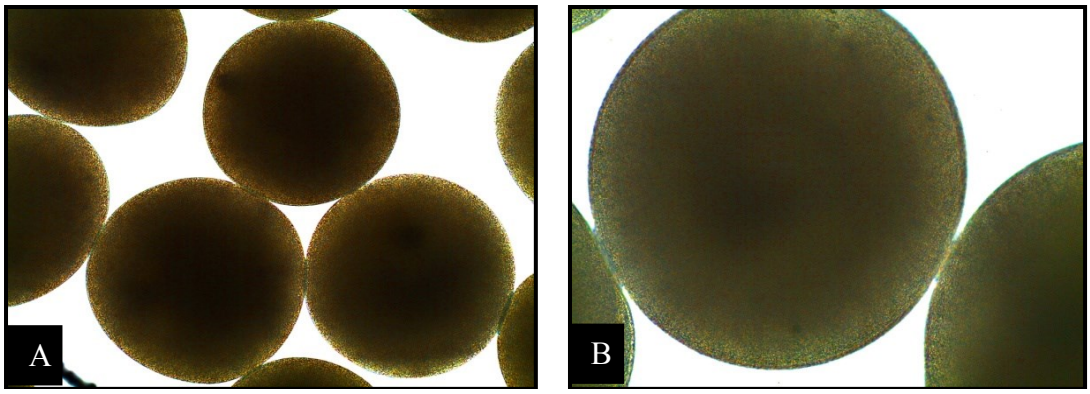
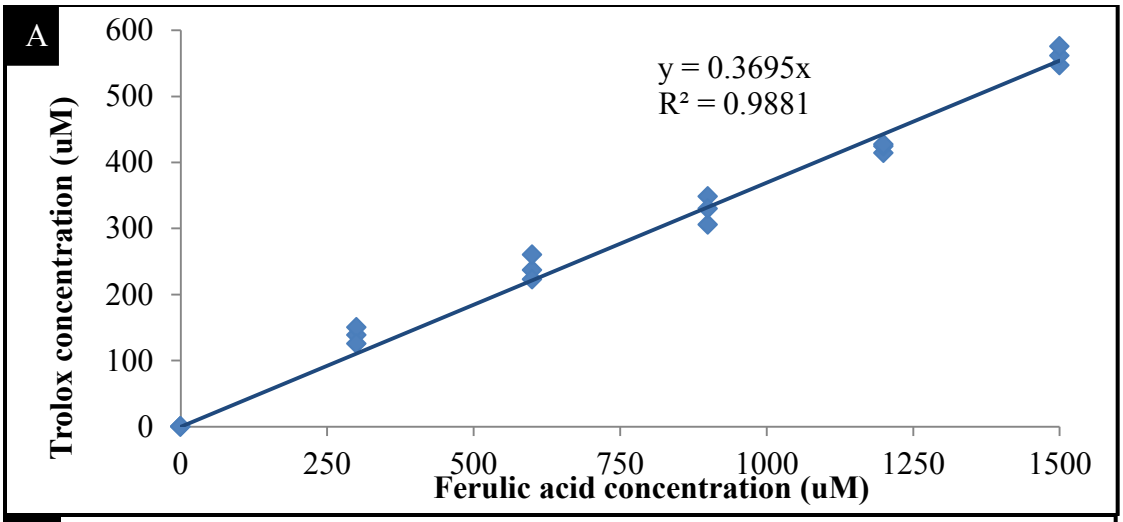
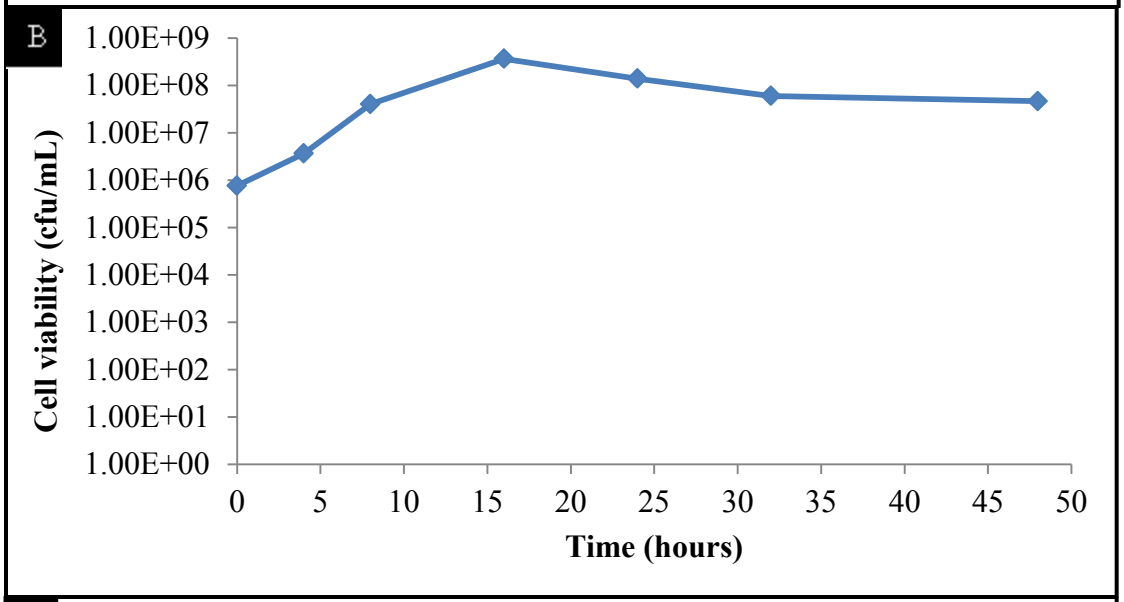
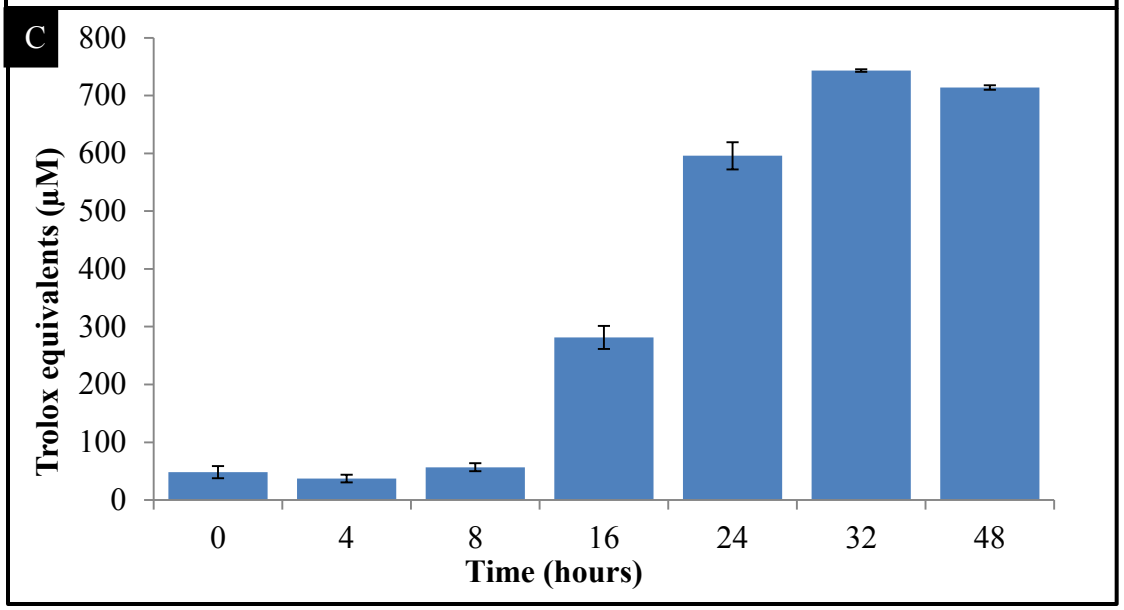


Figure 7.1: Morphology of APA microcapsules containing *L.f.* 11976 taken by light microscope (A) 100x and (B) 200x. The approximate diameter of the microcapsules was 602±30μm in diameter.


A
Trolox concentration (uM)
y = 0.3695x
R² = 0.9881
Ferulic acid concentration (uM)
0
100
200
300
400
500
600
0
250
500
750
1000
1250
1500


B
Cell viability (cfu/mL)
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
0
5
10
15
20
25
30
35
40
45
50
Time (hours)


C
Trolox equivalents (µM)
0
100
200
300
400
500
600
700
800
0
4
8
16
24
32
48
Time (hours)

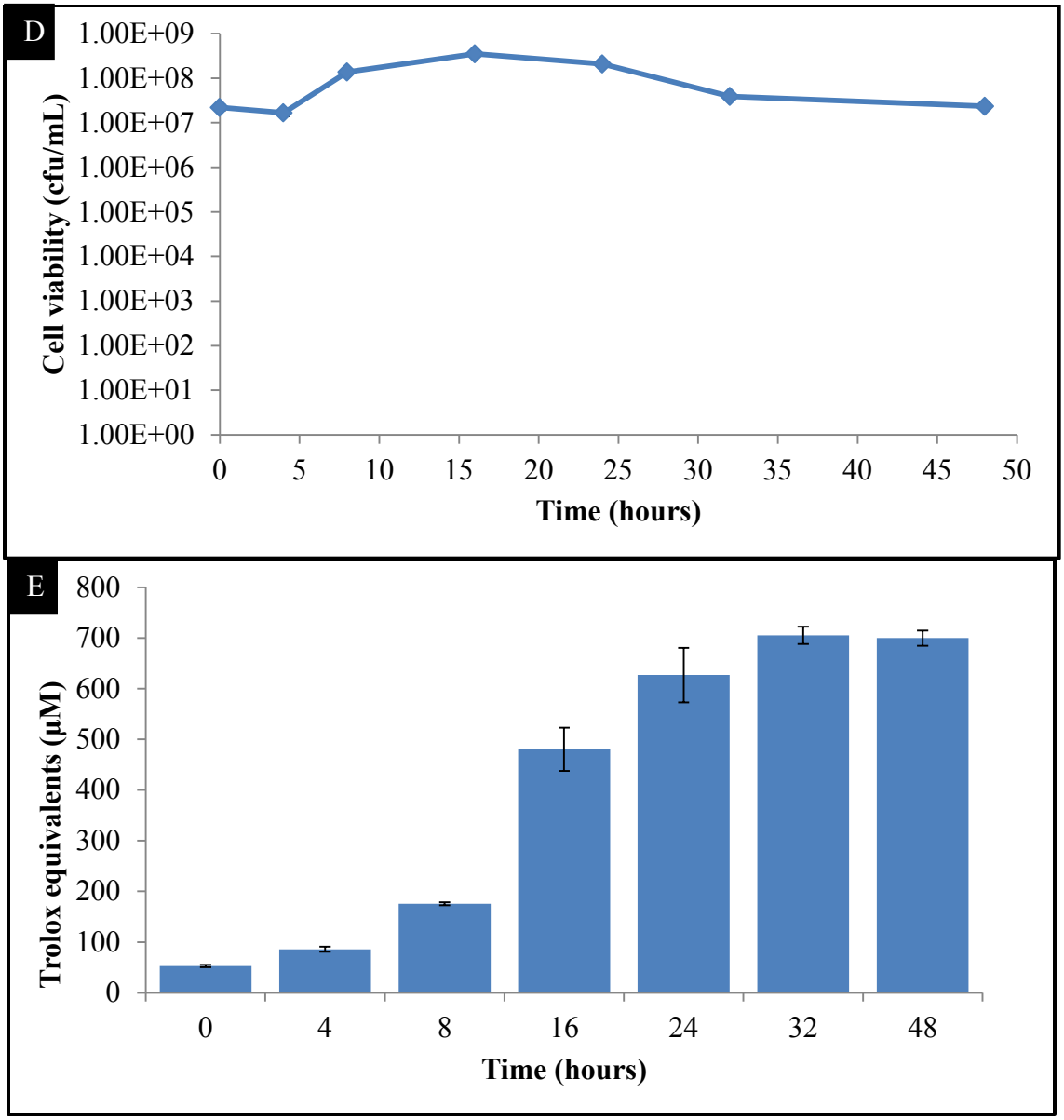


Figure 7.2: Antioxidant activity of ferulic acid produced by *L.f.* 11976. (A) Commercially purchased ferulic acid antioxidant activity correlated with Trolox concentration using a QuantiChrom™ Antioxidant Assay Kit ($R^2 = 0.9881$, $p < 0.0001$)). (B) Viability and (C) antioxidant production by free *L.f.* 11976 and (D) viability and (E) antioxidant production by APA microencapsulated *L.f.* 11976. Following 48 hours of incubation antioxidant activity of *L.f.* 11976 was 713.81±3.72µM Trolox equivalents for the free cells and 699.52±15.26µM Trolox equivalents for the microencapsulated cells (p=0.450).

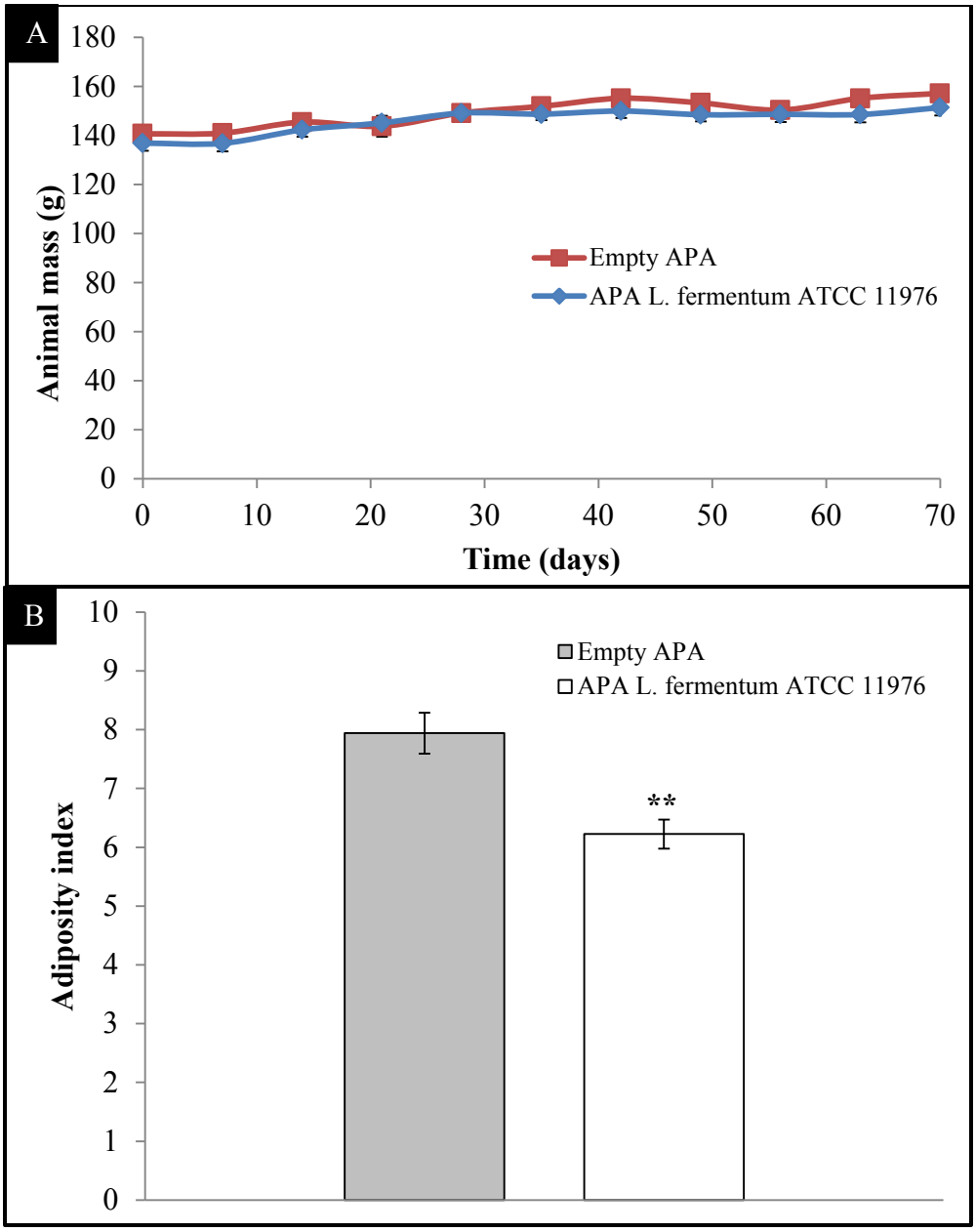


Figure 7.3: Effect of oral feeding of microencapsulated *L.f.* 11976 on the (A) animal mass over time and on the (B) adiposity index (**p<0.01) following 70 days of treatment in Golden Syrian hamsters. Data represents the mean ± SEM (n = 12).

