Master’s Thesis:

Isolation, in vivo characterization, and safety validation of nitrate reductase active Lactobacillus fermentum NCIMB 702342 and its implications in addressing metabolic disease

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ABSTRACT

*Lactobacillus* is a diverse genus of lactic acid bacteria with many strains that have been used for food fermentation. Given their historic use in foods and the ability of some strains to reduce nitrate, these bacteria are perfect candidates as a promising probiotic for treating metabolic disease. Herein, a nitrate reductase active *lactobacillus* was isolated from a library of candidate bacteria and an assay was developed to quantify its enzymatic activity. Moreover, a study was conducted to assess the strain’s nitrate reductase activity in vivo following induced hypertension using N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) in an animal study. The results indicated that the probiotics did not result in attenuation of hypertension; however, the isolated strain, *L. fermentum* NCIMB 72023, may potentially reduce systemic nitrate to nitrite. Furthermore, safety validation was performed on *Lactobacillus fermentum* NCIMB 702342. Species-level identity was achieved through metabolic and genetic techniques and confirmed that the strain was a *Lactobacillus fermentum*. Although *L. fermentum* NCIMB 702342 was found to be resistant to clindamycin, the strain was susceptible to 7 other antibiotics. Furthermore, formation of metabolic by-products and antimicrobial agents were not observed.

In summary, *L. fermentum* NCIMB 702342 was isolated from a library of candidate strains for its nitrate reductase activity, an assay was developed to quantify it enzymatic rate, an in vivo strain efficacy trial was conducted, and preliminary in vitro safety were assessed.

**Keywords**: *Lactobacillus fermentum*, bacterial screening, nitrate reductase, safety validation
RÉSUMÉ

*Lactobacillus* est un genre diversifié de bactéries d’acide lactiques avec de nombreuses souches qui sont utilisées pour la fermentation des aliments. Compte tenu de leur utilisation historique dans les aliments et la capacité de certaines souches de réduire les nitrates, ces bactéries sont des candidats parfaits pour leur utilisation comme probiotiques. Ici, un lactobacille exprimant une réductase du nitrate a été isolé à partir d’une bibliothèque de candidats bactériens et une méthode a été développé pour quantifier son niveau d’activité enzymatique. Une étude animal a été faite pour déterminer les effets *in vivo* des bactéries exprimant l’enzyme de réduction du nitrate dans un modèle d’hypertension induit par le L-NAME (N⁵-nitro-L-arginine méthyl ester). Les résultats indiquent que la souche sélectionnée ne réduit pas l’hypertension bien qu’elle puisse possiblement réduire le nitrate sanguin en nitrite. En outre, la sécurité alimentaire du *Lactobacillus fermentum* NCIMB 702342 a été validée. L'identité au niveau de l’espèce a été obtenue par des techniques génétiques et métaboliques et a confirmé que la souche était un *Lactobacillus fermentum*. Bien que *L. fermentum* NCIMB 702342 ait été démontré résistant à la clindamycine, la souche était sensible à 7 autres antibiotiques. De plus, la formation de métabolites et d’agents antimicrobiens n’a pas été observée.

En résumé, *L. fermentum* NCIMB 702342 a été isolé à partir d’une bibliothèque de souches candidates pour son activité de réduction du nitrate, une méthode d’analyse a été développé pour quantifier l’activité enzymatique, une évaluation *in vivo* de sa capacité à réduire l’hypertension a été faite et la sécurité alimentaire préliminaire a été évaluée.

**Mots-clés:** Lactobacillus fermentum, le dépistage des bactéries, réduction du nitrate, validation de la sécurité
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PREFACE

This thesis was prepared in accordance with the guidelines established by McGill University for thesis preparation and submission:

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

Hence, the experimental section of this thesis is compiled as a collection of original papers that are suitable for publication. These papers consist of an Abstract, Introduction, Materials and Methods, Results, and Discussion. Moreover, a common Abstract, General Introduction, Literature Review, General Discussion, Summary, Conclusions, Future Directions, References, and Appendix are also included in this thesis.
LIST OF ABBREVIATIONS

Terms

ANOVA  Analysis of variance
MRS  de Man, Rogosa and Sharpe media
NO$_3^-$  Nitrate
NO$_2^-$  Nitrite
NO  Nitric oxide
STD  Standard Deviation
oxyHb  Oxyhemoglobin
methHb  Methemoglobin
  U  Units of enzymatic activity
  L.  Lactobacillus
L-NAME  N$^G$-nitro-L-arginine methyl ester

Units

CFU  colony forming units
ml  milliliters
μl  microliters
mM  millimolar
g  grams
kg  kilograms
mg  milligrams
L  liter
h  hour
ppm(V)  Parts per million(volume)
w/v  weight/volume
v/v  volume/volume
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CHAPTER 1: GENERAL INTRODUCTION

1.1 Overview

Consumption of nitrate has been a controversial issue, associated with health benefits (gastroprotective effects, inhibits platelet aggregation, and increases cardiac health [4]) as well as health risks (bladder and gastric cancer, and methaemoglobinemia in infants [5]). Due to its ubiquitous presence in water as a consequence of a variety of environmental factors [5] and high content in vegetables and cured meats [6], consumption of nitrate is inevitable. A potential solution for high nitrate consumption is the use of nitrate reductase active bacteria. Several strains of Lactobacillus have demonstrated nitrate reductase activity [7] and other strains have commonly been used in food fermentation, earning these strains the GRAS designation in the United States [8]. Hence, use of a Lactobacillus strain as probiotics for reduction of nitrate to nitrite is an attractive cost effective and simple means of addressing aspects of metabolic disease.

To determine efficacy of a probiotic strain to elicit a certain response before use in the public, the use of an animal models is a prerequisite prior to conducting clinical trials. Several animal models have been used; however, rats are the most popular and their use in studies have been increasing annually [9]. Due to their short life cycles and the availability of several hypertension models, these animals have been widely used in metabolic disease studies [10-15]. Concurrent with determination of efficacy, in vitro safety validations must be performed on the probiotics to ensure that they do not produce harmful metabolic by-products and antimicrobial compounds or transfer antibiotic resistance to neighbouring bacterial

In this thesis, a strain of nitrate reductase active Lactobacillus fermentum was isolated from a library of candidate strains for its nitrate reductase activity and an assay using nitrate-rich beet root juice assay media was developed to quantify it enzymatic activity. Furthermore, an animal study was conducted to determine if the two highly nitrate reductase active bacteria could reduce nitrate in vivo. To assess strain safety for potential human consumption, preliminary in vitro safety tests were conducted.
1.2 Thesis Hypothesis

Using an *in vitro* chemiluminescence assay, a suitable nitrate reductase active probiotic can be isolated from a library of *Lactobacillus* strains that can then be identified as safe, through a battery of *in vitro* safety assays, as well as efficacious at reducing ingested nitrate for potentially treating symptoms of metabolic disease in a hypertension-induced animal model.

1.3 Thesis Research Objectives

The thesis objectives are to:

i. Identify a nitrate reductase active strain of *Lactobacillus* from a library of candidate strains and develop an assay for quantification of its enzymatic activity

ii. Perform an animal study to determine the *in vivo* efficacy of *Lactobacillus fermentum* NCIMB 7230 and *Lactobacillus fermentum* NCIMB 702342 to reduced dietary nitrate resulting in an increase of plasma nitrite concentration and subsequent attenuation of L-NAME induced hypertension

iii. Investigate the *in vitro* safety profile of *Lactobacillus fermentum* NCIMB 702342: establish strain identity using genetic and metabolic techniques; establish production of metabolic by-products and antimicrobial compounds; and determine the strain’s antibiotic resistance

1.4 Thesis Outline

This thesis consists of 6 chapters. Chapter 1 provides a general introduction and states the research objectives of this thesis. An extensive literature review is outlined in Chapter 2 and allows the reader to understand the fundamental concepts discussed in Chapters 3-5. Chapters 3-5 are presented as original papers that aim to address the aforementioned research objectives. A general discussion of the key findings will be presented in Chapter 6 whereas Chapter 7 will consist of the summary, conclusion, and future directions.
CHAPTER 2: LITERATURE REVIEW

Scientific and medical interest in the effects of nitrate consumption has grown considerably over the past decades. As a ubiquitous inorganic compound, consumption of nitrate has been linked to both health benefits and risks. Hence, understanding the factors that can contribute to a specific outcome has been a topic of intense research. It is widely known that atmospheric nitrogen is converted to ammonium via nitrogen fixation. As the ammonium is serially oxidized, it ultimately produces nitrate. In the reverse process, denitrification, nitrate (NO$_3^-$) is serially reduced to nitrite (NO$_2^-$), nitric oxide (NO), nitrous oxide and finally to nitrogen gas [16]. Due to the integral role that nitrogen plays in organisms, both of these components of the nitrogen cycle are pervasive in nature and can be facilitated by bacteria that use the nitrogen oxide species as terminal electron acceptors or as nutrients during bacterial growth [16]. Over the years, it has been possible to establish a link between this process and human nitrate/nitrite/nitric oxide balance.

2.1 Background about nitrate, nitrite, and nitric oxide

Exogenous consumption of nitrate accounts for approximately 1-2 mmol nitrate/day through a typical western diet [17]. Although nitrate and its derivatives are completely absorbed in the upper gastrointestinal tract, 60% is excreted in urine [18]. In fact, 25% of the plasma nitrate is uptaken by the salivary glands and concentrated in saliva at 10 times higher concentrations than in plasma [19-22]. Circulating plasma nitrate levels are approximately 20-40 μM and have a half-life of 5-6 hours before being reduced to nitrite [1]. Upon ingestion (24 mmol KNO$_3$), plasma nitrate levels increase significantly after 30 minutes and peak at 3 hours post-consumption. Plasma nitrite levels, on the other hand, increase significantly following 1.5 hours post-consumption and peak following 2.5 hours [23]. Also, nitrite has a circulating plasma concentration of 0.01-0.6 μM and has a half-life of 20 minutes and is more reactive than nitrate [18]. It is produced through bacterial reduction of nitrate, as well as, oxidation of nitric oxide in the presence of molecular oxygen. To facilitate the latter process, multicopper oxidase ceruloplasmin catalyzes the reaction in plasma [24]. Nitrite has been shown to act as an
endocrine signalling molecule by modulating cyclic guanosine monophosphate (cGMP) production and cytochrome P450 activity, as well as regulating gene expression of heat shock protein 70 and heme oxygenase-1 in a variety of tissues [25]. These activities have also been observed by nitric oxide; however, due to its millisecond short half-life, it primarily serves as an autocrine or paracrine signal. Furthermore, nitric oxide can be produced by the reduction of nitrite through both enzymatic and non-enzymatic means. Enzymatic nitrite reduction pathways involve deoxyhemoglobin [26], deoxymyoglobin [27], xanthine oxidase [28], cytochrome P450 enzymes [29], aldehyde oxidase [30], carbonic anhydrase [31], mitochondrial enzymes [32], whereas non-enzymatic reduction of nitrite occurs in acidic environments and is enhanced by vitamin C [33] and polyphenols.

2.2 Nitrate, nitrite, and nitric oxide production

Nitrate is obtained through both exogenous consumption and endogenous production (Figure 2.1). Exogenous consumption of leafy-green, nitrate-rich vegetables accounts for 60-80% of nitrate intake and can range from 53-300 mg/day based on the location and harvest time of the vegetable (Table 2.1) [6, 34]. Moreover, an additional source of nitrate comes from drinking water [1]. Upon consumption, nitrate is reduced to nitrite by commensal facultative bacteria, present on the posterior dorsal surface of the tongue, during anaerobic bacterial respiration in the hypoxic environment [16, 35]. The resultant nitrite is protonated and subsequently forms nitrous acid (pKa 3.2–3.4) when introduced to the acidic stomach environment [4, 36]. Simultaneously, nitrous acid generates dinitrogen trioxide, a powerful nitrosating agent that can form nitric oxide as well as S-nitroso compounds and N-nitrosamines [4]. In fact, concentration of nitric oxide in the stomach can exceed 100 ppm; however, most of the nitrite in the stomach enters the blood stream through the mesenteric circulation and is reconverted to nitrate [37]. Due to the short half-life of nitric oxide within a biological fluid, it is readily oxidized to nitrite in simple aqueous systems; however, in the presence of oxyhemoglobin it is rapidly oxidized to nitrate [38-40]. Nitrate reduction in the body is predominantly due to the presence of the nitrate reductase active bacteria in the oral cavity. In both animal and human studies, it has been shown that disturbance of the oral microenvironment
through the use of antibacterial mouthwash can result in a decrease of plasma nitrite following consumption of nitrate [15, 41].

<table>
<thead>
<tr>
<th>Vegetable</th>
<th>Nitrate (mg/100g fresh weight)</th>
<th>Nitrite (mg/100g fresh weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinach</td>
<td>23.9-387.2</td>
<td>0.000-0.073</td>
</tr>
<tr>
<td>Bok choy</td>
<td>102.3-309.8</td>
<td>0.009-0.242</td>
</tr>
<tr>
<td>Lettuce</td>
<td>12.3-267.8</td>
<td>0.008-0.215</td>
</tr>
<tr>
<td>Carrot</td>
<td>92-195</td>
<td>0.002-0.023</td>
</tr>
<tr>
<td>Chinese cabbage</td>
<td>42.9-161.0</td>
<td>0.000-0.065</td>
</tr>
<tr>
<td>Cole</td>
<td>76.6-136.5</td>
<td>0.364-0.535</td>
</tr>
<tr>
<td>Cabbage</td>
<td>25.9-125.0</td>
<td>0.000-0.041</td>
</tr>
<tr>
<td>Mustard lead</td>
<td>70-95</td>
<td>0.012-0.064</td>
</tr>
<tr>
<td>Wax gourd</td>
<td>35.8-68.0</td>
<td>0.001-0.006</td>
</tr>
<tr>
<td>Eggplant</td>
<td>25.0-42.4</td>
<td>0.007-0.049</td>
</tr>
<tr>
<td>Cucumber</td>
<td>1.2-14.3</td>
<td>0.000-0.011</td>
</tr>
</tbody>
</table>

An alternative pathway leading to nitrate production is through endogenous formation of nitric oxide. A class of nitric oxide synthase (NOS) enzymes, in the presence of the molecular oxygen, and cofactors such as nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), tetrahydrobiopterin (BH₄), calmodulin, and calcium catalyze a five-electron reduction of L-arginine to nitric oxide [1]. There are three isoforms of these NOSs: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). Whereas endothelial NOS (eNOS) and neuronal NOS (nNOS) are responsible for constitutive NO synthesis in endothelial cells and neurally associated cells, the inducible form (iNOS) is found in epithelial, endothelial, and inflammatory cells, whose expression is upregulated by cytokines, microbes or bacterial products. Although eNOS was initially identified in vascular endothelial cells, it is present throughout the body and serves an important role in plasma nitrate concentrations. In fact, eNOS knockout mice have exhibited a 70% reduction in plasma nitrate concentrations because eNOS produces 0.1 mmol·kg⁻¹·day⁻¹ of nitrate [42]. Remarkably, eNOS can function in the absence of molecular oxygen to produce endothelial nitric oxide by using nitrite as a substrate. This mechanism allows for NO production in ischemic tissues during hypoxic/ischemic conditions [43, 44]. Akin to eNOS, nNOS is also
found throughout the body - initially found in the brain - and is calcium and calmodulin dependent [16]. iNOS is also found throughout the body; however, it is selectively activated during infections.

Figure 2.1: Schematic representation of the nitrate-nitrite-nitric oxide pathway within the body [1].

Both sources of nitrate-nitrite-nitric oxide production promote a steady-state circulating concentration of the substituents. These systems converge at the enterosalivary circulation of nitrate which maintains homeostasis of the nitrate/nitrite/nitric oxide balance.

2.3 Nitrate Reductase

As an integral component of the nitrogen cycle, nitrate reduction is performed by organisms due to three main reasons: assimilatory ammonification (use of nitrogen as a source of nitrogen for synthesis of biomolecules), respiration (production of metabolic energy during cellular metabolism), and dissimilatory ammonification (elimination of excess generated energy to maintain the oxidation-reduction balance) [2, 3, 45-48]. Specifically, the first step in the
reduction of nitrate to nitrite is facilitated by a class of molybdenum-containing nitrate reductases (NRs) [2]. Due to the important role these enzymes serve within organisms, there are four main groups of NRs: cytoplasmic eukaryotic nitrate reductases (Euk-NR), assimilatory cytoplasmic nitrate reductases (Nas), respiratory membrane-bound nitrate reductase (Nar), and periplasmic nitrate reductases (Nap) [2, 3]. The latter three, prokaryotic, NRs fall under the dimethyl sulfoxide (DMSO) reductase family whereas the eukaryotic NRs are members of the sulphite oxidase (SO) family (Figure 2.2) [49, 50].

![Figure 2.2: Enzymatic active site of representative organisms within the dimethyl sulfoxide (DMSO) reductase and sulphite oxidase (SO) families [2].](Image)

Specifically in an anaerobic environment, the respiratory nitrate reductase enzyme is preferentially synthesised during cellular respiration; instead of oxygen, nitrate can be used as an electron acceptor [51]. During cellular respiration, the membrane-bound nitrate reductases generate energy by coupling their three subunits - NarG (\(\alpha\)), NarH (\(\beta\)), and NarI (\(\gamma\)) – with the transmembrane proton gradient (Figure 2.3) [2, 52, 53]. The NarI, a transmembrane protein, and the cytoplasmically anchored NarH both contain an electron transfer center, whereas the NarG, anchored to the cytoplasmic side of the inner membrane, contains the enzymatic active site. Reduction of nitrate to nitrite is accomplished by the transfer of electrons from the quinol pool, at the membrane’s periplasmic side, through the electron transfer pathway and ultimate reduction
in the inner cytoplasmic membrane. The electron transfer pathway originates in the two \( b \)-type hemes of the NarI, where low-potential heme on the periplasmic side obtains the electrons and transfers them to the high-potential heme at the cytoplasmic side via histidine ligands [54-56]. Transfer of the electrons continues to the three \([4Fe-4S]\) clusters and one \([Fe-4S]\) cluster of the NarH. Finally, the electrons are transferred to the one \([4Fe-4S]\) cluster and Mo site of the NarG \([2, 57, 58]\). Nitrate is used as a final electron acceptor, and the Mo catalyzes the reduction of nitrate to nitrite through its versatile redox properties (Equ. 2). An important consideration of the enzymatic active site is to note that the Mo atom is coordinated by two molybdopterin guanine dinucleotide (MGD) cofactors that each donates four thiolate ligands. Moreover, a monodentate or bidentate coordination can be achieved by the carboxylate side chain of an aspartate residue \([2, 57, 58]\).

\[
NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O \tag{Eq. 2}
\]

In \textit{E. coli}, it has been shown that during anaerobiosis and in the presence of nitrate, expression of the narGHIJ gene is initiated [3]. However, in the presence of oxygen, the \([4Fe-4S]\) cluster is broken down, resulting in inactivation of the active enzyme. In fact, even a 5\% oxygen tension results in a 35\% decrease of nitrate reductase activity [59].
2.4 Health Effects

2.4.1 Health Risks

Although ever-present within the environment, over the past 50 years, inorganic nitrate has been considered a harmful compound due to its association with development of cancer and methemoglobinemia. Primarily, its reduction from this stable state, with a LD$_{50}$ of 1600–9000 mg per kg of body weight, to a relatively unstable oxidative state of nitrite, with LD$_{50}$ of 85–220 mg per kg of body weight, has been associated with complications [5]. Nitrite reduction to nitrosonium ions can result in the formation of N-nitrosamines, a carcinogenic compound, as dietary amines are nitrosylated [60, 61]. These compounds have been cited as potential causes of gastric and bladder cancer by resulting in chromosomal aberrations of the surrounding cells [19, 62]. Although cell culture and animal studies have demonstrated the carcinogenic properties of N-nitroso compounds, nitrate uptake in human has not been directly linked to gastric cancer [36, 60, 63-66]; this effect may be due to the elevated concentrations of vitamin C/E and other antioxidants present within nitrate-rich foods that minimize production of N-nitrosoamines [35].