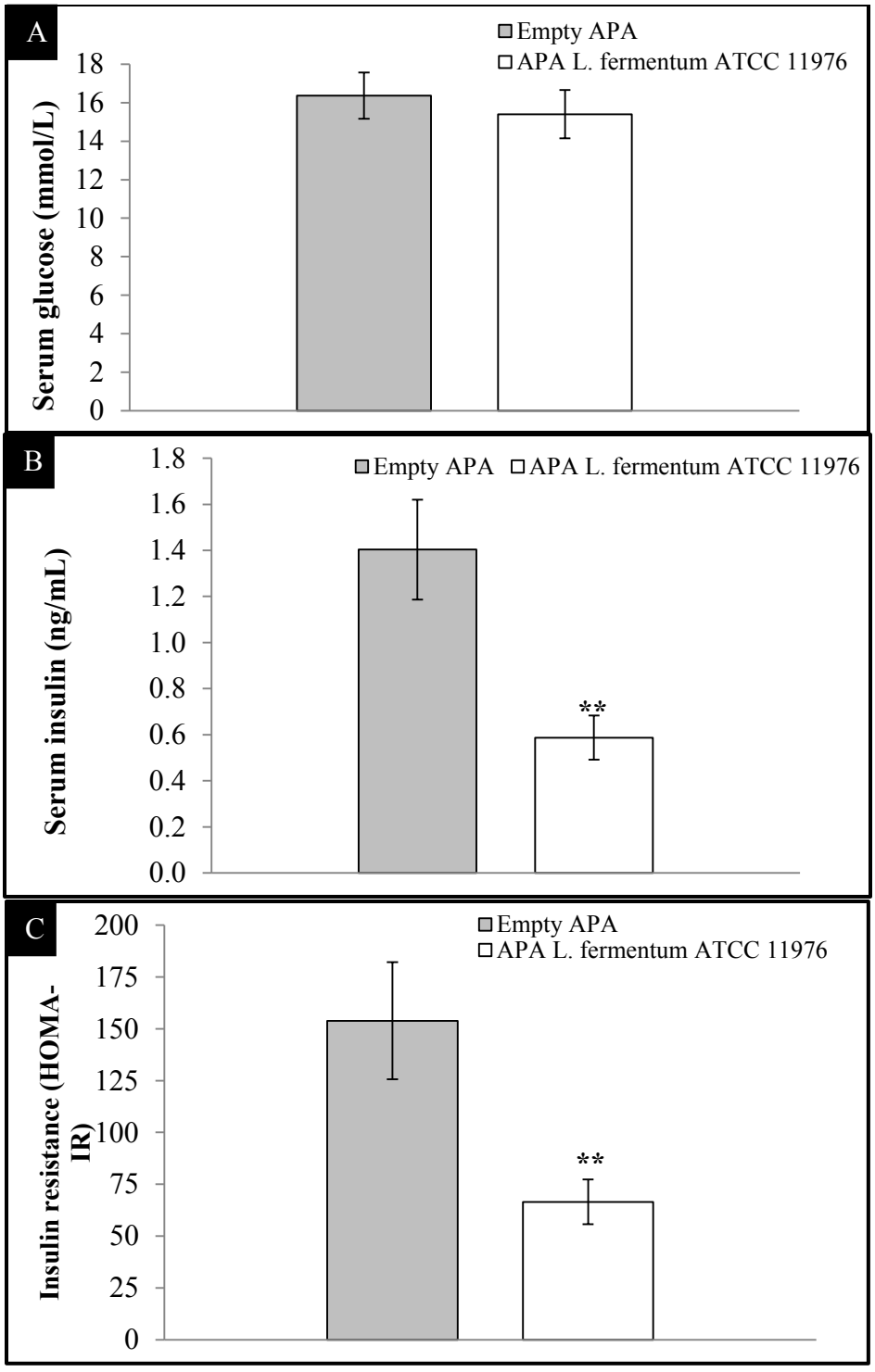
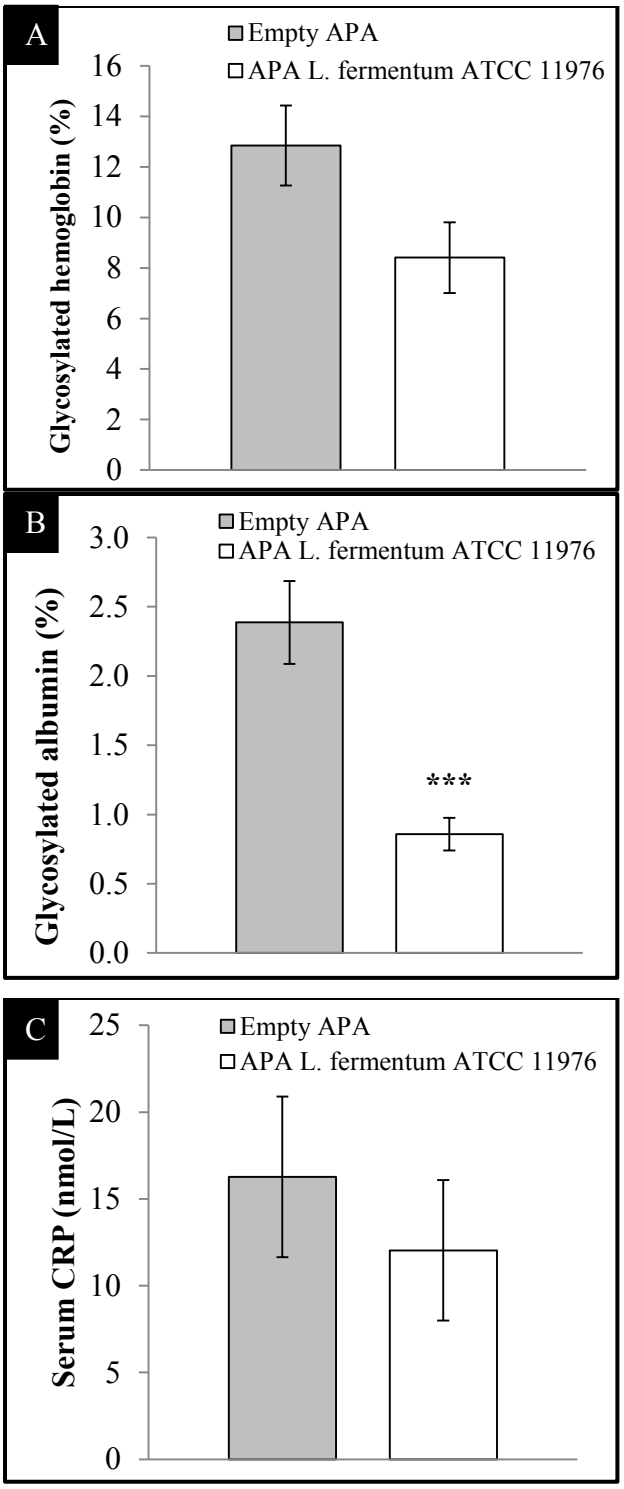


Figure 7.4: Treatment of diabetic obese Golden Syrian hamsters with microencapsulated *L.f.* 11976 resulted in a non-significant reduction in (A) serum glucose levels following 10 weeks of treatment, but showed a significant reduction in (B) serum insulin levels (**p<0.01). (C) Insulin resistance was quantified using Homeostasis Model Assessment (HOMA-IR). The treated group showed HOMA-IR values significantly lower than untreated control animals (**p<0.01). Data represents the mean ± SEM (n = 12).


A
Empty APA
APA L. fermentum ATCC 11976
Glycosylated hemoglobin (%)
16
14
12
10
8
6
4
2
0
B
Empty APA
APA L. fermentum ATCC 11976
Glycosylated albumin (%)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
C
Empty APA
APA L. fermentum ATCC 11976
Serum CRP (nmol/L)
25
20
15
10
5
0

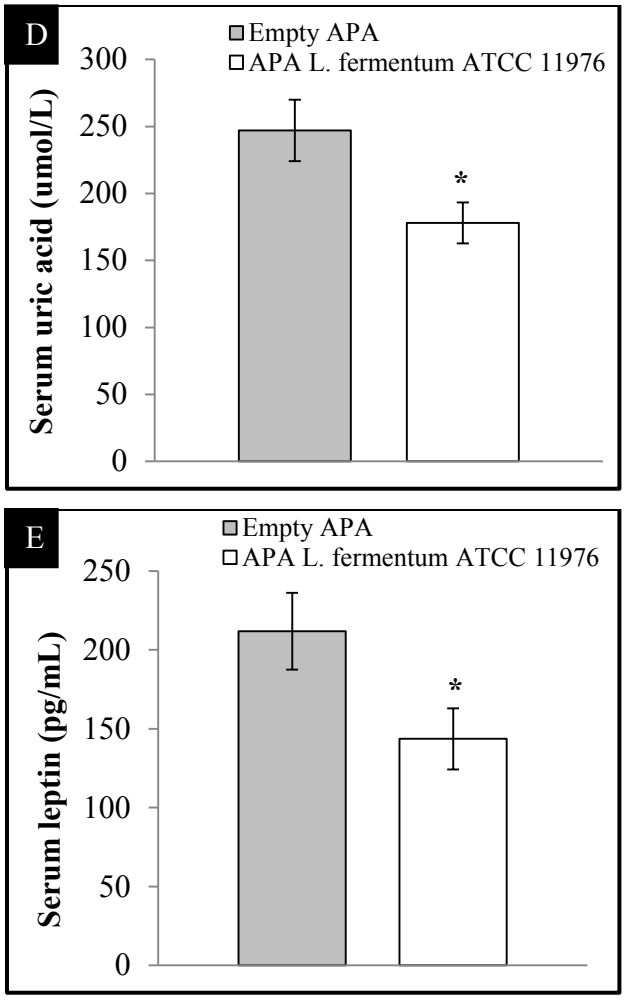


Figure 7.5: Changes in (A) serum HbA1c, (B) serum GA (***p<0.001), (C) serum CRP, (D) serum uric acid (*p<0.05) and (E) serum leptin (*p<0.05) levels in hamsters treated with microencapsulated *L.f.* 11976. Data represents the mean ± SEM (n = 12).

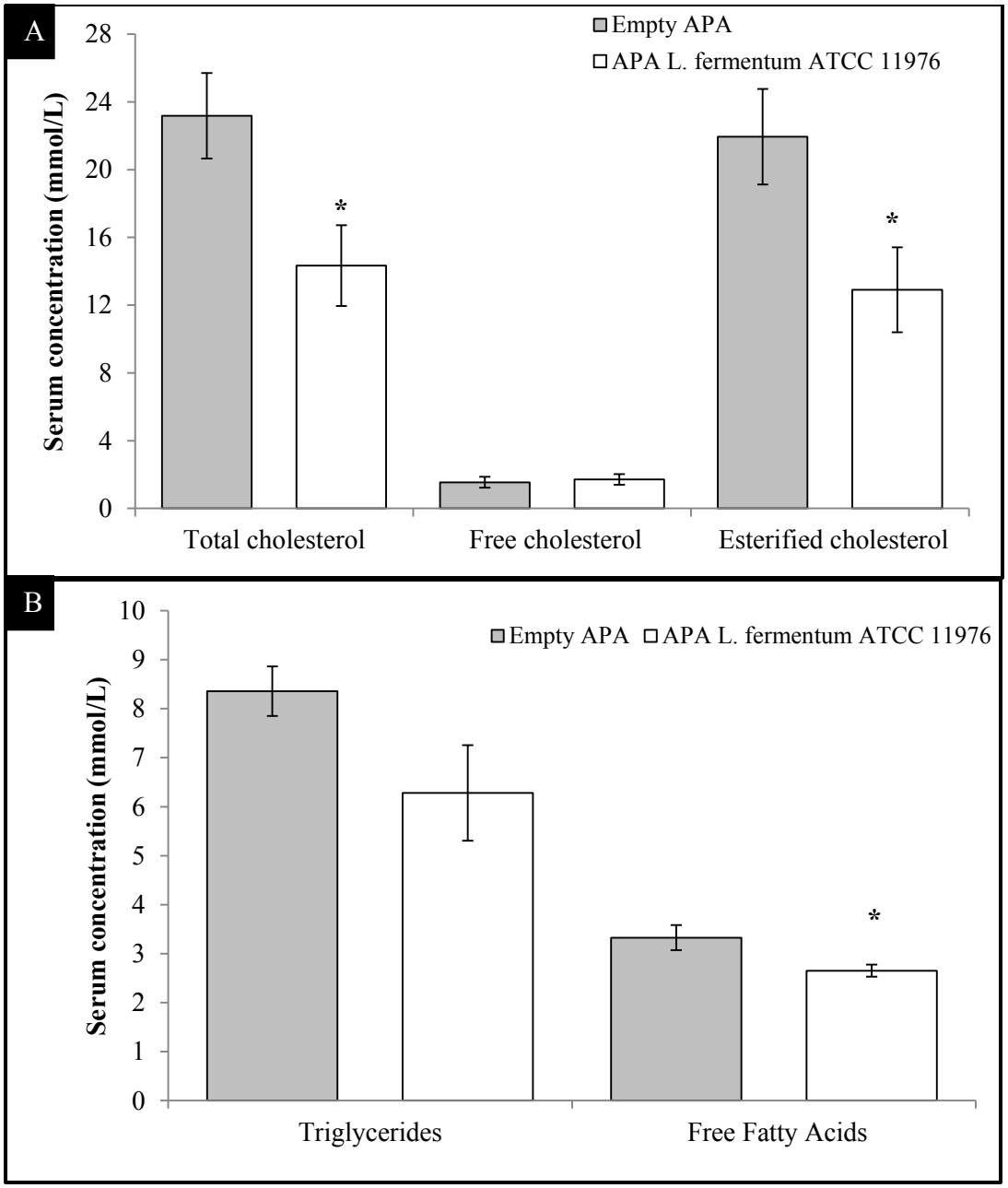


Figure 7.6: Clinical effects of microencapsulated *L.f.* 11976 on lipid metabolism in Golden Syrian hamsters; (A) serum total cholesterol (*p<0.05), serum free cholesterol and esterified cholesterol (*p<0.05) and (B) serum triglycerides and non-esterified (free) fatty acids (*p<0.05). Data represents the mean ± SEM (n = 12).