In terms of bladder cancer, chronic urinary tract infections due to the parasite *Schistosoma haematobium* may readily reduce nitrate, resulting in N-nitrosamine production [67, 68]. Finally, nitrate concentration of $>10$ mg/L have been associated with methemoglobinaemia in infants as nitrite oxidizes the heme subunit in hemoglobin from Fe$^{2+}$ to Fe$^{3+}$ (Equ. 1) resulting in tight...
oxygen binding that prevents oxygen release and its subsequent reduced availability [5, 64]. Normally, methemoglobin levels in three month old infants are less than 3% of normal hemoglobin; however, when methemoglobin levels exceed 10%, clinical effects such as cyanosis or asphyxia appear [5]. A higher concentration of fetal haemoglobin, which can be easily oxidized, and a lack of methemoglobin reductases, which reform haemoglobin, puts infants under three months at a greater risk of methemoglobinemia [5]. Excessive nitrate contamination of water can occur predominantly through air pollution, run-off of agricultural fertilizer use, oxidation of nitrogenous human and animal waste, and reduction of nitrate-rich, oxygen-poor drinking water in galvanized steel pipes by *Nitrosomonas* bacteria [5]. Due to these fears, most countries closely regulate nitrate concentrations in drinking water [69].

\[ \text{NO}_2^- + \text{oxyHb(Fe}^{2+}\text{)} \rightarrow \text{metHb(Fe}^{3+}\text{)} + \text{NO}_3^- \quad \text{(Equ. 1)} \]

### 2.4.2 Health Benefits

However, recent lines of evidence have demonstrated considerable beneficial effects of nitrate consumption (Table 2.2). As up to one liter of saliva is swallowed per day, it lead to a baseline concentration of 20 parts per million (ppm) of NO in the stomach; this along with the consumption of nitrate-rich foods amounts to >400 ppm of NO [4, 70]. Concentrations of 200 ppm have been shown to effectively kill pathogenic bacteria *in vitro* without any cytotoxic effect on surrounding human dermal fibroblasts [71]. Nitric oxide has been shown to be bactericidal against a wide range of both Gram-positive and Gram-negative bacteria by targeting bacterial cellular components such as cell membranes, structural proteins, metabolic enzymes, or DNA by inducing either nitrosative or oxidative stress[72-76].

Nitrosylation of the cellular proteins thiols (S-nitrosation) can disrupt cellular activity [77, 78]. Else, nitric oxide can accumulate within the membrane, where it is oxidized to dinitrogen trioxide (N₂O₃) and cause nitrosative stress [79]. Also, NO can react with O₂ or peroxynitrous acid (HOONO) can decompose to produce nitryl radical (NO₂•), which accelerates membrane decomposition via lipid peroxidation [78, 80]. Finally, NO-mediated deamination through the formation of N-nitrosating intermediate, such as N₂O₃, or oxidative cleavage by OONO⁻ or NO₂• can result in bacterial DNA damage [77, 81]. Although NO can be cytotoxic to
bacterial cells, it can also elicit positive functions within the body, where different outcomes are due to its relative concentration. In a recent review by Thomas et al. it was shown that elevated concentrations of NO can result in cell cycle arrest, senescence and even apoptosis, whereas lower concentrations promote cell survival and proliferation [82, 83].

NO is a lipophilic molecule, hence it can diffuse through the cellular membrane [77, 79], resulting in increased gastric mucosal blood flow and mucosal secretion that result in gastroprotective effects [11, 84, 85]. Moreover, NO has been shown to exert its effects by inhibiting platelet aggregation [86, 87] as well as leading to increased cardiac health [17, 88].

A key contribution for use of nitric oxide has been in cardiovascular diseases. Due to problems with vasodilation, hypertension and atherosclerosis can result as blood flow is hindered and blood pressure increases (50-56). Historically, nitroglycerine, an organic nitrate, has been used as a vasodilator for cardiac health; however, its continuous use has been attributed to nitrate tolerance (15). Inorganic nitrate, on the other hand, does not show tolerance and can be administered over several days and result in attenuation of systolic blood pressure (92, 93). Similar to nitroglycerine, inorganic nitrate infusions of into the fore-arm increased blood flow (29). It is believed that this response is due to NO activating soluble gyanylyl cyclase in smooth muscle cells (87). However, inorganic nitrate is less potent than organic nitrite presumably due to the charge on the inorganic ion that hinders its transit across biological membranes [89]. Hypertension is manifested by a >140/90 mmHg systolic/diastolic blood pressure relative to a normal blood pressure of 120/80 mm Hg. Repeatedly the effects of nitrate administration have been shown to attenuate hypertension: 0.1 mmol nitrate/ kg/day decreased diastolic blood pressure by 4 mm Hg [90]; Webb et al. demonstrated that ingestion of 500 mL of beetroot juice can result systolic and diastolic blood pressure by 19 and 8 mmHg, respectively, within 3 hours and produced sustained effects up to 24 hours later [91]. Moreover, nitrite administration has been show to decrease myocardial ischemia-reperfusion (IR) injury, which occurs when myocardial cells are damaged by oxidative stress as blood flow returns to the ischemic areas [92, 93]. However, the effects of nitrite have been shown to be more pronounced during hypoxia and acidosis, which may contribute to its effects in IR injury [94]. Specifically, Webb et al. showed that rat heart preparations treated with nitrite resulted in more than a 60% reduction in the infarcted size. This mechanism of action was interesting because they suspected that the nitrite
reduction in the hypoxic conditions was due to xanthine oxidoreductase, an enzyme that has can actually result in I/R injury during non-hypoxic conditions through production of superoxides [92].

Furthermore, nitrate administration has been shown to reduce platelet function [86, 95]. This is significant because platelet aggregation can lead to thrombosis and result in myocardial infarction or stroke [1]. The antiplatelet aggregation properties of nitrate ingestion are believed to be due to several factors. Formed nitric oxide may activate soluble guanylate cyclase that can produce cGMP that in turn inhibits platelet function [95, 96]. Also, formation of nitrotyrosine, as a product of peroxynitrite (formed by reaction of nitric oxide with superoxide) with tyrosine residues in protein, can inhibit platelet aggregation stimulus [95, 97, 98]. Moreover, nitric oxide or nitrite may produce S-nitrosothil that may function to inhibit platelet function through both cGMP-dependent and -independent means [99, 100].

2.5 Lactobacillus and Safety

A pure culture of lactic acid bacteria (LAB) were first isolated in 1873 by J. Lister; however, prior to their identification, LABs such as Lactobacillus, Leuconostoc and Pediococcus species had been widely used in food microbiology and fermented foods ranging from yogurt and cheese to salami and olives [101-103]. Through the production of lactic acid and subsequent acidification of the environment, these strains prevent food spoilage [103]. Moreover, some LAB strains exhibit specific enzymatic activity that elicits a beneficial effect on the gut microbiota, thereby establishing these bacteria as probiotics [103, 104].

These Gram-positive rods are primarily found in carbohydrate-rich environments such as in dairy products, vegetables, sewage, and human and animal cavities (oral, intestinal, and vaginal) [105]. Due to their widespread used and ubiquitous presence within foods, members of the Lactobacillus genus have been recognized as safe for consumption under the generally recognized as safe (GRAS) status in the United States [8] and qualified presumption of safety (QPS) by the European food safety authority (EFSA) [106]. In fact, nearly all reports of infection associated with probiotic use have been in patients with conditions predisposing them to infection [107], thereby making the overall risk of infection by these species low [108-110].
Despite their widespread presence and general safety, before new strains are approved for use in the public, a series of safety tests must be performed. Following isolation of a novel strain, a polyphasic approach must be applied using phenotypic and genotypic tests. These approaches combine simple metabolic tests (API Zym and API 50 CHL) with molecular-based PCR tests (16S rRNA, whole-genome sequencing) for strain identification [111]. Moreover, production of metabolic by-products and antimicrobial agents such as bacteriocin, hydrogen peroxide, reuterin, biogenic amines, organic acids, and D(-) and L(-)-lactic acid must be quantified as they may lead to complications, especially at high concentrations. For instance, bacteriocin and hydrogen peroxide are considered non-toxic and beneficial for food preservation by preventing growth of other bacterial strains in fermented products [112-114]; however, a review by Halliwell demonstrated that elevated concentrations of hydrogen peroxide potentially lead to tissue damage [115]. Also, D-lactate or biogenic amines can accumulate in the host and result in adverse health consequences [105, 116]. Finally, antibiotic-resistance can pose a serious public health concern. Although *Lactobacillus* have natural antibiotic resistance (bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, gentamicin, metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin, trimethoprim/sulphamethoxazole, and vancomycin), these may be intrinsic and not of concern [105, 117]. However, antibiotic resistance on mobile-elements pose a greater risk because they can be transmitted to pathogenic bacteria through horizontal gene transfer, resulting in multi-drug resistant strains [118]. Hence, identification, and screening for production of metabolic by-products and antimicrobial resistance are required for safety validation of a novel *Lactobacillus* strain.

Furthermore, the mucin degradation activity and translocation ability of the bacteria must be examined. Some pathogen bacteria degrade the protective mucosal barrier within the intestinal wall, translocate through the barrier, and invade the epithelial cells [119]. Moreover, an animal study must be run to validate the *in vitro* safety results and demonstrate that ill-effects were not observed in the animals’ health due to administration of the bacteria [120].

Recently, some strains of *Lactobacillus* have garnered great interest due to their nitrate reductase activity under anaerobic conditions [7, 121]. These strains can be implemented for a variety of uses: NO release in a medical device [122], waste-water treatment [123], and
probiotics [35]. Particularly, use of nitrate reductase active bacteria is of great interest for addressing metabolic disease.

2.6 Animal models

There are multiple animal models for hypertension utilizing rats, mice, dogs, and cats. Rat models are by far the most popular and their use is slightly increasing in proportion every year [9]. Some of the current rat model of hypertension include spontaneously hypertensive rats, two-kidney one-clip rat models [9], and L-NAME treated rats.

The spontaneously hypertensive rat (SHR) was developed by inbreeding Wistar rats with the highest blood pressure [14] and it is described that this indicator rises around 5-6 weeks of age, increasing steadily to reach systolic pressure of 180-200 mmHg. The SHR present many of the landmarks of hypertensive end organ damage including cardiac hypertrophy, cardiac failure, and renal dysfunction. The aetiology of this model is not completely known but it is of genetic origin and is related to a kidney disorder [124, 125]. Since the hypertension in SHR rats is sensitive to ACE inhibitors, calcium antagonists, and vasodilators, the use of this model is suitable to test probiotics that enhance NO release. As an example, the administration of nitroglycerin lowered blood pressure in SHR [126].

The two-kidney one-clip model ensures that one of the renal arteries is clipped to allow for hypertension formation. This invasive model is sensitive and can result in a range of 25-50% hypertrophy based on the clip size and rate age. Since this model is also sensitive to calcium antagonists and vasodilators, it is comparable to SHR [9].