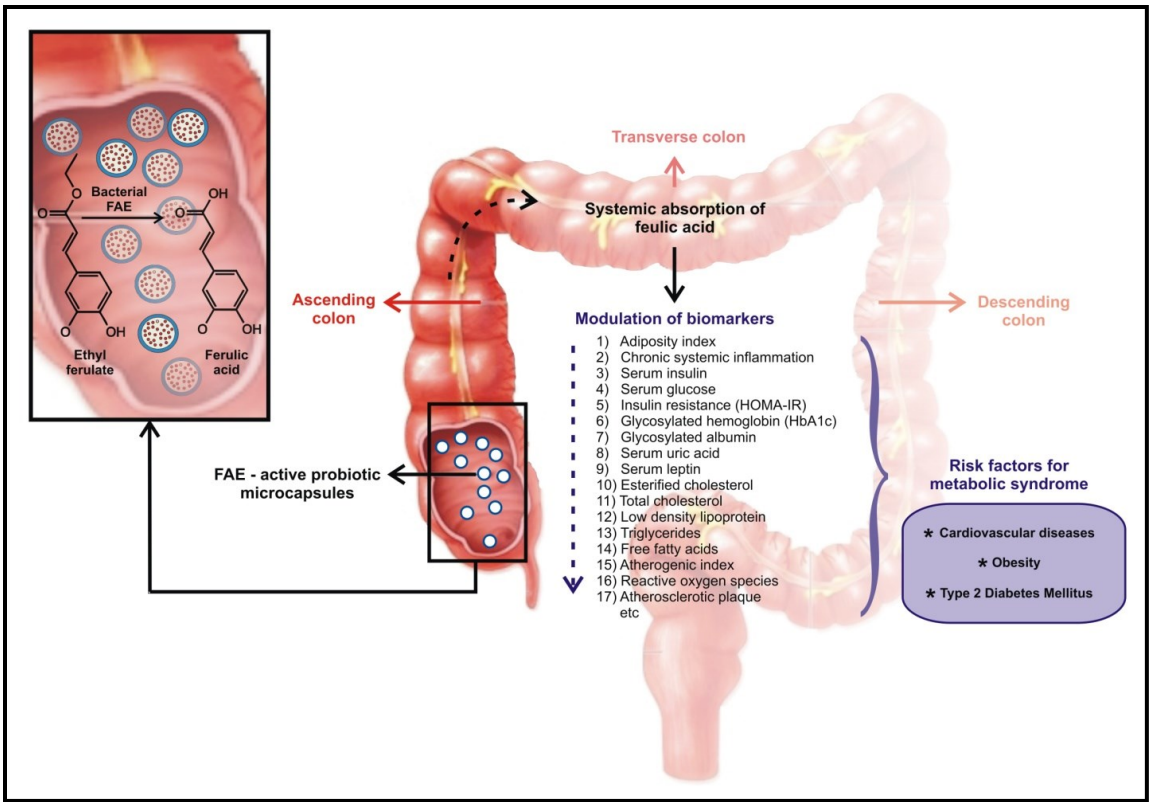


Figure 7.7: FA is proposed to modulate markers of metabolic syndrome, presenting a number of different mechanisms of action by which it can prove beneficial. Probiotic microcapsules, in the ascending colon, release FA due to FAE enzymatic activity, leading to the systemic absorption of FA. FA has been shown, in the presented research and in other works, to modulate a number of markers that play an important role in metabolic syndrome—cardiovascular diseases, obesity and T2DM.

Original Research Article 6

CHAPTER 8: EFFECT OF ORALLY ADMINISTERED *L. FERMENTUM* NCIMB 5221 ON MARKERS OF METABOLIC SYNDROME: AN IN VIVO ANALYSIS USING ZDF RATS

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Preface: Following investigations using a diet-induced model of MetS, we investigated the formulation's effect on a genetic model of MetS, the Zucker Diabetic Fatty rat, presented in this chapter. We investigated the probiotic effect on insulin levels, insulin resistance, serum triglycerides, serum low-density lipoprotein cholesterol, serum cholesterol, serum high-density lipoprotein cholesterol and the atherogenic and atherosclerosis index. This research indicates that the administration of the ferulic acid-producing *L. fermentum* can reduce insulin resistance, hyperinsulinemia, hypercholesterolemia, and other markers involved in the pathogenesis of MetS, in a genetic model of the syndrome.

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8.1 Abstract

Metabolic syndrome, encompassing type 2 diabetes mellitus and cardiovascular disease, is a growing health concern of industrialized countries. Ferulic acid (FA) is a phenolic acid found in foods normally consumed by humans that has demonstrated antioxidant activity, cholesterol-lowering capabilities and anti-tumorigenic properties. Selected probiotic bacteria, including *Lactobacillus fermentum* NCIMB 5221, produce FA due to intrinsic ferulic acid esterase (FAE) activity. The aim of the presented research was to investigate a FA-producing probiotic, *L. fermentum* NCIMB 5221, as a biotherapeutic for metabolic syndrome. The probiotic formulation was administered daily for 8 weeks to Zucker Diabetic Fatty (ZDF) rats, a model of hyperlipidemia and hyperglycemia. Results show that the probiotic formulation reduced fasted insulin levels and insulin resistance, significantly reduced serum triglycerides (p = 0.016), lowered serum low-density lipoprotein cholesterol levels (p = 0.008) and significantly reduced the atherogenic (p = 0.016) and atherosclerosis (p = 0.012) index, as compared to the control animals. In addition, the probiotic formulation significantly increased high-density lipoprotein cholesterol levels (p = 0.041), as compared to the control animals. This research indicates that administration of the FA-producing *L. fermentum* NCIMB 5221 can reduce insulin resistance, hyperinsulinemia, hypercholesterolemia, and other markers involved in the pathogenesis of metabolic syndrome. Further studies are required to investigate the exact mechanism(s) of action by which the probiotic formulation is affecting the markers and pathogenesis of metabolic syndrome.

8.2 Introduction

Metabolic syndrome, encompassing Type 2 Diabetes Mellitus (T2DM) and cardiovascular diseases, is a significant public health concern of industrialized countries (Ford et al. 2002a). In the United States in 2005, the incidence of T2DM was estimated at 16.2 million individuals and is projected to rise to 48.3 million by 2050 (Narayan et al. 2006a). T2DM is characterized by insulin resistance, hyperglycemia, systemic low-grade inflammation and hyperinsulinemia. Cardiovascular disease, responsible for an estimated 16.7 million deaths worldwide, is the leading cause of global mortality and morbidity (Tarride et al. 2009). Cardiovascular disease factors include dyslipidemia, hypercholesterolemia, hypertension, atherosclerosis and obesity. Metabolic syndrome encompasses factors associated with the pathogenesis of T2DM and cardiovascular diseases. The Zucker Diabetic Fatty (ZDF) rat is a model of metabolic syndrome, initiated with a mutation in the leptin receptor gene (Scarda et al. 2010). ZDF rats develop hyperglycemia (Mizuno et al. 2002), hyperinsulinemia (van den Brom et al. 2009), hypertriglyceridemia (van den Brom et al. 2009; Xu et al. 2009), hypercholesterolemia (Sparks et al. 1998), systemic low-grade inflammation (Lu et al. 2010), and diastolic and systolic dysfunction (van den Brom et al. 2009), all characteristic of metabolic syndrome. Current treatment methods for metabolic syndrome primarily involve lifestyle modifications as well as combinations of pharmacologic agents, all of which remain inefficient. The majority of patients remain far from achieving target goals for lipid, cholesterol and glucose levels (Lebovitz 2011).

Ferulic acid (FA) is a naturally found phenolic acid abundantly bound to foods consumed by humans (wheat bran, fruits, coffee, etc.). FA is a potent antioxidant able to

neutralize free radicals, such as Reactive Oxygen Species (ROS) (Rice-Evans et al. 1996) implicated in DNA damage (Lombard et al. 2005b), cancer (Hu et al. 2011), accelerated cell aging (Ishii et al. 1998), obesity (Sonta et al. 2004) and T2DM (Donath and Shoelson 2011; Sonta et al. 2004). FA has been shown to regulate blood glucose levels by modulating insulin secretion, by promoting pancreatic beta-cell survival and by reducing inflammatory markers linked to antioxidant activity (Adisakwattana et al. 2008). Interestingly, a number of gastrointestinal tract (GIT) bacterial strains have the enzyme feruloyl esterase (FAE) (Bhathena et al. 2007; Bhathena et al. 2008; Bhathena et al. 2009; Lai et al. 2009b; Tomaro-Duchesneau et al. 2012a; Tomaro-Duchesneau et al. 2012b), that hydrolyses and releases free FA from its bound state. *Lactobacillus fermentum* NCIMB 5221 has been shown to produce large quantities of FA that possess significant antioxidant activity (Tomaro-Duchesneau et al. 2012a). Metchnikoff, in the early 1900s, established the concept of probiotics, the ingestion of live microorganisms in foods (Metchnikoff 1907). Since then, probiotic bacteria have gained interest for the treatment of a number of disorders, including colon cancer, inflammatory bowel disease, allergies, oral diseases and hypercholesterolemia (Bhathena et al. 2013; Jones et al. 2013; Prakash et al. 2011b; Prakash et al. 2013; Saha et al. 2012a; Tomaro-Duchesneau et al. 2013b; Whelan and Quigley 2013). Probiotic bacteria have shown promising results for the management of metabolic syndrome, but are yet to prove complete clinical/therapeutic efficacy (Bogsan et al. 2011). We hypothesise that a FA-producing probiotic bacterium should prove beneficial for the treatment and prevention of metabolic syndrome.

Considering the beneficial effects of FA and probiotic bacteria, the delivery of FA-producing probiotic bacteria in the GIT should lead to metabolic changes which can

prove beneficial for the treatment of metabolic syndrome, including hyperglycemia, hyperinsulinemia, insulin resistance, hypercholesterolemia and hypertriglyceridemia. The presented research investigates, *in vivo*, the effects of daily administration of the probiotic FA-producing *L. fermentum* NCIMB 5221 on the pathogenesis and progression of metabolic syndrome using the ZDF rat animal model.

8.3 Materials and Methods

8.3.1 Bacterial growth media and chemicals

De Man-Rogosa-Sharpe (MRS) broth was obtained from Fisher Scientific (Ottawa, ON, Canada). Water was purified with an EASYpure Reverse Osmosis System and a NANOpure Diamond Life Science (UV/UF) ultrapure water system from Barnstead/Thermoline (Dubuque, IA, U.S.A.). All other chemicals were of analytical or high-performance liquid chromatography (HPLC) grade and purchased from commercial sources.

8.3.2 Bacterial strain and culture conditions

L. fermentum NCIMB 5221 was purchased from NCIMB (Aberdeen, Scotland, UK). The bacterial strain was stored at -80°C in MRS containing 20% (v/v) glycerol. An MRS-agar plate was streaked for isolation from the frozen stock and incubated at 37°C with 5% CO_2 for 24 h to ensure purity. One colony from the MRS-agar plate was inoculated into 5 mL of MRS broth and incubated at 37°C for 24 h. A 1% (v/v) inoculum was then used for subculturing and incubated at 37°C for 24 h immediately before use. To prepare the doses for daily administration to the animals, the overnight culture was centrifuged at 4000 rpm for 10 min at 4°C and the pellet was resuspended in 0.85% (w/v)

NaCl to a concentration of 5 x 10^9 cfu/mL of *L. fermentum* NCIMB 5221, determined by colony forming units (cfu) on MRS agar plates.

8.3.3 Ferulic acid production by *L. fermentum* NCIMB 5221

L. fermentum NCIMB 5221 was subcultured from MRS broth at 1% (v/v) to MRS-ethyl ferulate (EFA) broth at an EFA concentration of 1.33 mM (0.2956 mg/mL) and incubated at 37°C during the course of the experiment. A HPLC assay, modified from Mastihuba et al., was used to measure FA production (Mastihuba et al. 2002), as used previously by our group (Tomaro-Duchesneau et al. 2012a; Tomaro-Duchesneau et al. 2012b). Following 24 h of incubation, 500 µL of the culture was centrifuged at 10,000 rpm for 7 min at 4°C. The resulting supernatant (300 µL) was acidified with 0.35 M H_2SO_4 (100 µL) and briefly vortexed. 1 mM benzoic acid (300 µL) was added, as an internal standard, to each tube followed by the addition of 0.7 M NaOH (100 µL). The samples were filtered with a 0.45 µm syringe filter. HPLC analysis was performed on a reverse-phase C-18 column: LiChrosorb RP-18, 25 x 0.46 cm (Grace Davison Discovery Sciences, ON, Canada), with a 25 µL injection volume. The HPLC system consists of a ProStar 335 diode array detector (DAD) set at 280 nm and 320 nm, a ProStar 410 autosampler, and the software Star LC workstation version 6.41. The mobile phase (solvent A) was 37% (v/v) methanol and 0.9% (v/v) acetic acid in water. Solvent B consisted of 100% (v/v) methanol. The HPLC run was initiated with solvent A at 100% for 16 min. This was then followed by a 1 min linear gradient to reach 100% of solvent B, attained at the 17th min. Solvent B was isocratically held at 100% for 12 min, until the 29th min. This was then followed by a 1 min linear gradient to reach 100% of solvent A by the 30th min. A FA standard curve was generated using the concentrations 100, 300,

500, 960 and 1100 μM plotted against peak area (R^2 = 0.9869). The EFA standard curve was generated using the concentrations 100, 300, 500, 700, 1000, 1400 and 1800 μM plotted against peak area (R^2 = 0.9785).

8.3.4 Antioxidant production by *L. fermentum* NCIMB 5221

The antioxidant production of *L. fermentum* NCIMB 5221 was measured using a QuantiChrom™ Antioxidant Assay Kit. The protocol provided with the assay kit was followed. A standard curve was generated for Trolox, at concentrations of 0, 300, 600 and 1000 μM plotted against absorbance at 570 nm (R^2 = 0.9970). *L. fermentum* NCIMB 5221 was subcultured from MRS broth at 1% (v/v) to MRS-EFA broth. Uninoculated MRS-EFA broth was used as a negative control. Each sample was treated in triplicate and incubated at 37°C during the course of the experiment. Samples were removed at every time point and were stored at -20°C until the assay was performed.

8.3.5 Animals

Seven week old male ZDF (*Lepr^{fa}*) rats were obtained from Charles River Laboratories (Wilmington, MA, USA) and housed one per cage in a room with controlled temperature (22-24°C), humidity and an alternating light and dark cycle (12:12-hour light:dark cycle, lights on at 7am). All experimental protocols complied with the Animal Care Committee of McGill University and the Canadian Council on Animal Care guidelines.