Recently line of evidence has shown that $N^G$-nitro-L-arginine methyl ester (L-NAME) inhibits NOS thereby elevating blood pressure [13, 127]. This is achieved through the hydrolysis of the methy ester by cellular esterases resulting in L-NNA, a functional inhibitor. This inhibitor has been shown to inhibit cyclic guanosine monophosphate (cGMP) formation in endothelial cells and counteract the effects of acetylcholine in rat aorta rings at IC$_{50}$ of 3.1 µM and EC$_{50}$ of 0.54 µM, respectively [128-130]. Moreover, low doses of oral nitrate have been shown to potentially protect against L-NAME induced kidney injuries without affecting blood pressure [131].
<table>
<thead>
<tr>
<th>Topic of Study</th>
<th>Study Results</th>
<th>Experimental Model</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Platelet function</td>
<td>KNO₃ (2 mmol) administration increased gastric RSNO levels and significantly inhibited platelet function</td>
<td>Human</td>
<td>[95]</td>
</tr>
<tr>
<td>Cellular ischemic stress response</td>
<td>Chronic nitrite administration significantly protects the liver against ischemia and reperfusion</td>
<td>Mice</td>
<td>[93]</td>
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<td>Metabolic syndrome</td>
<td>Dietary nitrate administration (1mM) to endothelial nitric oxide synthase-deficient mice for up to 10 weeks decreased components of metabolic syndrome Nitrate administration resulted in a dose-dependent increase in plasma nitrite concentration and subsequent blood pressure decrease</td>
<td>Mice</td>
<td>[88]</td>
</tr>
<tr>
<td>Blood pressure</td>
<td>Concurrent administration of nitrate (0.1 or 1 mmol nitrate/kg/day) with a nitrate content rich diet resulted in reduction of oxidative stress, prevention of cardiac and renal injuries, and reduction of blood pressure</td>
<td>Human</td>
<td>[23]</td>
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<tr>
<td>Renal and cardiovascular disease</td>
<td>Nitrite infusion (400 nM for 5 minutes at 1 mL/min) resulted in increased fore-arm blood flow Ingestion of 500 mL of beetroot juice resulted in a decrease of systolic and diastolic blood pressure by 19 and 8 mm Hg within 3 hours and produced sustained effects up to 24 hours</td>
<td>Rats</td>
<td>[132]</td>
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<td>Vasodilation</td>
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<td>Human</td>
<td>[26]</td>
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<tr>
<td>Blood pressure</td>
<td></td>
<td>Human</td>
<td>[91]</td>
</tr>
<tr>
<td>Renal injury</td>
<td>L-NAME induced renal injury was ameliorated by a dose dependent administration of nitrite for 8 weeks Nitrite and nitrate administration resulted in protective effects in hepatic I/R and decreased myocardial infarct size by 67%</td>
<td>Rats</td>
<td>[133]</td>
</tr>
<tr>
<td>Liver ischemic/reperfusion</td>
<td></td>
<td>Mice</td>
<td>[134]</td>
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LIST OF CONTRIBUTIONS

I) Contributions Listed in the Thesis
   a. Original research article
      ii. **Imran Sulemankhil**, Jorge Gabriel Ganopolsky, Chris Anthony Dieni, Mitchell Lawrence Jones, and Satya Prakash. Evaluation of *in vivo* administration of *Lactobacillus fermentum* NCIMB 7230 and *Lactobacillus fermentum* NCIMB 702342 for metabolic syndrome. To be submitted.
   b. Research Presentation
      i. Screening, Isolation, Optimization, and Safety Validation of a Nitrate-Reductase Active Bacteria. Seminar in Biomedical Engineering. Seminar. March 21, 2012

II) Contributions Not Listed in the Thesis
   a. Original research article
   b. Research Presentation

Note: Publications in the section entitled “Contributions Not Listed in the Thesis” were omitted from the thesis since it did not directly lead to the conclusions of the thesis.
PREFACE TO CHAPTERS 3 TO 5

In order to determine the efficacy of nitrate reductase active bacteria to test in a hypertension induced animal model, a strain positive for nitrate reductase activity must be first isolated. As outlined in Chapter 3, a nitrate reductase active strain was isolated from a library of lactobacillus strains and an assay was developed for quantification of its enzymatic activity.

To determine the efficacy of nitrate reductase active bacteria to reduce dietary nitrate and address a symptom of metabolic disease (hypertension), a rat preclinical study was conducted using L-NAME hypertension-induced rats as outlined in Chapter 4.

Chapter 5 allowed for partial in vitro safety validation of Lactobacillus fermentum NCIMB 702342 through strain identification, detection of antibiotic resistance, and detection of formation of metabolic by-products and antimicrobial agents.
CHAPTER 3: STRAIN ISOLATION AND ASSAY DEVELOPMENT

IDENTIFICATION AND OPTIMIZATION OF ASSAY CONDITIONS FOR A NITRATE REDUCTASE ACTIVE LACTOBACILLUS STRAIN*

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Preface

The present study outlines the development of an assay for quantification of nitrate reductase enzymatic activity. Both a synthetic and natural assay media were used to screen a library of *Lactobacillus* strains to identify the strain demonstrating maximum enzymatic activity. Moreover, incubation time, bacterial cell load, and assay media concentration were optimized. The co-authors have contributed to the experimental design and execution, as well as provided guidance with technical aspects of the experimentation.

Abstract

*Lactobacillus fermentum* NCIMB 702342 was isolated from a library of *Lactobacillus* strains for demonstrating optimal nitrate reductase activity. Nitrate reduction by the test strains was assayed in MRS media supplemented with nitrate, and natural nitrate-rich beet root juice assay media. Growth conditions were optimized and indicated that a 5% (v/v) final bacterial suspension in a 5% (w/v) beet root juice assay media cultured 8 hours resulted in maximum nitric oxide production, an indicator of nitrate reduction to nitrite. This study identified *Lactobacillus fermentum* NCIMB 702342 as a nitrate reductase activity bacterium and optimized its assay conditions to examine this activity.

**Key words:** Nitrate reductase, *Lactobacillus fermentum*, beet root juice
Introduction

For prokaryotic and eukaryotic cells, cellular respiration is an important metabolic reaction that allows for conversion of nutrients to energy [135]. Specifically, certain bacterial cells can utilize oxygen during aerobic respiration; however, in its absence, inorganic molecules such as nitrate can be used during anaerobic cellular respiration [48]. This task is achieved by nitrate reducing bacterial cells in low oxygen tension through the up-regulated expression of a respiratory membrane-bound nitrate reductase [136]. This enzyme drives ATP production by using nitrate as an electron-acceptor and subsequently reducing it to nitrite.

Within industry, the reduction of nitrate can serve a potential role in water treatment by addressing nitrate contamination. A possible use of nitrate reductase active bacteria is to reduce nitrate that contaminates water, to nitrite. Although nitrite consumption has been linked to potentiating gastric and bladder cancer in animal models, epidemiological studies in humans have not demonstrated a correlation [5, 34, 65, 66]. However, a direct health risk has been associated with nitrate concentration of >10 mg/L by causing methemoglobinemia in infants [5, 64]. Use of low-cost, highly-scalable nitrate reductase active bacteria is a potential approach for water treatment. Potential candidate strains capable of nitrate reduction are from the Lactobacillus genus [7]. These strains have historically been used in a variety of food products; they have obtained the generally recognized as safe (GRAS) status [8] and have been used as probiotics [137]. Hence, use of Lactobacillus bacteria is a possible solution for addressing public concerns of treating nitrate-contaminated water.

Due to the diverse background of these strains they can be found in a variety of environments [103]. Therefore, during media production, the primary goal is to prepare synthetic media mimicking the nutrients available in the native environment. Often vegetable derived media are selected for bacterial growth, as was the case with initial Lactobacillus media composed of tomato juice [138]. An ideal assay media for nitrate reductase active bacteria is beet root juice. This has essential components such as a reducing agents, antioxidants, and high carbohydrate content that allow for bacterial growth, as well as a high nitrate concentration [139].
In this paper, a library of lactic acid bacteria (LAB) was screened for nitrate reductase activity using both synthetic and natural assay media. Since all of the strains tested were LABs, the rich, non-selective MRS media was idea for initial growth and screening to assess whether nitrate reductase enzyme was expressed. The strain with the highest nitric oxide (NO) production was selected and its growth profile was constructed. After establishing the optimal harvest time, the cell concentration and natural assay media concentrations were titrated to determine the conditions necessary for optimal NO production.
Methods and Materials

Microorganism

For screening of nitrate reductase active bacteria, the following 92 strains of Lactobacillus were assayed: *Lactobacillus acidophilus* strains 702173, 1723, 4504, 8821, 702470, 702471, 702472, 702473, 702658, 702659, 702660, and 702662; *L. buchneri* ATCC 4005; *L. casei* subsp. *casei* strains 8822, 11970, and 700173; *L. delbrueckii* DN111244; *L. delbrueckii* subsp. *bulgaricus* ATCC 11842; *L. farcininis* ATCC 29644; *L. fermentum* strains LMG 18251 and 18252; *L. fermentum* strains ATCC 14932, 11976, and 14931; *L. fermentum* strains NCIMB 5220, 5221, 6991, 7230, 8028, 8828, 8829, 8830, 8961, 8962, 12116, 700335, 700479, 700927, 700928, 701052, 701068, 702341, 702342, 2797; *L. helveticus* strains 76, 2889, 8652, 9949, 700030, 700101, 701209, and 701844; *L. johnsonii* strains 8795, and 702241; *L. parabuchneri* LMG 11457; *L. paracasei* subsp. *paracasei* strains 1407, 9713, and 700151; *L. paracasei* subsp. *tolerans* strains 9709 and 701974; *L. plantarum* LP80; *L. reuteri* strains ATCC 23272, PTA-4659, 53608, 53609, 55148, and 55739; *L. reuteri* strains NCIMB 11951, 701359, 702656, 701089, 702655; *L. reuteri* strains LMG 9213, 18238, 22877, 22878, and 22879; *L. reuteri* strains CCUG 32271, 32305, 37470, 44001, 44144, and 47824; and *L. rhamnosus* strains 6375, 6557, 7473, 8824, 9282, 12561, 702965, 702966, and 53103. All strains were obtained from the National Collection of Industrial Food and Marine Bacteria (Aberdeen, Scotland).

Source plates were prepared by streaking frozen master stocks of the appropriate strain onto MRS agar plates and grown anaerobically overnight at 37°C. A single well-isolated colony was picked and anaerobically incubated overnight in 15 mL of MRS (MRS, Becton Dickenson, Canada) broth at 37°C.