8.3.6 Animal experimental protocol and animal diet

Upon arrival, the rats were allowed free access to food (Purina 5008 from Ren's Pet Depot, ON, Canada) and water for one week to allow their adaptation to the environment. The composition of this diet, by weight, is 23% protein, 58.5%

carbohydrate and 6.5% fat. Following the acclimatization period, non-fasting blood glucose baseline values were obtained from the tail vein, as well as determination of body mass. The obtained values were used to randomly assign animals into two groups (n = 8). The experiment lasted 8 weeks with a daily gavage of 2 mL ($1x10^{10}$ cfu of *L. fermentum* NCIMB 5221 dissolved in 0.85% (w/v) NaCl). The control group was administered 2 mL of 0.85% (w/v) NaCl (n = 8). Food consumption, water intake and animal mass were monitored routinely. Blood, serum and whole blood, were collected weekly from non-fasted animals by the lateral saphenous vein with a 23 gauge/19 mm needle, into Microtainer® serum separator tubes from Becton Dickinson (Franklin Lakes, NJ, USA). Serum was obtained by allowing the blood to clot for a minimum of 30 minutes and centrifugation for 5 minutes at 10,000 g. Serum samples were stored at -80°C until analysis. To obtain non-clotted whole blood, blood was also collected in Microtainer® tubes with K2E (K_2EDTA) from Becton Dickinson and stored at 4°C until analysis. At the end of the experimental period (8 weeks), the ZDF rats were euthanized by carbon dioxide asphyxiation and blood was withdrawn by cardiac puncture.

8.3.7 Non-fasted glucose monitoring

Non-fasted blood glucose levels were monitored daily using an Accu-Chek® Aviva blood glucose meter with Accu-Chek® Aviva test strips (Roche Diagnostics, Quebec, Canada). Approximately 10µ L of blood was obtained from the tail vein of each rat using a 23 gauge/19 mm needle and read immediately using the glucose metre. The time of glucose measurement was kept consistent daily to ensure that hourly fluctuations were not affecting the data.

8.3.8 Analysis of fasted clinical markers

Fasted serum samples were obtained at 11, 39 and 53 days, following 16 h of fasting, with free access to water. Triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL) cholesterol and free fatty acid (FFA) levels were assayed by conventional enzymatic methods on a Hitachi 911 automated clinical chemistry autoanalyzer (Roche Diagnostics, U.S.A.) using reagent kits supplied by Roche Diagnostics (Laval, QC, Canada). Low-density lipoprotein (LDL) levels were calculated using the Iranian formula: LDL = TC/1.19 + TG/0.81 – HDL/1.1 – 0.98 (mmol/L) (Ahmadi et al. 2008). Fasted insulin and Glucagon-like peptide-1 (GLP-1) levels were measured from serum using a Multi-Spot® Assay System with a Sector® Imager 2400 (Meso Scale Discovery®, Maryland, USA). Insulin resistance was calculated using the homeostatic model assessment for insulin resistance (HOMA-IR) equation: HOMA-IR = (Glucose x Insulin) / (22.5) (Matthews et al. 1985).

8.3.9 Determining percentage glycosylated hemoglobin and fructosamine levels

Hemoglobin (Hb), glycosylated hemoglobin (HbA1c) and fructosamine levels were measured from serum collected by the cardiac puncture at sacrifice (non-fasted). They were assayed by conventional enzymatic methods on a Hitachi 911 automated clinical chemistry autoanalyzer using reagent kits supplied by Roche Diagnostics. % HbA1c was calculated using the formula: % HbA1c = 91.5 x HbA1c/Hb + 2.15, as recommended by the reagent kit manufacturer.

8.3.10 Determining the Atherogenic and Atherosclerosis Index

The atherogenic and atherosclerosis index for the treatment and control groups were determined on days 11, 39 and 53 of probiotic treatment. The atherogenic index was calculated from fasted serum samples using the formula: log(TG/HDL) (Dobiasova 2004). The atherosclerosis index was calculated from fasted serum samples using the formula: LDL/HDL (Mertz 1980).

8.3.11 Oral sucrose tolerance tests

The oral sucrose tolerance tests were performed on weeks 2, 5 and 8 of probiotic treatment. The rats were fasted for 16 hours, with free access to water, prior to the test. Sucrose was orally administered to each rat at a concentration of 2 mg/kg body weight. Glucose measurements were obtained, using the Accu-Chek® Aviva blood glucose meter with Accu-Chek® Aviva test strips, at times 0, 30, and 120 minutes following sucrose administration. Blood was also collected in serum separator tubes and processed as aforementioned. GLP-1 and insulin were measured using a Multi-Spot® Assay System with a Sector® Imager 2400. The trapezoidal rule was used to calculate areas under the curve (AUC).

8.3.12 Statistical analysis

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, USA). Statistical comparisons were carried out using two-way analysis of variance (ANOVA) comparing the means of the treatment and control groups over time, followed by multiple comparisons of the means

using Tukey's post-hoc analysis. Statistical significance was set at $p < 0.05$ and p-values less than 0.01 were considered highly significant.

8.4 Results

8.4.1 *L. fermentum* NCIMB 5221 ferulic acid and antioxidant production

The production of FA and other antioxidants by *L. fermentum* NCIMB 5221 was determined following 24 h in its substrate *in vitro* (**Table 8.1**), as an indicator of potential *in vivo* activity. For a bacterial count of 1 x 10^{10} cfu, the associated FA production is 1.338 ± 0.090 mg. Similarly the antioxidant production of *L. fermentum* NCIMB 5221 is 3783.32 ± 76.11 μmoles of Trolox equivalents.

8.4.2 Effect of *L. fermentum* NCIMB 5221 on animal mass and non-fasted glucose

Dietary intake, food consumption, water consumption and non-fasted blood glucose were monitored throughout the 8 weeks. The animals, of both groups, gained significant amounts of body weight over the course of the treatment period (**Figure 8.1**). Following the 8 week treatment period, there was no significant difference in body mass between the control (460.5 ± 11.8 g) and the probiotic treated (461.1 ± 14.3 g) animals ($p = 0.974$). In addition, throughout the 8 weeks, there was no significant difference in food and water consumption between the treated and control groups (data not shown). Daily non-fasted glucose monitoring demonstrated a significant increase in blood glucose levels in both the treatment and control groups over the treatment period compared to baseline (**Figure 8.2**). No significant difference ($p = 0.490$) in non-fasted blood glucose levels was

measured following the 8 week treatment period between the control (19.54 ± 1.89 mM) and the treated (22.23 ± 3.33 mM) animals.

8.4.3 Effect of *L. fermentum* NCIMB 5221 on serum insulin, insulin resistance and GLP-1 levels

Fasted serum insulin levels were measured at days 11, 39 and 53. The insulin levels significantly increased during the treatment period compared to baseline (**Figure 8.3A**). At day 39, the fasted serum levels for the treatment group (3932.7 ± 969.8 pg/mL) were non-significantly (p = 0.153) lower than the control group (6024.7 ± 985.6 pg/mL). At day 53, the fasted serum insulin levels had no significant difference (p = 0.343) between the control (6309.8 ± 1355.5 pg/mL) and the treated (4314.1 ± 1514.5 pg/mL) animals. Insulin resistance was determined at the end of 53 days, and showed a non-significant reduction (p = 0.111) in the *L. fermentum* NCIMB 5221 treated animals (42.66 ± 12.77) as compared to the control animals (72.51 ± 10.90) (**Figure 8.3C**). Fasted GLP-1 levels were also measured at days 11, 39 and 53. At day 39, GLP-1 levels were not significantly different (p = 0.554) between the control (29.92 ± 2.53 pg/mL) and treated (28.05 ± 1.72 pg/mL) animals (**Figure 8.3B**). Similarly, at day 53, the GLP-1 levels were not significantly different (p = 0.734) between the treated (27.70 ± 3.81 pg/mL) and control (29.66 ± 4.16 pg/mL) animals.

8.4.4 Effect of *L. fermentum* NCIMB 5221 on fructosamine and HbA1c serum levels

Fructosamine and HbA1c levels were measured at sacrifice from non-fasted samples. Fructosamine levels were not significantly different (p = 0.527) in the control (195.16 ± 14.48 μM) and treated (207.81 ± 13.04 μM) animals (**Figure 8.4A**). Similarly,

HbA1c levels were not significantly different (p = 0.284) at sacrifice between the control (4.68 ± 0.27 %) and treated (5.23 ± 0.41 %) groups (**Figure 8.4B**).

8.4.5 Effect of *L. fermentum* NCIMB 5221 on fasted serum TG, TC, HDL, LDL, FFA levels

Fasted TG, TC, HDL, LDL and FFA serum levels were measured at days 11, 39 and 53. Serum TG levels increased significantly for both the control and probiotic treated animals throughout the course of the 8 weeks (**Figure 8.5A**). At day 53 of the treatment period, the fasted serum TG levels were significantly lower (p = 0.016) in the treated (12.18 ± 0.97 mM) as compared to the control (15.21 ± 0.54 mM) animals.

Fasted serum TC levels also significantly increased throughout the course of the 8 weeks, in both the control and probiotic treated groups (**Figure 8.5B**). By day 53, there was a non-significant decrease (p = 0.328) in TC levels in the *L. fermentum* NCIMB 5221 treated animals (4.97 ± 0.32 mM) compared to the control (5.34 ± 0.16 mM) animals.

Fasted serum HDL levels decreased throughout the course of the 8 weeks for both the control and probiotic-treated animals (**Figure 8.5C**). At day 39, the *L. fermentum* NCIMB 5221 treated animals (1.198 ± 0.195 mM) had non-significantly (p = 0.247) higher levels of HDL compared to the control animals (0.939 ± 0.088 mM). In addition, by day 53, the *L. fermentum* NCIMB 5221 treated animals (1.026 ± 0.174 mM) had significantly higher (p = 0.041) fasted serum HDL levels (0.525 ± 0.128 mM).

As the time progressed, fasted serum LDL levels increased in both the control and probiotic-treated animals (**Figure 8.5D**). At day 39, the control animals (18.15 ± 1.03 mM) had non-significantly (p = 0.145) higher serum LDL levels than the *L. fermentum* NCIMB 5221 treated animals (15.61 ± 1.28 mM). More importantly, at day 53, there was

a highly significant difference (p = 0.008) in serum LDL levels between the treated (16.70 ± 1.41 mM) and the control (21.80 ± 0.76 mM) animals.

Fasted serum FFA levels were also monitored at days 11, 39 and 53. There was no significant difference detected between the control and probiotic-treated animals at any of the time points measured (**Figure 8.5E**).

8.4.6 Effect of *L. fermentum* NCIMB 5221 on atherogenic and atherosclerosis index

The atherogenic and atherosclerosis index were calculated at days 11, 39 and 53 from the fasted serum levels of TG, LDL and HDL. The atherogenic index significantly increased throughout 8 weeks, for both the control and probiotic-treated animals (**Figure 8.6A**). At day 53, there was a significant difference (p = 0.016) between the control (1.53 ± 0.10) and the *L. fermentum* NCIMB 5221 treated (1.10 ± 0.11) animals. The atherosclerosis index also significantly increased throughout the 8 weeks, for both the control and probiotic-treated animals (**Figure 8.6B**). At day 53, the *L. fermentum* NCIMB 5221 treated animals (22.29 ± 5.53) had a significantly lower (p = 0.012) average atherosclerosis index than the control animals (57.10 ± 9.96).

8.4.7 Effect of *L. fermentum* NCIMB 5221 on sucrose tolerance

Oral sucrose tolerance tests were performed at days 11, 39 and 53 with glucose, insulin and GLP-1 monitoring (**Table 8.2**). There was no significant difference in sucrose tolerance between the *L. fermentum* NCIMB 5221 treated and the control animals, throughout the 8 weeks. No significant difference in AUC was demonstrated at any time between the treated and control animals. Interestingly, a significant difference (p = 0.034) in fasted serum glucose was observed early (day 11 of treatment) between the control

(9.78 ± 0.43 pg/mL) and treated (8.43 ± 0.38 pg/mL) animals, with no significant differences as the time progressed.

8.5 Discussion

Metabolic syndrome encompasses a number of medical disorders linked to the development of cardiovascular disease and T2DM, including raised triglyceride levels, reduced levels of HDL-C and elevated fasted glucose levels (National Cholesterol Education Program (NCEP) Expert Panel on Detection Evaluation and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) 2002). The ZDF rat is an inbred model of hyperlipidemia and hyperglycemia with administration of the Purina 5008 diet. These animals develop a metabolic syndrome phenotype through genetic mutation and manipulation of the diet, similar to the etiology of metabolic syndrome in humans of the Western World. Current treatment for metabolic syndrome primarily involves lifestyle changes (diet and exercise), but may also involve the use of drug therapies aimed at lowering blood pressure, LDL-C and serum glucose levels (Grundy et al. 2005). These therapies remain ineffective and pose important side effects, as such, a novel therapeutic is required.

Probiotic bacteria, when administered in adequate amounts, can confer important health benefits on the host (FAO and WHO 2001). Probiotic bacteria have shown great interest for the treatment and prevention of a wide spectrum of health disorders (Prakash et al. 2011b). Recent research with probiotic bacteria focuses on the microbial secretion and production of beneficial biologically active enzymes and proteins. These include: bile salt hydrolase for hypercholesterolemia (Jones et al. 2012b; Jones et al. 2013; Tanaka et

al. 1999b), ornithine decarboxylase for the treatment of autoimmune diseases and accelerated cell apoptosis (Mates et al. 2002), and bile tolerance and transport proteins for the efficient delivery of probiotic bacteria (Pfeiler and Klaenhammer 2009). Another microbial protein, cinnamoyl esterase, has gained interest in recent studies (Bhathena et al. 2012; Tomaro-Duchesneau et al. 2012a). Of these, FAE make up a large group of hydrolases that can produce the phenolic acid FA. FA is a component of plant cell walls, and is naturally present in its bound state in foods, such as wheat bran, whole grains and fruits. Previous research by our group has demonstrated that *L. fermentum* NCIMB 5221 is a FAE-active probiotic that produces large amounts of FA (Tomaro-Duchesneau et al. 2012a). Furthermore, the administration of a microencapsulated FA-producing *L. fermentum* formulation in a high-fat fed hamster model has demonstrated important effects on markers of metabolic syndrome (Bhathena et al. 2012).

We first determined the FA and antioxidant production of *L. fermentum* NCIMB 5221 when incubated with substrate (EFA). This was determined as an indicator of the potential activity *in vivo*. It is clear, however, that investigations into the exact quantities produced *in vivo* and the mechanisms of action of these are required. Albeit, both FA and antioxidants have great potential as biotherapeutics for metabolic syndrome (Adisakwattana et al. 2008; Balasubashini et al. 2004; Ford et al. 2003; Sri Balasubashini et al. 2003).

Based on the FA and antioxidant production results and previous probiotic screening by our group (Tomaro-Duchesneau et al. 2012a), we then investigated the effect of *L. fermentum* NCIMB 5221 on markers of metabolic syndrome in the ZDF rat model. Following the 8 weeks of treatment with the probiotic bacteria, there was no

significant difference in fasted glucose levels nor animal mass between the treated and control groups, throughout the 8 weeks. This potentially suggests the need for a longer treatment period, or the administration of a higher probiotic dose or a probiotic that produces more FA, to have a significant impact on hyperglycemia and obesity. In terms of long-term glycemia monitoring, there was no significant difference in percent HbA1c and fructosamine following the 8-week treatment period. In terms of percent HbA1c, one must keep in mind that these levels only reveal the percentage of circulating hemoglobin that has chemically reacted with glucose and, as such, reflects the blood glucose levels over the 120 days preceding the test (Gabbay et al. 1977). It is unaffected by short-term fluctuations in glycemia present in a short-term study (56 days) like this one. Serum fructosamine levels represent the reaction between fructose and ammonia (or an amine), and the glucose levels over the last 2-3 weeks. It may also be used to calculate HbA1c using the equation: Fructosamine = ((HbA1c - 1.61) x 58.82). The measured values of fructosamine and percent HbA1c do correspond with this equation, and demonstrate no difference between the control and probiotic-treated groups in terms of long-term glycemia levels. Furthermore, no difference in sucrose tolerance levels was observed between the probiotic-treated and control animals, as investigated in the oral sucrose tolerance test.

On the other hand, there was a difference in insulin levels, although not significant, between the control (6309.8 ± 1355.5 pg/mL) and treated (4314.1 ± 1514.5 pg/mL) animals at the end of the 8-week treatment period. These results suggest that *L. fermentum* NCIMB 5221 may moderately control hyperinsulinemia. Hyperinsulinemia results in increased levels of intracellular sodium and decreased intracellular potassium

levels, important contributors of hypertension (Modan et al. 1985). Hypertension is a key component of metabolic syndrome, as it is associated with obesity, dyslipidemia and glucose intolerance (Modan et al. 1985). The *L. fermentum* NCIMB 5221 formulation also demonstrated potential to decrease insulin resistance, as determined using the HOMA-IR, although not significantly ($p = 0.111$). These results demonstrate the ability of the FA-producing probiotic to improve the sensitivity of the peripheral tissues to insulin.

The effects on hyperlipidemia and hypercholesterolemia by the daily administration of *L. fermentum* NCIMB 5221 were also investigated. Serum TG levels were shown to be significantly reduced ($p = 0.016$) following 53 days of probiotic administration, as compared to the placebo animals. Hypertriglyceridemia, as well as being a key component of metabolic syndrome, is closely associated with insulin resistance (Kissebah et al. 1976). The probiotic formulation was also shown to decrease serum TC levels, although not significantly. Indeed, previous research by our group has demonstrated that a microencapsulated *L. fermentum* formulation could lower cholesterol levels in a hamster model of hypercholesterolemia (Bhathena et al. 2009). Of interest, while lowering TC levels, the *L. fermentum* NCIMB 5221 formulation significantly increased ($p = 0.041$) HDL cholesterol levels, as compared to the animals administered placebo. As detailed by the National Institutes of Health (NIH), low HDL levels are an important criteria for diagnosing a patient with metabolic syndrome (National Institutes of Health 2001). In addition, *L. fermentum* NCIMB 5221 had a highly significant impact ($p = 0.008$) on fasted LDL cholesterol levels, another factor of metabolic syndrome

(Grundy 2011). On the other hand, the probiotic formulation had no effect on serum FFA levels.

The daily administration of *L. fermentum* NCIMB 5221 to the ZDF rats significantly lowered the atherogenic index ($p = 0.016$). Indeed, as aforementioned, HDL cholesterol levels have been shown to have an inverse relationship with cardiovascular risk. However, the contribution of serum TG levels to cardiovascular disease and metabolic syndrome has been underestimated (Dobiasova 2004). The atherogenic index considers both the HDL and the TG levels, and hence, is an important marker of metabolic syndrome. In addition, the daily administration of *L. fermentum* NCIMB 5221 significantly reduced the atherosclerosis index ($p = 0.012$) following 8 weeks of treatment, as compared to the control animals. Although atherosclerosis was not investigated directly in the rats, the atherosclerotic index simply provides an indication of the LDL/HDL ratio. This suggests further control of hyperlipidemia, a key component of metabolic syndrome, by the *L. fermentum* NCIMB 5221 probiotic formulation.

The consumption of probiotic bacteria has shown great potential for the treatment and prevention of metabolic syndrome, specifically T2DM and cardiovascular diseases. Indeed, previous research has demonstrated that dahi, a probiotic formulation, can control hyperglycemia, hyperinsulinemia, dyslipidemia, glucose intolerance and oxidative stress (Yadav et al. 2007; Yadav et al. 2008). In this study, a FA-producing *L. fermentum* NCIMB 5221 demonstrated similar potential, controlling hyperinsulinemia, hypertriglyceridemia, insulin resistance and hypercholesterolemia. The exact mechanism(s) of action behind the observed effects is not clear. Further investigations, both *in vitro* and *in vivo*, will focus on determining the mechanism(s) of action in terms

of the metabolic effects of FA and the probiotic. One proposed mechanism of action, that remains to be investigated, is the role of FA and probiotics on the low-grade systemic inflammatory profile of metabolic syndrome (Donath and Shoelson 2011), as FA has been characterized as an antioxidant molecule (Tomaro-Duchesneau et al. 2012a) capable of diminishing inflammation (Srinivasan et al. 2007). In addition, probiotic bacteria have, themselves, demonstrated anti-inflammatory potential which may prove beneficial for the treatment and prevention of metabolic syndrome (Isolauri et al. 2002).

The presented study demonstrated the capability of *L. fermentum* NCIMB 5221 to modulate hyperinsulinemia, insulin resistance, hypertriglyceridemia and hypercholesterolemia, all key components of metabolic syndrome. Further investigations are required to elucidate the precise mechanism(s) of action behind the observed effects. A longer treatment period may also be necessary to observe greater probiotic effects on the metabolic syndrome markers, as well as to be relevant to the management of metabolic syndrome in humans. Studies investigating the levels of FA production as well as its biodistribution may also prove interesting in the future. Nonetheless, the presented research provides strong evidence for the role of a dietary FA-producing probiotic as a biotherapeutic for the modulation of markers of metabolic syndrome.

8.6 Acknowledgements

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8.7 Conflict of interest

The authors declare that they have no conflict of interest.

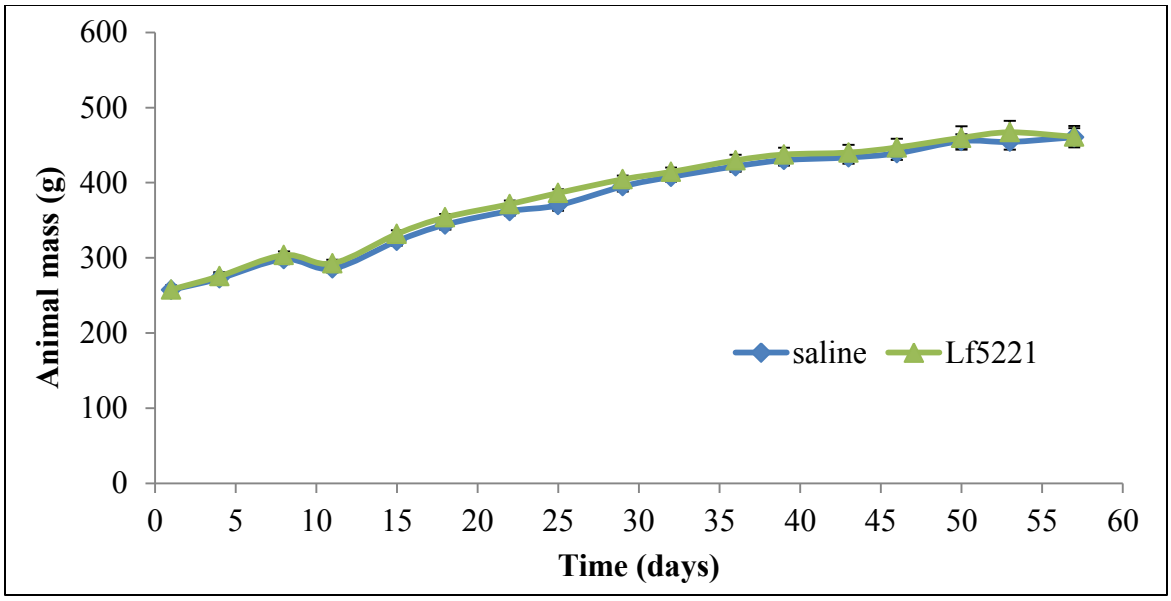


Figure 8.1 Animal mass was monitored throughout the 8 weeks, with the probiotic *L. fermentum* NCIMB 5221 (Lf5221) administered daily. Following the 8-week treatment period, there was no significant difference (p = 0.974) in body mass between the control (460.5 ± 11.8 g) and the treated (461.1 ± 14.3 g) animals. Data is presented as mean ± SEM, n = 8

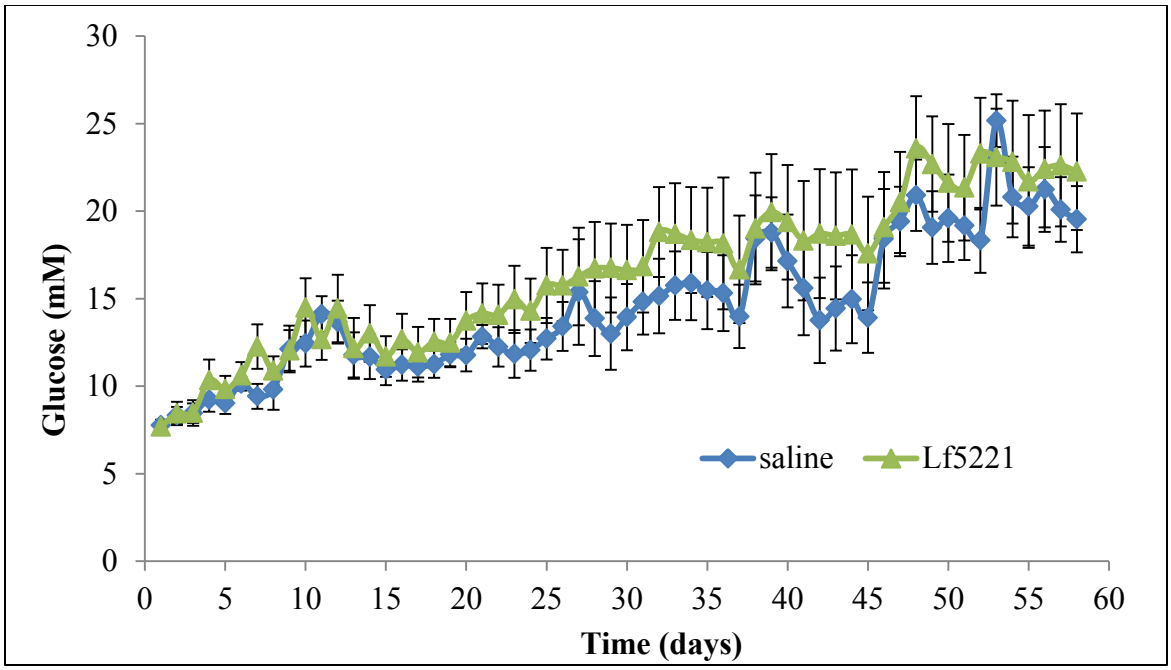
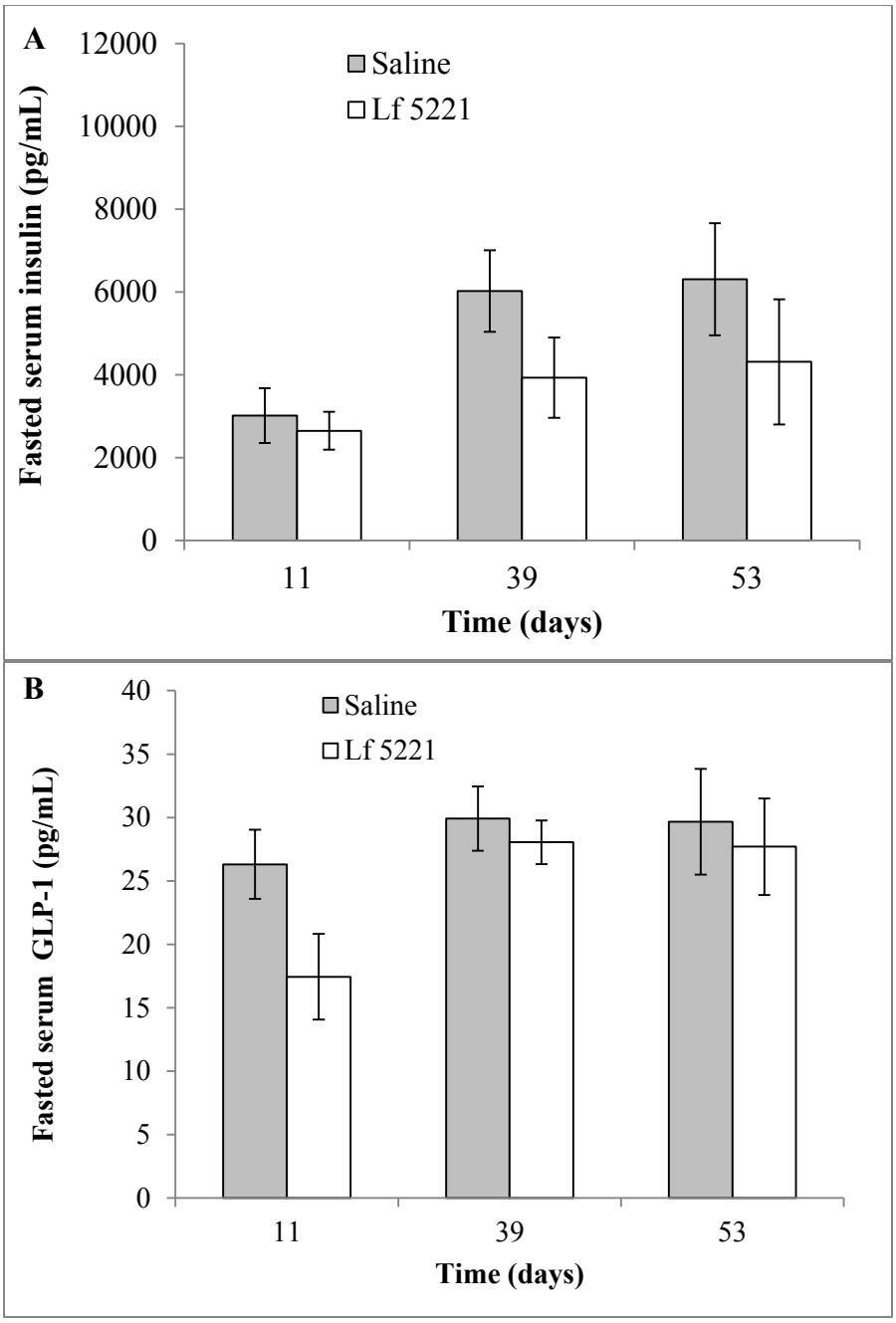


Figure 8.2 Non-fasted blood glucose was monitored daily during the 8 weeks with daily administration of *L. fermentum* NCIMB 5221 (Lf 5221), with an increase in glycemia in both the treated and control groups over time. No significant difference (p = 0.490) was measured at the end of the 8 week treatment period between the control (19.54 ± 1.89 mM) and the treated (22.23 ± 3.33 mM) animals. Data is presented as mean ± SEM, n = 8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Fasted serum insulin (pg/mL)
12000
10000
8000
6000
4000
2000
0
Saline
Lf 5221
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Time (days)
B
Fasted serum GLP-1 (pg/mL)
40
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Time (days)