Nitrate reductase-active bacteria screening assay

Overnight bacterial strains were diluted to 1:10 in MRS and supplemented with 30 mM sodium nitrate resulting in an artificial assay media. A 2 mL solution of the resulting suspension was transferred to a 4 mL sterile glass vials equipped with septa. The suspensions were incubated at 37°C for up to 7.5 h. A head gas sample (100 μl) was
collected at 1.5 h intervals with a Hamilton syringe and the produced NO was determined by chemiluminescence with a NO analyzer (Siever, GE). The detection limit was 0.001 ppmV (one part per million parts in volume). Following the initial strain screening in the artificial assay media, a subsequent screening of the top NO producing strains was performed using a natural nitrate-rich media to identify the strain of interest. Herein, the samples were made anaerobic prior to incubation and the samples’ head space was injected 2 and 3 h post-incubation for NO quantification. Beet root juice powder was reconstituted in water (5% w/v) and autoclaved; the supernatant was used as the source of natural nitrate-rich assay media. A NO standard curve was prepared by injecting known volumes of a 200 ppmV NO standard gas. Bacteria that presented detectable NO production were selected as nitrate reductase active. Controls using solely MRS supplemented with nitrate and beet root juice were used. Experiments were performed in triplicates.

**Bacterial growth**

The strain of interest resulting in maximum NO production was grown as described above. However, following overnight growth of picked colony in 15 mL MRS, the inoculum was passaged in 40 mL of MRS and grown anerobically at 37⁰C for 10 h. To identify the bacterial growth profile of the selected strain, cultures were inoculated in 40 mL MRS (1% inoculum) in triplicate. Volumes of 100µL were transferred to a 96-well plate and the cell cultures were assayed for cell density over a 16 h time course. Cell density was evaluated spectrophotometrically at 600nm using a Beckman™ spectrophotometer (DU®520 UV/VIS Spectrophotometer, Beckman Coulter). Moreover, from 4-18 h post-inoculation at 2 h intervals, the cell suspension was assayed in the beet root juice assay media following 2 and 3 hours of incubation to determine the optimal growth time. Values were reported as enzymatic activity using a log transform as outlined in equation 1.

\[
\text{Enzymatic activity} = \frac{\ln(\text{NO Production } 3\text{ h}) - \ln(\text{NO Production } 0\text{ h})}{(3\text{ h})-(0\text{ h})}
\]

(Equ. 1)
**Bacterial and natural nitrate-rich media titration**

To determine the maximum enzymatic activity that could be achieved using the optimal concentration of the bacterial strain of interest and of the beet root juice assay media, the concentrations of both components were titrated from 1.25%, 2.5%, 5%, and 10% (v/v for bacterial strains and w/v for beet root juice assay media) while maintaining the other concentration constant.

**Statistical analysis**

All data were presented as the mean ± SE (standard error mean). Moreover, statistical analyses were performed using SPSS software package version 17·0 (SPSS Inc., Chicago, IL, USA). Significance was set at $P < 0.05$. 
Results

Nitrate reductase-active bacteria screening assay

The screening assay for nitrate reductase active bacteria yielded 12 nitrate reductase active strains out of the 98 strains screened. *L. fermentum* ATCC 11976; *L. fermentum* NCIMB strains 6991, 7230, 8028, 8828, 12116, 702342, 700335, and 700479; *L. reuteri* LMG 18238; and *L. reuteri* CCUG strains 37470 and 32305 resulted in NO product ion, with the *L. fermentum* NCIMB 7230, *L. fermentum* NCIMB 702342, and *L. fermentum* NCIMB 6991 resulting in the greatest NO production (Figure 3.1). These strains were subsequently screened using a natural nitrate-rich media and *L. fermentum* NCIMB 702342 was shown to produce maximum NO production following 2 h of incubation (Figure 3.2).

Bacterial growth

*L. fermentum* NCIMB 702342 produced a sigmoidal growth profile when incubated in MRS media (Figure 3.3). Following inoculation (1% v/v), exponential growth occurred at 3 h, and reached post-exponential and stationary phase after approximately 7 and 8 hours of growth, respectively.

Concurrent analysis of *L. fermentum* NCIMB 702342 enzymatic activity with varying harvest times resulted in a statistically significant maximum occurring at 8 h post-incubation with a subsequent marginal decrease at subsequent time points (Figure 3.4).

Natural nitrate-rich assay media screen

Titration of the beet root juice assay media and final bacterial cell solution demonstrated that maximum enzymatic activity was achieved following 2 and 3 h of incubation using a 5% beet root juice solution (Figure 3.5a) when inoculated with a 5% final bacterial solution (Figure 3.5b).
**Discussion**

To screen for nitrate reductase activity amongst a library of candidate *Lactobacillus* strains, bacterial cultures were assayed in either synthetic media consisting of MRS media supplemented with 30mM of nitrate or in natural nitrate-rich assay media composed of reconstituted beet root powder. The rational for supplementing MRS with 30mM nitrate was to match the nitrate content present in the beet root powder (data not presented). From the list of 92 bacterial strains, 12 strains were detected as nitrate reductase active bacteria using the synthetic assay media. The three strains with maximum NO production were further screened using the beet root juice assay media. The final screen yielded that *L. fermentum* NCIMB 702342 achieved maximal NO production following 2 h of incubation in the natural assay media. Since *L. fermentum* are anaerobic bacteria, these results demonstrate that the strain is capable of using nitrate as an electron acceptor during anaerobic respiration, resulting in nitrite formation, which is subsequently reduced to nitric oxide in the acidified environment [7].

The NO producing capability of beet root powder was due to its high nitrate content (450-725 mg/kg) [140]. Moreover, the presence of reducing agents, antioxidants, and high carbohydrate content present within the beet root powder [139], made it an ideal assay medium since it could sustain bacteria growth during the 2-3 hour assay. Previously, beet root juice has been shown to be fermentable by *Lactobacillus* strains while maintaining bacteria viability above 10^6 CFU/mL [141]. Since beet roots have been shown to maintain a wide variety of microorganisms, the beet root assay media were autoclaved prior to use to ensure artefacts would not be present due to other nitrate reductase active bacteria. Furthermore, when the beet root juice was inoculated with bacteria, it was shown that the activity resided in the solution supernatant rather than in the fibrous components of the beet root, and use of the fibrous components would result in increased variability (data not presented). Hence, the reconstituted beet root powder was centrifuged at 1,500 rpm for 5 minutes to isolate the supernatant for further use.

To ensure that the bacterial cells were being assayed following an appropriate harvest time, bacterial cells were passaged three-fold to reduce variability amongst runs, then a time course was run for both bacterial cell growth and enzymatic activity of NO
production. These experiments demonstrated that the bacterial cells would enter post-exponential growth and exhibit maximum enzyme rate following 8 h of growth. After exponential growth, as nutrients are depleted from the growth media, there will be a decrease in the growth rate and cell lysis; hence, following 8 h of harvest, the cell solution transitions from active cell division to maintenance of aging cell. Upon incubation of these cells in the fresh nutrient-rich assay media, it is assumed that de novo synthesis of the nitrate reductase protein occurs.

Bacterial and natural assay media titration ensured that the ideal concentration of both components would be used resulting in an optimal enzymatic activity. A 5% bacterial final concentration to 5% beet root juice concentration was shown to result in maximum enzymatic activity.

These results demonstrate a method for detection and isolation of nitrate reductase active bacteria. Furthermore, through the titration of beet root juice assay media and bacterial cells, as well as determination of the ideal harvest time, it was possible to achieve maximum nitric oxide production, which was found to correlate with nitrite formation.
**Figure 3.1:** Screening profile of the nitrate reductase activity bacteria as indicated by NO production as assayed using MRS medium supplemented with 30 mM inorganic nitrate. Note: Only the 12 strains demonstrating NO production are reported. The reported values represent the mean of two independent trials ± SE.
Figure 3.2: Screening profile of the nitrate reductase activity *Lactobacillus* bacteria as indicated by NO production as assayed using beet root juice assay media. The reported values represent the mean of two independent trials ± SE. * indicates statistical significance $P<0.05$. 
Figure 3.3: Shows the growth curve of *L. fermentum* NCIMB 702342 grown in MRS broth measured from 0 to 16 h at OD$_{600}$ following media inoculation. The reported value at each time point represents the mean of three independent trials ± SE.
Figure 3.4: Enzymatic activity of *L. fermentum* NCIMB 702342 from 4-18 h post-inoculation at 2 h intervals. Samples were assayed at 2 and 3 h following incubation in beet root juice assay media. The reported value at each time point represents the mean of two independent trials ± SE. * indicates statistical significance $P<0.05$. 
**Figure 3.5:** Enzymatic activity of *L. fermentum* NCIMB 702342 following titration (%) of beet root assay media (a), and cell concentration (b). NO production analysed following 2 and 3 h incubation. The reported values represent the mean of two independent trials ± SE. * indicates statistical significance relative to all other values *P*<0.05.
CHAPTER 4: IN VIVO MODEL FOR NITRATE REDUCTION

EVALUATION OF IN VIVO ADMINISTRATION OF LACTOBACILLUS FERMENTUM NCIMB 7230 AND LACTOBACILLUS FERMENTUM NCIMB 702342 FOR METABOLIC SYNDROME*

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Preface

The two lactobacillus strains demonstrating maximum enzymatic activity, as quantified by the assay developed in Chapter 3, were tested in an animal study to evaluate their nitrate reductase activity in vivo. Mean arterial, dyostolic, and systolic blood pressure as well as plasma nitrate and nitrite concentrations were evaluated to quantify the efficacy of the nitrate reductase activity of the bacteria. The co-authors have contributed to the experimental design and provided guidance with technical aspects of the experimentation.

Abstract

Through the course of the past 50 years, nitrate consumption has been attributed to eliciting both harmful and beneficial effects [4, 5]. As a key component of the enterosalivary system, nitrate reductase active bacteria are also found in a variety of organism within nature and serve the essential role of reducing nitrate to nitrite [142]. Though this process occurs naturally in the anaerobic microniches of the tongue and functions to reduce nitrate within a food matrix to contribute to the nitrate/nitrate/nitric oxide balance, probiotics can also be used to complement this system. The probiotics use inorganic nitrate as a substrate and ultimately produce nitric oxide in the lower gastrointestinal tract. Potential benefits of this delivery system can lead to treatment of hypertension, inflammatory bowel disease, diabetes, and improve the risk of thrombosis. Herein, two nitrate reductase active strains (Lactobacillus fermentum NCIMB 7230 and Lactobacillus fermentum NCIMB 72023) were administered to male Wistar-Kyoto rats that had been made hypertensive through the administration of NG-nitro-L-arginine methyl ester. The results indicate that the probiotics did not result in attenuation of hypertension; however, L. fermentum NCIMB 72023 may potentially reduce systemic nitrate to nitrite.