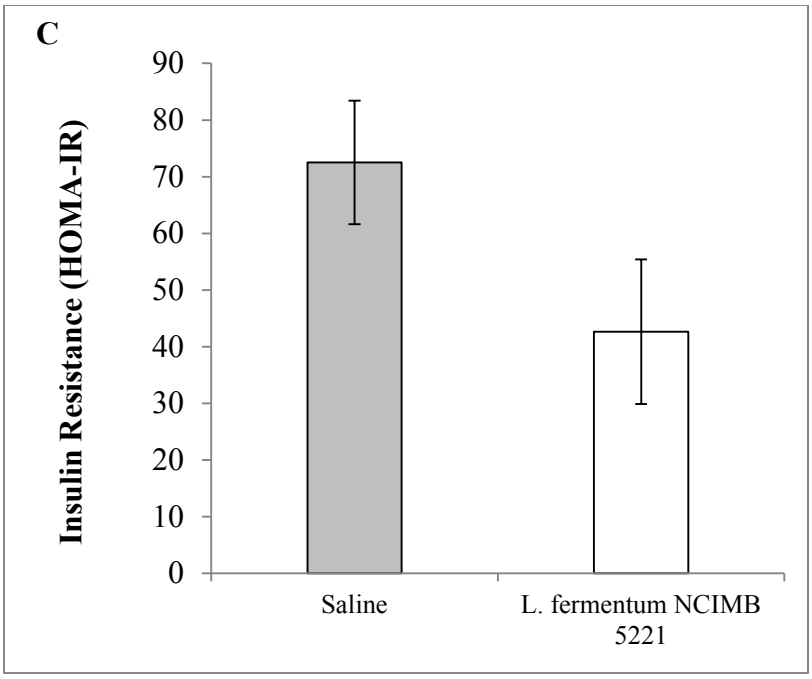


Figure 8.3 (A) Fasted serum insulin and (B) GLP-1 were measured at days 11, 39 and 53 following *L. fermentum* NCIMB 5221 (Lf5221) daily administration. At day 39, the fasted insulin serum levels for the treated group (3932.7 ± 969.8 pg/mL) were non-significantly ($p = 0.153$) lower than the control group (6024.7 ± 985.6 pg/mL). At day 53, there was no significant difference ($p = 0.343$) in insulin levelsbetween the control (6309.8 ± 1355.5 pg/mL) and the treated (4314.1 ± 1514.5 pg/mL) groups. At day 53, the GLP-1 levels were not significantly different ($p = 0.734$) between the treated (27.70 ± 3.81 pg/mL) and control (29.66 ± 4.16 pg/mL) animals. **(C)** Insulin resistance was determined using HOMA-IR, with a non-significant reduction ($p = 0.111$) in the treated animals (42.66 ± 12.77) as compared to the control animals (72.51 ± 10.90). Data is presented as mean ± SEM, n=8.

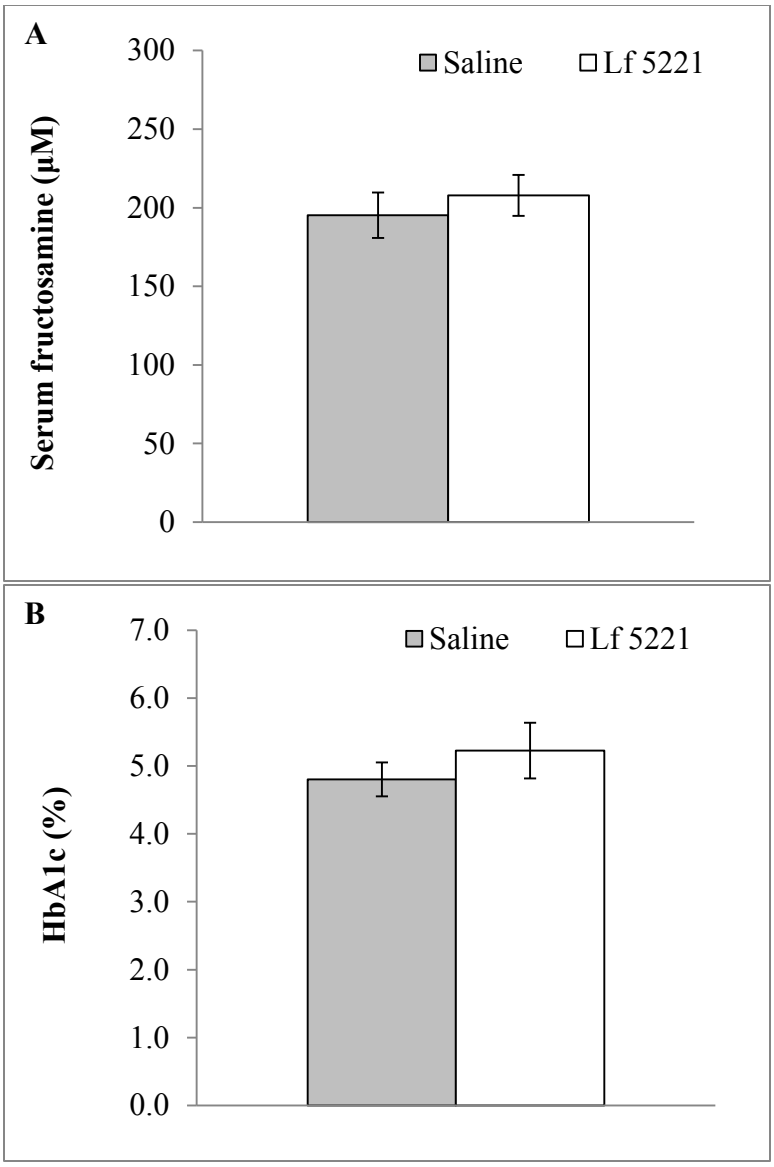
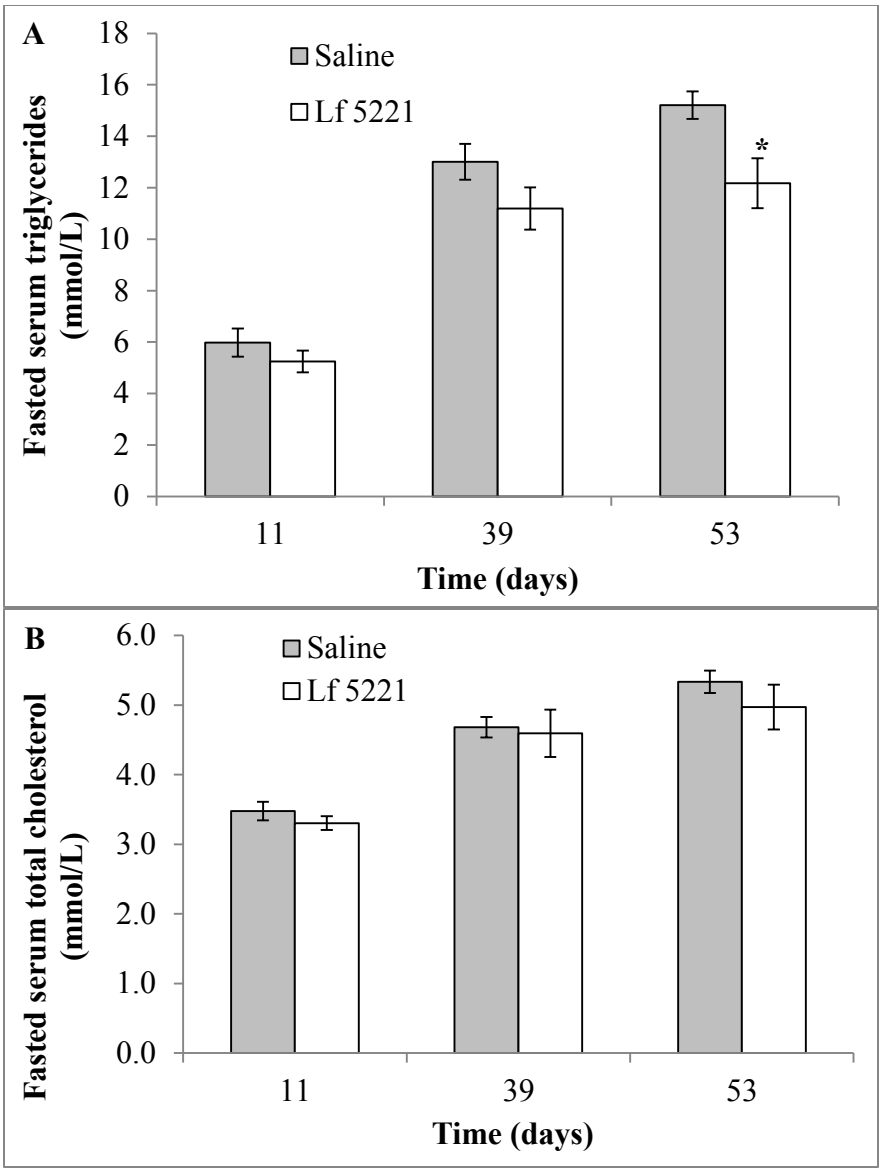


Figure 8.4 Serum (A) fructosamine and (B) % HbA1c following 8 weeks of treatment with *L. fermentum* NCIMB 5221 (Lf5221). Fructosamine levels were not significantly different (p = 0.527) in the control (195.16 ± 14.48 µM) and treated (207.81 ± 13.04 µM). Similarly, % HbA1c levels were not significantly different (p = 0.284) between the control (4.68 ± 0.27 %) and treated (5.23 ± 0.41 %) groups. Data is presented as mean ± SEM, n = 8


A
Fasted serum triglycerides (mmol/L)
18
16
14
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8
6
4
2
0
Saline
Lf 5221
*
11
39
53
Time (days)
B
Fasted serum total cholesterol (mmol/L)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Saline
Lf 5221
11
39
53
Time (days)

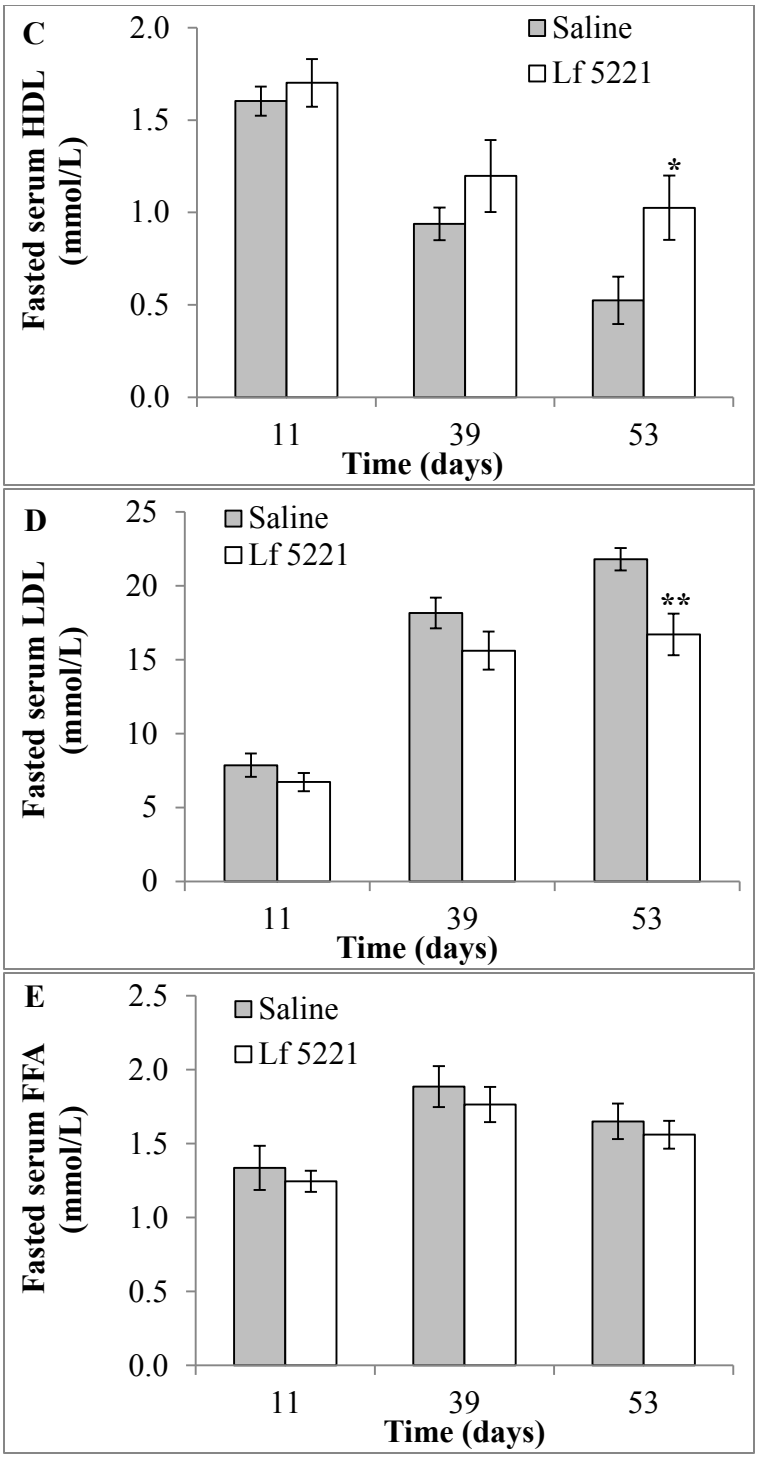

Figure 8.5 Fasted serum (A) TG, (B) TC, (C) HDL, (D) LDL and (E) FFA levels were measured following treatment with *L. fermentum* NCIMB 5221 (Lf5221). Following 53 days, TG levels were significantly lower (*p = 0.016) in the treated (12.18 ± 0.97 mM) compared to control (15.21 ± 0.54 mM) animals. As well, the treated animals (1.026 ± 0.174 mM) had significantly higher (*p = 0.041) HDL levels and significantly lower (**p = 0.008) LDL levels (16.70 ± 1.41 mM) compared to control animals. Data is presented as mean ± SEM, n = 8