Keywords: nitrate reductase active bacteria, hypertension, and plasma nitrite and nitrate


**Introduction**

Nitric oxide (NO) is a free radical that plays a role as an autocrine/paracrine and endocrine messenger in a variety of tissues, regulating cellular processes [143]. In particular, NO initiates a signal transduction pathway in the endothelium, regulating vascular tone and blood pressure, modulating of hemostasis and proliferation of vascular smooth muscle cells [144]. NO signalling in leukocytes attenuates inflammatory responses through regulation of cytokine expression. In the gastrointestinal tract, NO not only functions as a protective agent against early inflammatory insults but also as a protector of a normal, intact mucosal barrier [145, 146]. NO is synthesized *in vivo* by a family of NO synthases (NOS) from arginine as a donor. Whereas endothelial NOS (eNOS) and neuronal NOS (nNOS) are responsible for constitutive NO synthesis in endothelial cells, and neurally associated cells, there is an inducible form (iNOS) found in epithelial, endothelial and inflammatory cells, whose expression is up-regulated by cytokines, microbes or bacterial products.

There is vast evidence of the physiologic role of NO in vascular diseases such as hypertension [91], portal hypertension [147], in platelet activation during thrombotic events, [148], in atherosclerosis [149], as well as in inflammatory bowel diseases [150] including ulcerative colitis, Crohn’s disease, collagenous colitis, lymphocytic colitis, ischaemic colitis, diversion colitis, Behçet's syndrome, and infective colitis. In vascular diseases, NO functions as a vasodilator, and retards atherogenesis by inhibiting inflammatory cell recruitment and platelet aggregation [148]. In intestinal inflammation, administration of NO protects the mucosa by maintaining blood flow, reducing intestinal epithelial permeability, inhibiting platelets, inhibiting leukocyte adhesion and aggregation, downregulating mast cell reactivity, and modulating oxidative stress [145, 150-153]. In addition, NO may alleviate inflammatory bowel disease due to its antimicrobial activity against infectious bacteria such as *E. coli*, *Salmonella*, or *Shigella*. One of the plausible mechanisms of antimicrobial activity of NO involves the interaction of this free radical (and a reactive nitrogen intermediate) with reactive oxygen intermediates, such as hydrogen peroxide (H$_2$O$_2$) and superoxide (O$_2^-$) to form a variety of antimicrobial molecular species. In addition to NO itself, these reactive antimicrobial
derivatives include, S-nitrosothiols (RSNO), nitrogen dioxide (NO₂), dinitrogen trioxide (N₂O₃), and dinitrogen tetroxide (N₂O₄) [77].

NO could be also generated in the gastrointestinal system of mammals from inorganic nitrate and nitrite by the action of commensal bacteria [35]. Under anaerobic conditions, oral bacteria presenting nitrate reductase activity can generate nitrite anions from nitrates present either in saliva or ingested. The produced nitrite will become NO once it reaches the stomach due to the acidic environment. Consequently, NO will enter the blood stream through the mesenteric circulation and get reconver ted to nitrate, which could be then eliminated by urine, or get recycled to the saliva where it is concentrated and becomes available for further NO generation [154]. Lactic acid bacteria can generate high amounts of NO from nitrites due to the acidification of the environment as a result of fermentation [7]. Production of NO in the gastrointestinal tract from nitrites and nitrates was observed in the presence of probiotics such as lactobacilli and bifidobacteria [155]. Finally, ammonia-oxidizing bacteria such as nitrosomonas produce NO from ammonia and hydroxylamine.

The ingestion of fruits and vegetables rich in nitrates and nitrites, such as leafy greens or beet root may benefit health by the blood pressure lowering effects of the Dietary Approaches to Stop Hypertension (DASH) diet [15, 156]. In fact, oral administration of nitrates increases mucosal blood flow and mucosal defense in a rat model of gastric injury [153]. In addition, dietary nitrates and nitrites lower blood pressure and present gastroprotective effects in a rat model of gastric injury [15]. These results were abolished by spraying the rats with an antibacterial mouth wash, indicating that oral bacteria are indispensable for the beneficial effects of NO derived from dietary nitrates. Moreover, a study in humans showed that provision of a nitrate-rich drink such as beet root juice significantly reduced blood pressure and had vasoprotective and antiplatelet properties when compared to the individuals that were treated with water controls [91].

As mentioned above, nitrate conversion to nitrite is achieved in the mouth by the action of resident bacteria. The oral delivery of probiotics could be utilized as a method to colonize the gastrointestinal tract, and thus facilitate and improve the conversion of
dietary nitrates not only in the mouth but in the whole digestive system. The administration of nitrate-reducing probiotics, in combination with a nitrate rich diet, presents a potential opportunity to generate physiologically relevant amounts of NO in the upper and lower gastrointestinal tract. This therapy can thus provide health benefits to individuals suffering or with a propensity of developing cardiovascular or inflammatory bowel diseases. Herein, an in vivo 7 week study was conducted to evaluate the efficacy of nitrate reductase active bacteria in attenuating $\text{N}^\text{G}$-nitro-L-arginine methyl ester-induced hypertension.
Methods and Materials

Microorganism growth, storage, and use

*Lactobacillus fermentum* NCIMB 7230 and *Lactobacillus fermentum* NCIMB 72023, herein referred to as *L. fermentum* 23 and *L. fermentum* 38, respectively, were obtained from the National Collection of Industrial Food and Marine Bacteria (Aberdeen, Scotland) and used for all experiments. Source plates were prepared by streaking a frozen glycerol stock of the bacteria on de Man-Rogosa-Sharpe (MRS, Becton Dickenson, Canada)-agar plates and grown overnight at 37°C under anaerobic conditions. A single colony was picked and anaerobically incubated in MRS broth for 10 h at 37°C; a 1% inoculum was used to subsequently passage MRS to be anaerobically incubated 10 h at 37°C. For experimental use, a 1% inoculum of the culture was growth in supplemented MRS (Table 4.1) for *L. fermentum* 23 and in modified growth media (Table 4.1) for *L. fermentum* 38 for 8 h at 37°C. The bacteria were harvested by centrifugation at 1,800 rpm for 15 min at 25°C. The cell pellet was resuspended in filter-sterilized maltodextrin supplemented with filter-sterilized cysteine (final concentrations: 2.5% maltodextrin and 0.05% cysteine) in a 1:1 ratio with bacterial pellet. Care was taken not to excessively airate the pellet.

The cell suspension was aliquoted with 2 ml volumes dispensed in 15 ml falcon tubes for storage at -80°C until use. For use in experiment, the aliquots of cell suspension were allowed to thaw at room temperature and centrifuged at 1,800 rpm for 13 min at 25°C. The supernatant was discarded and the cell pellet was resuspended in either 0.85% (w/v) autoclave sterilized saline (Phase 1) or 5% (w/v) autoclaved spinach juice (Phase 2).

Animals

Twenty-four, 3-month old normotensive male Wistar-Kyoto rats (230-280 g) were obtained from Charles Rivers laboratories. The animals were housed at the UQAM animal center two per cage and given food and water ad libitum. Moreover, the animals were kept on a 12h00 daylight cycle in a temperature controlled room (25°C). Prior to 7-week intervention period, the animals were left to acclimatize for 9 days and randomized
into three groups: control \((n = 8)\), \(L. fermentum\ 23 (n = 8)\), or \(L. fermentum\ 38 (n = 8)\) by orogastic tube during phase 1 and 2 of the experiment. Experimental procedures were approved by the “Commité Institutionnel de protection des animaux” of Université du Québec à Montréal and were performed in compliance with guidelines outlined by the Canadian Council on Animal Care.

**General conditions, food intake, and animal weight**

The general conditions of the animals were monitored daily. Food and water intake as well as animal body weights were measured throughout the duration of the experiment. Food and water consumption per animal were reported using equations 1 and 2, respectively.

\[
\text{Daily food intake per cage (g)} = \frac{\text{Food consumed each week by 2 animals}}{\text{2 animals}}
\]

\[
\text{Daily water intake per cage (mL)} = \frac{\text{Water consumed each 3 [or 4] days by 2 animals}}{\text{2 animals}}
\]

**Animal manipulation during Phase 1 and 2 of the animal trial**

Normotensive Wistar-Kyoto (WKY) male rats were gavaged twice daily (6 h interval between gavages). Control animals were administered 1 mL of sterile saline whereas treated animals received 1 mL of sterile saline with a 10\% (w/v) of bacterial suspension of either \(L. fermentum\ 23\) or \(L. fermentum\ 38\) during phase 1 (weeks 1-5) of the intervention period. Phase 2 (weeks 6-7) of the trial was conducted similar to phase 1, however, 5\% (w/v) spinach juice was used to substitute saline in all groups. Also, 0.6 mg/mL of \(\text{N}^\text{G}\)-nitro-L-arginine methyl ester (L-NAME) was chronically administered in the water of the animals 3 days prior to the start of week 2.

Once a week, the animals’ blood pressure was measured using tail cuffs (Kent Scientific Coda System, CT) immediately pre-gavage, as well as 2 and 6 h post-gavage. Moreover, blood samples were collected pre-gavage and multiple times post-gavage in potassium EDTA Microtainer tubes (Becton, Dickinson and Company, Canada) as follows: midday during the acclimatization period (Week 0); pre-gavage as well as 2 h, 4
Determinations of nitrite and nitrate concentrations in serum and plasma

A nitric oxide analyzer (Siever GE, CO) was used to determine nitrate and nitrite concentrations in the blood samples at each time-point in order to evaluate the efficacy of nitrate reduction by the probiotic. Whole blood samples were centrifuged at 10,000 x g for 15 min at room temperature. Plasma samples were collected and immediately injected into the reaction vessel for nitrite analysis. At a cell pressure of 6 torr and chamber pressure of 6.0 psig, 3 mL of 45 mM NaI, 10 mM I₂ in glacial acetic acid was used to reduce the plasma samples in the reaction vessel. Moreover, the jacket of the reaction vessel was heated to 56⁰C using a heated water bath and cold tap water was run through the condenser to avoid loss of evaporated reaction mixture. A 1M NaOH solution was utilized as an acid trap for neutralization of acetic acid and trapping I₂ gas. A calibration curve was constructed through injections of varying concentrations of a NaNO₂ solution.

For nitrate analysis in the samples, blood plasma could be stored at -80⁰C for future use. Upon thawing at room temperature, plasma samples were added to chilled 95% ethanol (1:3) and stored at -20⁰C for at least 1 h. Following storage, the samples were centrifuged at 10,000 x g for 10 min. The supernatant was injected into the reaction vessel for nitrate analysis. A solution of 8 mg/mL VCl₃ was dissolved in 1 M HCl and transferred to the reaction vessel that was adjusted to a cell pressure of 6.0 torr and chamber pressure of 3.0 psig. The reaction vessel was heating to 96⁰C using a heated water bath, cold tap water was run through the condenser, and a 1M NaOH solution was used as an acid trap. A calibration curve was constructed through injections of varying concentrations of NaNO₃ solution.

Euthanasia and end point sample collection

Three hours post-gavage during week 7 of the intervention period, animals were anaesthetized using isoflurane (30% v/v in propylene glycol). When unconscious, the peritoneum of the animal was perforated to expose the underlying organs. EDTA
microfuge tubes were used to obtain blood from the inferior vena cava followed by a terminal cardiac bleed.

**Biochemical analysis**

Serum samples from the control, *L. fermentum* 23, and *L. fermentum* 38 groups were analysed at the endpoint of the experiment following the terminal bleed at week 7. Serum separator tubes were filled with blood drawn from the inferior vena cava. The samples were centrifuged at 2,000 x g for 15 min to isolate the serum. A Hitachi 911 automated analyzer (Roche Diagnostics, Laval, QC) was used to perform the biochemical analysis on the serum samples, where the following biochemical markers were measured: alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) served as markers of liver function; high sensitivity C-reactive protein (hs-CRP) as served a marker for inflammation; urea and creatinine served as markers of kidney function; lipase served as a marker of pancreas function; calcium (Ca^{2+}) and phosphate (PO_{4}^{3-}) served as markers for endocrine function; and sodium (Na^{+}), chloride (Cl^-), potassium (K^+) and bicarbonate (HCO_{3}^-) allowed for the assessment of electrolyte balance.

**Statistical Analysis**

Data were presented as the mean ± SE (standard error mean). Blood pressure was analysed using one-way analysis of variance (ANOVA) and the Bonferroni Post Hoc Test, and the hematological data were analysed by the Student’s unpaired 2-tailed t-test. SPSS software package version 17.0 (SPSS Inc., Chicago, Illinois, USA) was used for statistical analyses and significance was established at *P* < 0.05. Microsoft Excel 2010 (Microsoft Corp., CA) and GraphPad Prism 5 (GraphPad Software, Inc., CA) was used for production of the figures.
Results

Blood Pressure

Chronic administration of L-NAME, commencing 3 days before week 2 of the experiment, exhibited elevated mean arterial, systolic, and diastolic blood pressure in control and treated groups (Figure 1). To monitor the acute effects of treatment, animal blood pressure was measured pre-gavage followed by 2 and 6 h postgavage. Due to a drop in blood pressure for all experimental groups at 2 h post-gavage, presumably due to animal relaxation within the restrainer, this data point is omitted. Pre-gavage (0 h) and 6h post-gavage data was observed for mean arterial (Figure 4.1a), systolic (Figure 4.1b), and diastolic (Figure 4.1c) blood pressure in control and treated groups to study the chronic effects of L-NAME and treatment administration. Statistical analysis of the data did not show statistical significance amongst the group in any week of measurement.

Blood Chemistry (Nitrate and Nitrite)

Analysis of blood chemistries did not yield conclusive results regarding general trends within the data set (Figures 4.2). Although, an elevation of nitrate was observed across the weeks in all animals upon administration of 5% spinach juice, conclusions could not be made regarding the nitrate reductase activity of *L. fermentum* 23 or *L. fermentum* 38.

Animal Weight

Initial animal body weight was approximately 250 g and gradually increased over the experimental trial. A decrease was observed within the last two weeks of the trial in all of the experimental groups (Figure 4.3).

Food and Water Consumption

Food consumption per animal was measured at ~16g/day. However, upon week 6 of the study (day 40) food consumption decreased amongst all groups (Figure 4.4). Water consumption was shown to be ~25ml/daily/animal across the experimental trial (Figure 4.5). Significant differences were not observed amongst the various groups during each week. For daily food and water intake calculation per animal, equation 1 and 2 was used, respectively.
**Overall Animal Behaviour**

All animals within the various groups were observed to be lethargic within a period of 3 days following L-NAME administration. During the progression of the study, two animals (one animal in each of the *L. fermentum* 23 or *L. fermentum* 38 groups) were sacrificed before the terminal bleed at week 7 due to substantial weight loss.

**Biochemical analysis**

Serum samples biomarkers were analysed and did not show a significant difference except for serum sodium concentrations between control treated animals and *L. fermentum* 38 groups treated animals ($P = 0.013$) (Table 4.2). Moreover, ALP, ALT, and AST concentrations were elevated in all groups relative to literature values.
Discussion

The use of nitrates within food products has been pervasive within the food industry. It was shown that an elevation in blood nitrates could result in the formation of $N$-nitroso compounds, a potential carcinogen that has been cited as potentially causing gastric and bladder cancer through chromosomal aberrations of the surrounding cells [19, 62]. But recent lines of data pioneered by Lundberg, Webb, and Weitzberg had demonstrate the use of natural nitrate sources for attenuation of blood pressure, a key symptom of metabolic disease [91, 154]. Sobko has shown that the dorsal tongue has a microenvironment of anaerobic bacteria capable of nitrate reductase activity [35]. Hence upon consumption of a nitrate-rich food, the oral bacteria are readily able to convert nitrate into nitrite which upon ingestion will be converted into the highly reactive nitric oxide in the acidic environment of the stomach. The nitrate reductase enzyme, responsible for the reduction of nitrate to nitrite, can be readily found in a variety of organisms including strains of *lactobacillus* [7]. Due to their GRAS status and simple growth, these stains can potentially be used as probiotics to address metabolic syndrome.

It was the belief of this project that upon ingestion of a nitrite reductase active probiotic, endogenous sources of nitrate present within the blood stream would be converted into nitrite during a transient interluminal interaction of the nitrate and the probiotic. To test this hypothesis, normotensive animals were administered $N^G$-nitro-L-arginine methyl ester (L-NAME), to induce hypertension by blocking endogenous nitrite production attributed to nitric oxide synthases (NOSs). Hence, the inhibition of the NOSs would result in an elevation of blood pressure that could be attenuated by administration a nitrate reductase active probiotic (phase 1). As expected, this did not result in attenuation of elevated blood pressure because one of the primary means of endogenous nitrate synthesis, NOSs, had been inhibited thereby limiting the relative concentration of substrate available for the nitrate reductase active probiotic. Hence, to ensure an adequate supply of nitrate was present for the probiotics to reduce, the probiotics were co-administered with a source of nitrate, spinach juice. Hence, within this system, L-NAME would inhibit nitrate synthesis by NOSs but nitrate would be available through spinach juice and be readily reduced by the nitrate reductase active probiotic. During this phase of
the study (phase 2), an elevation of blood pressure, characteristic of L-NAME administration, was observed in all experimental groups. However, statistically significant differences were not observed during the study amongst control group and the *L. fermentum* 23 or *L. fermentum* 38 groups. These results may be due to several factors. First, although the bacterial load was shown to reduce nitrate *in vitro*, the bacterial load administered to the animals may have been limited. Moreover, the gavaged probiotics may not have been able to survive the harsh acidic conditions of the stomach. As a result, the relative viability of the probiotic may have been substantially decreased thereby leading to a decrease in bioavailability. This can be addressed in future potential experiments by encapsulating the probiotics in alginate-PLL-alginate microcapsules to increase survivability through the stomach and upper duodenum. Moreover, *in vitro* studies (data presented in chapter 3) have shown an ideal ratio of probiotic to nitrate source that resulted in optimal nitric oxide production. Although the *in vitro* results indicated that a 5% (w/v) bacterial cell to 5% (w/v) of nitrate source leads to optimal nitric oxide production, due to the bacterial survivability as a result of the acidic environment and harsh environmental conditions, a titration of the two components is necessary *in vivo*.

Ideally, analysis of blood chemistries would have shown that the plasma nitrate concentration would decrease as nitrite concentrations would increase for bacteria treated animals. Although there was a significant difference between the nitrite concentration of control animals and *L. fermentum* 38 animals at 2 h post-gavage during weeks 1 and 4, this phenomenon was not observed during the other measurements. Furthermore, analysis of serum biomarkers did not result in significant differences between the bacterial treated animals and the control animals except for sodium concentrations, which were significantly different in the *L. fermentum* 38 treated animals relative to the control animals. This phenomenon may be a consequence of the L-NAME induced hypertension resulting in liver damage - as was evident due to increases in ALP, ALT, and AST relative to normotensive animals [120] - that could have resulted in an increase in sodium retention [157].
During the experimental period, animal body weight generally increased and was directly correlated with food and water consumption by the animals. However, during the last two weeks of the experiment (weeks 6-7), food consumption and body weight decreased. This potential observation may be due to the increased deterioration of animal health due to chronic administration of L-NAME.