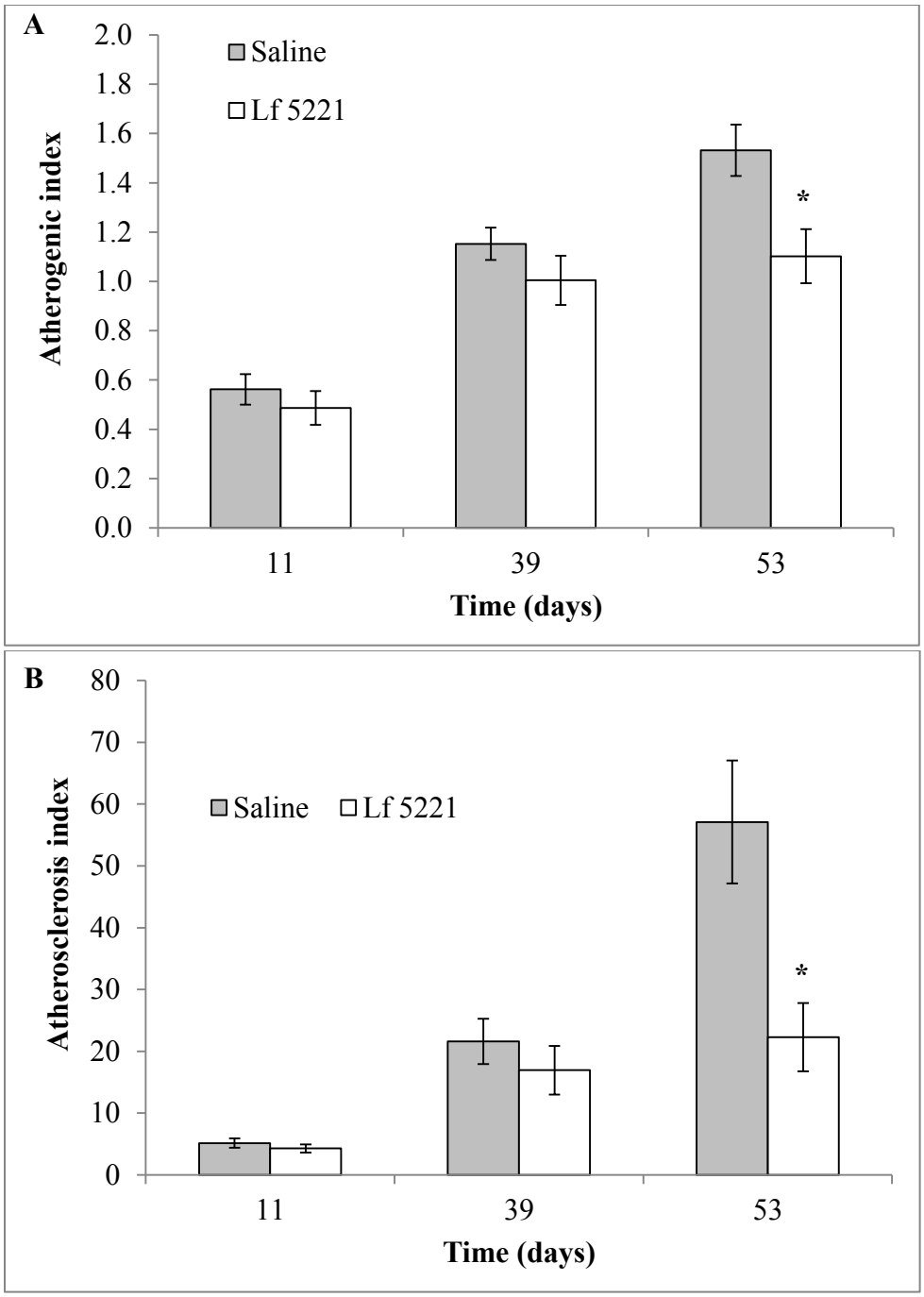

Figure 8.6 The (A) atherogenic and (B) atherosclerosis index were calculated following 11, 39 and 53 days of *L. fermentum* NCIMB 5221 (Lf5221) treatment. The atherogenic and atherosclerosis index increased throughout the course of the 8 weeks, for both the control and probiotic-treated groups. At day 53, there was a significant difference in the atherogenic index (*p = 0.016) between the control (1.53 ± 0.10) and the *L. fermentum* NCIMB 5221 treated (1.10 ± 0.11) animals. The *L. fermentum* NCIMB 5221 treated animals (22.29 ± 5.53) also had a significantly lower (*p = 0.012) atherosclerosis index than the control animals (57.10 ± 9.96). Data is presented as mean ± SEM, n = 8

Table 8.1: Ferulic acid and antioxidant production of *L. fermentum* NCIMB 5221 following a 24 h incubation in ethyl ferulate.

	in vitro	**Per bacterial cell**	***in vivo* dose**
Bacterial cell number (cfu)	$1.219 \times 10^{9} \pm 1.57 \times 10^{7}$	1.000 ± 0.0129	$1 \times 10^{10} \pm 1.29 \times 10^{8}$
EFA hydrolysis (mg)	0.273 ± 0.0013	$2.24 \times 10^{-10} \pm 1.10 \times 10^{-12}$	2.236 ± 0.011
FA production (mg)	0.163 ± 0.011	$1.34 \times 10^{-10} \pm 8.97 \times 10^{-12}$	1.338 ± 0.090
Antioxidant production (µmoles Trolox equivalents)	461.25 ± 9.28	$3.78 \times 10^{-7} \pm 7.61 \times 10^{-9}$	3783.32 ± 76.11

Data is presented as mean ± SEM, n = 3.

Table 8.2: Oral sucrose tolerance test results at days 11, 39 and 53 for glucose, insulin and GLP-1.

	Saline	**Lf5221**	***p*-value**
Serum glucose			
Day 11			
Fasting (pg/mL)	9.78 ± 0.43	8.43 ± 0.38	***p* = 0.034***
Post-prandial (pg/mL)	9.45 ± 0.75	8.20 ± 0.61	$p = 0.217$
AUC	1424 ± 84	1319 ± 112	$p = 0.468$
Day 39			
Fasting (pg/mL)	8.63 ± 1.51	10.81 ± 0.85	$p = 0.233$
Post-prandial (pg/mL)	10.45 ± 2.21	11.80 ± 2.153	$p = 0.668$
AUC	1411 ± 259	1710 ± 246	$p = 0.416$
Day 53			
Fasting (pg/mL)	10.08 ± 0.94	12.40 ± 1.66	$p = 0.249$
Post-prandial (pg/mL)	12.31 ± 1.99	13.16 ± 2.58	$p = 0.798$
AUC	1756 ± 255	1990 ± 329	$p = 0.584$
Serum insulin			
Day 11			
Fasting (pg/mL)	3017.8 ± 660.7	2650.5 ± 455.9	$p = 0.655$
Post-prandial (pg/mL)	2310.6 ± 299.4	3635 ± 517.6	$p = 0.054$
AUC	285 629 ± 28 329	452 432 ± 68 848	$p = 0.051$
Day 39			
Fasting (pg/mL)	6024.7 ± 985.6	3932.7 ± 969.8	$p = 0.153$
Post-prandial (pg/mL)	6283.8 ± 1160.0	4908.4 ± 1080.9	$p = 0.400$
AUC	950 608 ± 159 997	664 553 ± 146 206	$p = 0.208$
Day 53			
Fasting (pg/mL)	6309.8 ± 1355.5	4314.1 ± 1514.5	$p = 0.343$
Post-prandial (pg/mL)	5300.1 ± 1137.8	4150.5 ± 1413.7	$p = 0.537$
AUC	835 031 ± 179 956	654 769 ± 229 639	$p = 0.547$
Serum GLP-1			
Day 11			
Fasting (pg/mL)	26.31 ± 2.73	17.45 ± 3.38	$p = 0.061$
Post-prandial (pg/mL)	23.00 ± 2.06	32.48 ± 2.13	$p = 0.019$
AUC	3037.6 ± 178.8	3273.7 ± 166.4	$p = 0.369$
Day 39			
Fasting (pg/mL)	29.92 ± 2.53	28.05 ± 1.72	$p = 0.554$
Post-prandial (pg/mL)	36.70 ± 2.17	40.09 ± 2.77	$p = 0.352$
AUC	4124.8 ± 243.3	4577.7 ± 314.8	$p = 0.274$
Day 53			
Fasting (pg/mL)	29.66 ± 4.16	27.70 ± 3.81	$p = 0.734$
Post-prandial (pg/mL)	41.71 ± 3.37	48.67 ± 3.95	$p = 0.202$
AUC	4720.0 ± 451.3	5435.6 ± 366.0	$p = 0.239$

Data is presented as mean ± SEM, n = 8.

CHAPTER 9: GENERAL DISCUSSION

MetS, which encompasses T2DM and CVD, has become a significant health and economic concern, especially of industrialized countries (Ford et al. 2002b; Narayan et al. 2006b). MetS is characterized by its associated risk factors, specifically abdominal obesity, atherogenic dyslipidemia, elevated blood pressure, insulin resistance, proinflammatory state and prothrombotic state (Grundy et al. 2004). In patients diagnosed with MetS, the therapeutic focus lies primarily on lifestyle changes, including diet and exercise modifications. Pharmacologic agents focus on the primary risk factors of MetS and often include combinations therapies of statins, metformin and hypertension drugs. Many of these agents have important side-effects, as presented in **Table 2.2** and, regardless of their use, many patients simply fail to achieve the necessary goals for lipid, cholesterol and glucose levels set by the WHO, EGIR and NCEP/ATP III, presented in **Table 2.1** (Lebovitz 2011). It is evident that a novel therapeutic approach is required to efficiently prevent and treat MetS.

In recent years, the role of low-grade chronic systemic inflammation in MetS, termed "metaflammation" has been described (Hotamisligil 2006; Medzhitov 2008), rendering inflammation as a potential therapeutic target. In addition, the gut microbiota has been directly associated with MetS and its related disorders, initially demonstrated using germ-free rodents (Backhed et al. 2004). With the gut microbiota as an interesting therapeutic target, probiotic bacteria become of interest, as these have been shown as capable of modifying the composition of the microbes of the gastrointestinal tract to promote health (Prakash et al. 2011b). Of even greater interest is the fact that certain probiotic bacteria possess anti-inflammatory properties which may provide a synergistic

approach for the management of inflammation, as well as for the modulation of the contents of the microbiota. One such anti-inflammatory activity involves ferulic acid esterase (FAE) which allows for the production of ferulic acid (FA). This gives rise to the initial thesis research hypothesis, that a novel microencapsulated FAE-active *Lactobacillus fermentum* formulation can be designed and used as a MetS biotherapeutic, by reducing chronic systemic inflammation and hypercholesterolemia.

The first objective of the project, presented in Chapter 3, was to screen, select and characterize a FA-producing probiotic *Lactobacillus* bacterium. FA was chosen as a probiotic molecule of interest as it has shown a number of properties beneficial to the development of a MetS therapeutic, including its activity as an antioxidant (Kayahara et al. 1999; Ketsawatsakul et al. 2000; Ou et al. 1999), anti-inflammatory (Ou et al. 2003; Sakai et al. 1999), antimicrobial (Jeong et al. 2000), antidiabetic (Balasubashini et al. 2004) and cholesterol-lowering agent (Son et al. 2010). Previous research by our lab had demonstrated that a number of probiotic bacteria have FAE activity which allows for probiotic FA production (Bhathena et al. 2007). The screening and selection of a probiotic strain was initiated with this research, and *L. fermentum* NCIMB 5221 was selected as the probiotic strain that produces the greatest levels of FA, with *L. fermentum* NCIMB 2797 producing the second highest levels. Indeed, following the selection of *L. fermentum* NCIMB 5221 we demonstrated that the probiotic strain demonstrated significant antioxidant production, correlated to its FA production and growth kinetics. The selected strain was used in the remainder of the project.

The second objective presented in this thesis was to microencapsulated *L. fermentum* NCIMB 5221 and characterize the formulation for the viable delivery of the

probiotic cells through simulated gastrointestinal tract transit. An important concern of probiotic oral delivery is the harsh environment of the upper gastrointestinal tract characterized by acidic ph, digestive enzymes and bile (Prakash et al. 2011b). Microencapsulation is a method defined as "the entrapment of a compound or a system inside a dispersed material for its immobilization, protection, controlled release, structuration and functionalization" (Poncelet 2006) and can be used for the immobilization of microorganisms (Prakash et al. 2011b; Tomaro-Duchesneau et al. 2013b). The most commonly used microencapsulation method is the APA microcapsule which relies on the polyelectrolyte complexation between the polymers alginate and PLL. In the presented work, APA microencapsulation of *L. fermentum* NCIMB 5221 significantly increased, by 2.5 log, the number of viable cells remaining following an *in vitro* gastrointestinal transit as compared to the number of non-microencapsulated cells. In addition, our findings demonstrated that the APA microcapsule did not slow the mass transfer of the EFA substrate into and the FA product out of the microcapsules, as the FA production was not significantly different between microencapsulated and non-microencapsulated *L. fermentum* NCIMB 5221. These results suggested that APA microencapsulation could be used as an effective oral delivery method for the later animal studies.

The third objective of the thesis research was to investigate and characterize the anti-inflammatory properties of the probiotic *L. fermentum in vitro* using macrophage cells and a co-culture model of the colon epithelium. As aforementioned, chronic low-grade systemic inflammation is a key component of MetS pathogenesis. Research has demonstrated that changes in the human gut microbiota can alter the integrity of the

intestinal barrier, leading to an increased permeability to luminal contents. LPS, an endotoxin present in Gram-negative bacteria, promotes the secretion of pro-inflammatory cytokines, especially TNF-α, by macrophages, and has been associated with a number of features of metabolic diseases (Cani et al. 2007b). In the presented work we demonstrated that *L. fermentum* NCIMB 2797 produced high levels of FA, antioxidants and NO, all probiotic-originating modulators of inflammation. In addition, the probiotic *L. fermentum* cell-free supernatant, but not whole cells, significantly decreased TNF-α and NO production by LPS-stimulated RAW 264.7 macrophages. Using a co-culture model, adapted from work by Tanoue *et al.* (Tanoue et al. 2008), we also demonstrated that the cell-free supernatant of *L. fermentum* NCIMB 2797 prevented LPS-induced intestinal epithelial permeability. These results demonstrate the potential of a *L. fermentum* NCIMB 2797 biotherapeutic for managing MetS associated inflammation, as well as other inflammatory disorders.

The fourth objective of this work was to investigate and characterize the probiotic effects on cholesterol metabolism *in vitro*, by measuring probiotic cholesterol assimilation, probiotic adhesion to the colon epithelium and inhibition of cholesterol uptake by colon epithelial cells. One of the risk factors of MetS, described earlier, is atherogenic dyslipidemia, comprised of elevated LDL and triglycerides and reduced HDL (Musunuru 2010). Probiotic bacteria have demonstrated important hypocholesterolemic properties (Bhathena et al. 2013; du Toit et al. 1998; Huang and Zheng 2010; Jones et al. 2013; Yoon et al. 2011). Previous groups have demonstrated that certain probiotic bacteria can assimilate cholesterol when grown in its presence, contributing to cholesterol-lowering effects *in vivo* (Lin and Chen 2000; Tahri et al. 1996). In the

presented work, both *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221 successfully assimilated large quantities of cholesterol, potentially due to probiotic-cholesterol co-precipitation, however this is normally observed when oxgall or bile is supplemented in the growth media (Gilliland et al. 1985). Work by previous groups also demonstrated that certain probiotic strains can decrease cholesterol uptake by Caco-2 colon epithelial cells (Yoon et al. 2013). We investigated this mechanism in this thesis work and demonstrated that both *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 5221 significantly decreased cholesterol uptake by Caco-2 cells by a moderate 14%. Previous studies by Yoon *et al.* observed cholesterol uptake inhibition up to 40-45% for certain probiotic strains (Yoon et al. 2013), potentially attributed to the presence of bile salt hydrolase activity in the probiotic strains investigated (Jones et al. 2013), by the downregulation of Niemann-Pick C1-like (NPC1L1) (Yoon et al. 2013) and the upregulation of ATP-binding cassette sub-family G members 5 and 8 (ABCG5/G8) and Liver X receptor (LXR) (Yoon et al. 2011). The effects on cholesterol suggest the potential of the two *L. fermentum* strains for managing the atherogenic dyslipidemia associated with MetS.

The last two research chapters focus on the *in vivo* investigations of the probiotic formulations on markers of MetS. No studies have been presented investigating probiotic therapeutics for the management of MetS as a whole, as presented in this work. The *in vivo* work used both a dietary model of MetS, the BioF1B hamster fed a high fat diet, and a genetic model of MetS, the Zucker Diabetic Fatty rat. These studies demonstrated that the developed FAE-active probiotic *L. fermentum* formulations could delay the onset of insulin resistance, hyperglycemia, hyperinsulinemia, dyslipidemia, inflammation and

obesity and provide a lower risk for developing T2DM and cardiovascular disease. Specifically, *L. fermentum* NCIMB 2797, investigated in BioF1B Golden Syrian hamsters, successfully modulated cholesterol, esterified cholesterol, free fatty acids, leptin, insulin, uric acid, adiposity index, insulin resistance and glycosylated albumin. In addition, *L. fermentum* NCIMB 5221, in the Zucker Diabetic Fatty rat, reduced serum triglycerides, low-density lipoprotein cholesterol and the atherogenic and atherosclerosis index, while increasing high-density lipoprotein. These studies demonstrate the potential of a FAE-active probiotic formulation for the prevention and treatment of MetS.

As presented in this work, though probiotic formulations are demonstrating increasing potential as biotherapeutics for a number of health disorders, there are a number of important issues that remain. Importantly, a number of probiotic formulations may be found on the market, but many lack basic supporting research, including successful clinical trials with regards to safety and efficacy. For example, probiotic bacteria are generally regarded as safe, but side effects, including septicemia and fungaemia have been reported in some high-risk individuals. Several research groups are now undertaking comprehensive safety profiling of probiotic strains for clinical applications. Safety profiling of probiotic bacteria include determining the proper strain identification, DNA sequencing and annotating, metabolic profiling, antiobiotic resistance, bacteriocin production, hydrogen peroxide production, organic acid production and biogenic amine production. Of course, human clinical studies are also ideal to conclude probiotic safety. Thus, probiotic strains identified in the presented thesis work should be evaluated further for safety before any clinical use is undertaken. Indeed, when developing probiotic biotherapeutics, the dose is always controversial. In the

presented work, the probiotic dose administered is the highest possible based on microcapsule volume and oral administration volume permitted in rodents. For both safety and efficacy purposes, future studies should focus on dose-optimization. The duration of treatment is also an issue that needs to be investigated further.

As observed in the results of this thesis, it is also clear that not all probiotic strains are effective, and there is also considerable variation between strains within a bacterial species. In this work, probiotic strains were initially screened for FA production based on previous research and, as such, only a few strains were thoroughly investigated. For the development of the best FA-producing probiotic formulation, one can envision that a more thorough screening process may be required. In addition, the choice of EFA as a substrate may be questioned. More complex substrates, such as wheat bran, may be more relevant to *in vivo* studies where the enzyme is to act on a dietary matrix. Two different strains were used in this thesis work. *L. fermentum* NCIMB 5221 was the best FA producer and *L. fermentum* NCIMB 2797 (ATCC 11976) was the second best. However, during the studies investigating anti-inflammatory activity, *L. fermentum* NCIMB 2797 demonstrated anti-inflammatory properties, unlike *L. fermentum* NCIMB 5221.

In terms of FAE activity as the mode of action, with the *in vivo* investigations, one may question whether the observed effects on MetS markers are due to the probiotic or FA. Other mechanisms of action may involve probiotic modulation of the microbiota composition, the production of short-chain fatty acids or other secreted by products. Investigations using a lesser FA-producing strain, or a knock-out of FAE in the selected strain could be undertaken in the future. As well, investigations into the specific mechanism(s) of action of the probiotic are required, including gene expression studies.

For example, the cholesterol-lowering mechanism of action could be further investigated via analysis of markers such as NPC1L1, LXR and HMC-CoA reductase. Investigations into the probiotic modulation of the gut microbiota contents may also be of interest, as this is a primary goal of a number of probiotic studies. In addition, the presence of FA in the systemic circulation as well as in specific organ locations could be further investigated. One may also consider the use of a "cocktail" formulation, like VSL#3, of probiotic bacteria, one which contains different bacterial strains, each with their respective mechanism of action. The combination of a number of probiotic mechanism(s) of action may provide a more holistic approach for the treatment of a number of health disorders, like MetS, that are complex and multi-etiological.

It is clear that the development of probiotic formulations is inherently limited by the research into disease pathogenesis and etiology. As well, in terms of both probiotic and microencapsulation development, industrial scale-up technologies are required for future clinical studies. Additionally, preclinical and clinical studies must continue to ensure the efficiency and safety of any probiotic formulation.

CHAPTER 10: CLAIMED ORIGINAL CONTRIBUTIONS TO KNOWLEDGE AND CONCLUSIONS

10.1 Claimed original contributions to knowledge

The presented research findings show that a novel microencapsulated FAE-active *Lactobacillus* formulation can be used as a MetS biotherapeutic.

The specific novel findings are:

1. Probiotic bacteria were screened for ferulic acid esterase activity and the results demonstrated the successful hydrolysis of ethyl ferulate by a number of probiotic *Lactobacillus* strains, suggesting ferulic acid production by ferulic acid esterase.

2. The probiotic bacteria selected for the presence of ferulic acid esterase activity were screened to select the strain with the greatest ferulic acid production, *Lactobacillus fermentum* NCIMB 5221 which produced 0.168 ± 0.001 mg/mL FA following 48 h of incubation in ethyl ferulate.

3. Investigations into the antioxidant properties of *L. fermentum* NCIMB 5221, a potential probiotic anti-inflammatory mechanism of action, demonstrated that the strain has a significant antioxidant activity of 509.58 ± 13.23 μM Trolox equivalents, directly correlated to its ferulic acid production and growth kinetics.

4. Alginate-polylysine-alginate microcapsules, of a size of 400 ± 25 μm, were used to encapsulate *L. fermentum* NCIMB 5221, and these were tested for oral delivery efficacy. The microcapsules provided significant protection of the bacterial cell viability (2.5 log greater than non-microencapsulated cells) and ferulic acid esterase activity through a simulated gastrointestinal transit, demonstrating the microcapsule suitability for future *in vivo* investigations employing oral administration.

5. The anti-inflammatory properties of *L. fermentum* NCIMB 2797 were investigated and the results demonstrate that the strain produces significant levels of the antioxidant ferulic acid (262.38 ± 39.48 μM), the inflammatory modulator

nitric oxide (144.83 ± 7.95 μM) and total antioxidant molecules (595.71 ± 23.58 Trolox equivalents). In addition, *L. fermentum* NCIMB 2797 was shown to significantly decrease the secretion of pro-inflammatory TNF-α ($p < 0.0001$) and nitric oxide ($p < 0.0001$) by macrophage cells stimulated with lipopolysaccharide. These results suggest the suitability of this strain to reduce MetS-associated inflammation.

6. To further investigate the anti-inflammatory properties of *L. fermentum* NCIMB 2797 we investigated whether this strain could reduce inflammation (LPS)-linked loss of intestinal epithelial integrity. The results demonstrate that this probiotic strain significantly reduced ($p < 0.0001$) the inflammation-induced intestinal permeability using an *in vitro* co-culture epithelial model containing colon epithelial cells and macrophages.

7. Investigations with *L. fermentum* NCIMB 5221 and *L. fermentum* NCIMB 2797 were performed to investigate whether these strains could influence MetS associated dyslipidemia by assimilating cholesterol from a solution. The results demonstrate that both *L. fermentum* NCIMB 5221 ($p = 0.003$) and *L. fermentum* NCIMB 2797 ($p = 0.012$) were capable of assimilating significant amounts of cholesterol when incubated in media containing the latter.

8. Studies were undertaken to investigate whether *L. fermentum* NCIMB 5221 and *L. fermentum* NCIMB 2797 could induce toxic effects on the colon epithelium and results demonstrate no toxicity (loss of viability) of Caco-2 colon epithelial cells ($p > 0.05$) exposed to the two probiotic strains.

9. The cell surface hydrophobicity of *L. fermentum* NCIMB 5221 and *L. fermentum* NCIMB 2797 probiotic cells was investigated as an indicator of adhesion to the colon epithelium using a hydrocarbon adhesion assay. The results demonstrate that *L. fermentum* NCIMB 5221 and *L. fermentum* have hydrophobic cell surfaces of 70.30 ± 8.85% and 55.60 ± 2.59 %, respectively.

10. To further investigate the probiotic cell attachment to the colon epithelium, *L. fermentum* NCIMB 5221 and *L. fermentum* NCIMB 2797 cells were co-incubated with Caco-2 colon epithelial cells, and demonstrated attachments of 5.30 x 10^6 ± 6.45 x10^5 cfu/well and 1.42 x 10^6 ± 1.33 x10^5 cfu/well, respectively, after 6 h of co-incubation, potentially indicating a longer residence time *in vivo*, following oral delivery.

11. As a cholesterol-lowering mechanism of probiotic bacteria we investigated whether *L. fermentum* NCIMB 5221 and *L. fermentum* NCIMB 2797 could inhibit cholesterol uptake by Caco-2 colon epithelial cells. Both of the probiotic strains successfully inhibit Caco-2 cholesterol uptake, by 14.12 ± 2.07% and 13.55 ± 1.71%, repectively.

12. The *L. fermentum* NCIMB 2797 probiotic formulation was investigated as a therapeutic for MetS i*n vivo*, in a dietary model of MetS, BioF1B Golden Syrian hamsters. The probiotic formulation successfully modulated cholesterol (p = 0.024), esterified cholesterol (p = 0.0328), free fatty acids (p = 0.029), leptin (p = 0.048), insulin (p = 0.0042), uric acid (p = 0.025), adiposity index (p = 0.0014), insulin resistance (p = 0.0096) and glycosylated albumin (p = 0.00013).

13. The *L. fermentum* NCIMB 2797 formulation demonstrated the potential, *in vivo* and *in vitro* to delay the onset of insulin resistance, hyperglycemia, hyperinsulinemia, dyslipidemia, inflammation and obesity, indicating a lower risk of diabetes and cardiovascular disease, all important components of MetS.

14. The *L. fermentum* NCIMB 5221 probiotic formulation was investigated as a MetS therapeutic i*n vivo*, in a genetic model of MetS, the Zucker Diabetic Fatty rat. *L. fermentum* NCIMB 5221 reduced serum triglycerides (p = 0.016), low-density lipoprotein cholesterol (p = 0.008) and the atherogenic (p = 0.016) and atherosclerosis index (p = 0.012), while increasing high-density lipoprotein (p = 0.041).

15. The *L. fermentum* NCIMB 5221 probiotic formulation demonstrated the potential, *in vivo* and *in vitro* to reduce insulin resistance, hyperinsulinemia, inflammation and hypercholesterolemia, all key risk factors associated with MetS.

10.2 Conclusions

In recent years, there has been a great interest in the development of probiotic biotherapeutics for a number of health disorders. Metabolic syndrome (MetS) has become an increasing public health concern of industrialized countries. Interestingly, MetS is not considered a disease but, rather, a cluster of metabolic disturbances which include: abdominal obesity, atherogenic dyslipidemia, elevated blood pressure, insulin resistance, proinflammatory state and prothrombotic state. Therapeutic approaches for MetS focus on dietary and lifestyle modifications, as well as pharmacological interventions, all of which have proven ineffective. There is an urgent need for the development of a successful therapeutic for the prevention and treatment of MetS. In the presented thesis work we have developed a novel microencapsulated ferulic acid esterase-active *Lactobacillus fermentum* formulation capable of reducing inflammation and hypercholesterolemia, important for the development of a MetS biotherapeutic, and demonstrated its efficacy in dietary (BioF1B hamster) and genetic (Zucker Diabetic Fatty rat) models of MetS.

Specifically, ferulic acid producing *L. fermentum* NCIMB 5221 and *L. fermentum* NCIMB 2797 were selected because of their ferulic acid production. The effect of *L. fermentum* NCIMB 2797 on inflammation was determined *in vitro* using macrophage cells and a co-culture model of the colon epithelium. In addition, both *L. fermentum* NCIMB 5221 and *L. fermentum* NCIMB 2797 demonstrated important cholesterol

lowering properties, *in vitro* via cholesterol assimilation and by inhibition of colon epithelial cell cholesterol uptake. *In vivo* using a high-fat diet induced model of MetS, *L. fermentum* NCIMB 2797 demonstrated the potential to delay the onset of insulin resistance, hyperglycemia, hyperinsulinemia, dyslipidemia and obesity. In addition, in a genetic model of MetS, *L. fermentum* NCIMB 5221 reduced insulin resistance, hyperinsulinemia and hypercholesterolemia. This work demonstrates the immense potential of ferulic acid producing probiotic bacteria for the development of a successful orally-administered MetS probiotic therapeutic.

CHAPTER 11: RECOMMENDATIONS

The work presented in this thesis demonstrates the potential of ferulic acid producing probiotic *L. fermentum* bacteria for the development of a successful MetS therapeutic. However, a number of additional investigations are required to be able to realize its potential as a biotherapeutic for MetS and other human health benefits. One of these investigations is the need to broaden the screening process by including other bacterial strains, rather than the narrow selection presented in this thesis. For the selection of a probiotic strain producing FA from the diet, investigations should be performed using more real and complex FA substrates, such as wheat bran and other food components. In this work, probiotic strains were initially screened for FA production, based on EFA hydrolysis.

In terms of the delivery of live bacterial cells, although probiotic cells are generally recognized as safe, a detailed safety profiling of the selected probiotic bacteria should be undertaken, specifically with respect to proper strain identification, DNA sequencing and annotating, metabolic profiling, antiobiotic resistance, bacteriocin production, hydrogen peroxide production, organic acid production and biogenic amine production. Of course, human clinical studies are ideal to conclude probiotic safety.

Future *in vitro*, pre-clinical and clinical studies should focus on the probiotic dose, formulation stability and duration of the *in vivo* treatment period requires further optimization. In the presented work, the probiotic dose administered is the highest possible based on microcapsule volume and oral administration volume permitted in rodents. For both safety and efficacy purposes, future studies should focus on dose-optimization. With the *in vivo* investigations, one may also question whether the observed

effects on MetS markers are due to the probiotic or FA, demonstrating the need for mechanistic studies. Investigations using a lesser FA-producing strain, or a knock-out of FAE in the selected strain could be undertaken in the future.

Most importantly, the mechanism of action of FA producing probiotic cells in MetS should be further investigated. Investigations into the probiotic modulation of the gut microbiota contents may also be of interest, as this is a primary goal of a number of probiotic studies. These investigations may provide additional information with regards to the mechanism of action of FA-producing probiotic cells. One could also investigate the use of a FA-producing probiotic in combination with currently used pharmaceutic formulations.

As a whole, FA-producing probiotic bacteria have great potential as MetS biotherapeutics, especially since there is an immediate need for a safe and effective orally administered therapeutic approach. In addition, this novel formulation can be investigated for a number of other biomedical, industrial and pharmaceutical applications.

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