In conclusion, the goal of this study was to determine whether administration of a nitrate reductase active probiotic could result in attenuation of blood pressure in hypertension induced Wistar-Kyoto using L-NAME. The results indicate that the therapy was unsuccessful in attenuating hypertension; however, it is possible that nitrate may have been reduced to nitrite. This phenomenon can manifest itself in other clinical effects such as increasing gastric mucosal blood flow and mucosal secretion, which will result in gastroprotective effects [11, 84, 85], or inhibiting platelet aggregation [86, 87]. Future studies can aim to evaluate these endpoints as well as test the effects of cellular encapsulation and cell/nitrate titration.
Figures and tables

Figure 4.1: Mean arterial pressure (a), diastolic blood pressure (b), and systolic blood pressure (c) as measured pre-gavage (left panels) and 6 h post-gavage (right panels) in control, *L. fermentum* 23, and *L. fermentum* 38 groups during intervention period. From week 1-5, control animals were treated with sterile saline whereas the *L. fermentum* 23, and *L. fermentum* 38 groups were administered the respective bacteria resuspended in saline; for weeks 6-7, spinach juice was used to substitute saline in all groups. Values are reported as the mean±SE (week 0-6: all groups *n* = 8; week 7: *n* = 8 for control, *n* = 7 for *L. fermentum* 23, and *n* = 7 for *L. fermentum* 38).
### Figure 4.2: Nitrate (left panels) and nitrite (right panels) concentrations in rat blood plasma as measured during weeks 0-7 (a-h, respectively) in control, *L. fermentum* 23, and *L. fermentum* 38 groups during the experiment. Week 0 indicates the acclimitization period. From week 1-5, control animals were treated with sterile saline whereas the *L. fermentum* 23, and *L. fermentum* 38 groups were administered the respective bacteria resuspended in saline; for weeks 6-7, spinach juice was used to substitute saline in all groups. Values are reported as the mean±SE (week 0-6: all groups *n* = 8; week 7: *n* = 8 for control, *n* = 7 for *L. fermentum* 23, and *n* = 7 for *L. fermentum* 38). Significance was set at *P* < 0.05 (*, *P* < 0.05).
Figure 4.3: Body weight distribution across three experimental groups during the experimental trial. Values are reported as the mean±SE (week 0-6: all groups $n=8$; week 7: $n=8$ for control, $n=7$ for *L. fermentum* 23, and $n=7$ for *L. fermentum* 38).
Figure 4.4: Daily food intake per animal across three experimental groups during the experimental trial. Values are reported as the mean±SE (week 0-6: all groups $n = 8$; week 7: $n = 8$ for control, $n = 7$ for *L. fermentum* 23, and $n = 7$ for *L. fermentum* 38).
Figure 4.5: Daily water intake per animal across three experimental groups during the experimental trial. Values are reported as the mean±SE (week 0-6: all groups $n = 8$; week 7: $n = 8$ for control, $n = 7$ for *L. fermentum* 23, and $n = 7$ for *L. fermentum* 38).
Table 4.1: Media formulation based on basal de Man, Rogosa and Sharpe (MRS) media for optimal *L. fermentum* 23 and *L. fermentum* 38

<table>
<thead>
<tr>
<th></th>
<th>Media for <em>L. fermentum</em> 23</th>
<th>Media for <em>L. fermentum</em> 38</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto malt extract</td>
<td>2%</td>
<td></td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.50%</td>
<td>0.50%</td>
</tr>
<tr>
<td>Peptone No3 (bacto)</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Beef Extract</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Soy peptone N-Z</td>
<td></td>
<td>2%</td>
</tr>
<tr>
<td>RNA sodium salt</td>
<td>0.01%</td>
<td>0.01%</td>
</tr>
<tr>
<td>Ammonium Citrate</td>
<td>0.20%</td>
<td>0.20%</td>
</tr>
<tr>
<td>Sodium Acetate</td>
<td>0.50%</td>
<td>0.50%</td>
</tr>
<tr>
<td>Magnesium Sulfate</td>
<td>0.01%</td>
<td>0.01%</td>
</tr>
<tr>
<td>Manganese Sulfate</td>
<td>0.005%</td>
<td>0.005%</td>
</tr>
<tr>
<td>Dipotassium Phosphate</td>
<td>0.20%</td>
<td>0.20%</td>
</tr>
<tr>
<td>Polysorbate 80</td>
<td>0.10%</td>
<td>0.10%</td>
</tr>
<tr>
<td>Sucrose</td>
<td></td>
<td>2%</td>
</tr>
<tr>
<td>Glucose</td>
<td>2%</td>
<td></td>
</tr>
<tr>
<td>FeCl₂</td>
<td>30uM</td>
<td>30uM</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.30%</td>
<td>0.30%</td>
</tr>
</tbody>
</table>
Table 4.2: Serum biochemistry of male Wistar-Kyoto rats in the control, *L. fermentum* NCIMB 7230 treated or *L. fermentum* NCIMB 72023 treated groups.

<table>
<thead>
<tr>
<th></th>
<th>Control (n 8)</th>
<th>L. fermentum 23 (n 7)</th>
<th>L. fermentum 38 (n 7)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td>p</td>
</tr>
<tr>
<td>ALP (units/L)</td>
<td>147.44 ± 5.87</td>
<td>154.04 ± 12.57</td>
<td>130.52 ± 21.09</td>
<td>0.692</td>
</tr>
<tr>
<td>ALT (units/L)</td>
<td>75.25 ± 7.43</td>
<td>69.01 ± 4.63</td>
<td>68.22 ± 7.26</td>
<td>0.504</td>
</tr>
<tr>
<td>AST (units/L)</td>
<td>117.86 ± 13.79</td>
<td>114.76 ± 14.45</td>
<td>160.93 ± 38.48</td>
<td>0.879</td>
</tr>
<tr>
<td>hs-CRP (nmol/L)</td>
<td>19.01 ± 0.60</td>
<td>18.81 ± 0.65</td>
<td>17.12 ± 2.06</td>
<td>0.822</td>
</tr>
<tr>
<td>Lipase (mmol/min×10⁻²)</td>
<td>11.23 ± 0.47</td>
<td>11.62 ± 0.18</td>
<td>13.47 ± 2.19</td>
<td>0.476</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>8.42 ± 1.01</td>
<td>8.97 ± 1.28</td>
<td>16.17 ± 8.26</td>
<td>0.476</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>68.52 ± 5.65</td>
<td>69.21 ± 4.82</td>
<td>123.14 ± 53.94</td>
<td>0.929</td>
</tr>
<tr>
<td>Calcium (mmol/L)</td>
<td>2.75 ± 0.04</td>
<td>2.79 ± 0.05</td>
<td>2.84 ± 0.04</td>
<td>0.599</td>
</tr>
<tr>
<td>Phosphate (mmol/L)</td>
<td>2.11 ± 0.05</td>
<td>2.11 ± 0.10</td>
<td>3.12 ± 0.62</td>
<td>0.476</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>141.04 ± 0.87</td>
<td>146.03 ± 1.56</td>
<td>138.11 ± 4.45</td>
<td>0.013*</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>4.40 ± 0.15</td>
<td>4.28 ± 0.13</td>
<td>5.66 ± 1.08</td>
<td>0.155</td>
</tr>
<tr>
<td>Chloride (mmol/L)</td>
<td>101.60 ± 1.14</td>
<td>102.17 ± 2.18</td>
<td>96.81 ± 4.16</td>
<td>0.271</td>
</tr>
<tr>
<td>Bicarbonate (mmol/L)</td>
<td>29.01 ± 0.64</td>
<td>27.69 ± 0.98</td>
<td>24.07 ± 2.66</td>
<td>0.335</td>
</tr>
</tbody>
</table>

Statistical analysis was performed using Student's t-test (*P < 0.05*)

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CHAPTER 5: SAFETY VALIDATION

IN VITRO SAFETY VALIDATION OF LACTOBACILLUS FERMENTUM NCIMB 702342

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*To be submitted

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Preface

Chapters 3 and 4 outlined the development of an assay for quantification of an isolated nitrate reductase active bacteria and in vivo study testing the effects of *Lactobacillus fermentum* NCIMB 7230 and *Lactobacillus fermentum* NCIMB 72023 administration, respectively. To ensure that the identified stain is safe for potential use within the public domain, a preliminary study was designed to determine the safety profile of the bacterial strain. Metabolic and molecular techniques were used for strain level identification. Moreover, antibiotic resistance as well as production of metabolic by-products and antimicrobial compounds was evaluated. The co-authors have contributed to the experimental design and execution, as well as provided guidance with technical aspects of the experimentation.

Abstract

Safety validation of a novel bacterial strain must be conducted prior to its introduction for public use. *Lactobacillus fermentum* NCIMB 702342 was screened against a panel of in vitro safety tests. Initially, the strain was identified using metabolic (API Zym and API 50 CHL) and molecular (16S rRNA and RiboPrinting) techniques. To determine antibiotic resistance, the strain was screened against 8 antibiotics and found to be susceptible to all of the antibiotics except clindamycin. Moreover, an evaluation of the strain’s ability to produce potentially harmful metabolic by-products and antimicrobial compounds was evaluated and did not show production of either species. This safety assessment of *L. fermentum* NCIMB 702342 suggests that this strain may be safe for public use; however, further in vitro and in vivo studies must be performed.

**Keywords:** Strain safety, *Lactobacillus fermentum*, bacteria
Introduction

Lactic acid bacteria (LABs) are a clade of lactic-acid producing bacteria that are found widely in nutrient-rich environments. Historically, members of the genus *lactobacillus* have been commonly used in a variety of fermented food, ranging from sausage to cheese [103]. Along with the *lactococcus* genus, they have been recognized under the generally-recognised-as-safe (GRAS) status [8, 158]. In recent years, *lactobacillus fermentum* have garnered increasing interest as a potential probiotic linked with prevention of colonic inflammation [159], lowering of cholesterol [160], and enhancing the effects of the influenza vaccine [161]. Although this species has demonstrated safe use for a variety of health benefits, a careful case-by-case evaluation must be performed on novel stains to ensure strain safety.

A primary consideration prior for use of candidate strains is to identify their antibiotic resistance profile. Although LABs possess intrinsic resistance to some antibiotics, these are not transferable to pathogenic strains [158]. However, mobile element-associated antibiotic resistance, which can be transfer to other bacterial strains through horizontal gene transfer, can lead to formation of multidrug resistance (MDR) pathogens [118]. Consequentially, the rise of MDR pathogens pose a serious public risk, especially within the hospital setting [162].

Additionally, for strain safety validation, both metabolic and genetic processes must be implemented, for strain identification, in conjunction with a battery of tests employed to assess production of metabolic by-products and antimicrobial compounds. API 50 CHL and API Zym assays have widely been used for metabolic profiling for species-level characterization [163-165]. However, due to the low resolving power of these approaches, ribotying via RiboPrinting and 16S rRNA has successfully been used to differentiate bacteria at up to the subspecies level [166, 167]. These genetic molecular approaches are inspired by identification of the rRNA genes via southern blotting (RiboPrinting) or sequencing of the 16S rRNA sequence (16S rRNA). An additional consideration during strain safety validation is to determine the production of metabolic by-products or antimicrobial agents by the bacteria. Although these compounds - reuterin, bacteriocin, hydrogen peroxide, D(-) and L(-) lactate, organic acids, and biogenic amines-
are relatively benign in healthy individuals, health complications have been reported mainly in individuals with underlying medical conditions [168].

The primary focus of this paper is to provide an *in vitro* safety evaluation of *Lactobacillus fermentum* NCIMB 702342. Using metabolic and genomic methods, the strain identity was established. Moreover, the strain’s antibiotic resistance profile as well as its capacity for metabolic by-product and antimicrobial compound production was evaluated.
Methods and Materials

Bacterial Growth

*Lactobacillus fermentum* NCIMB 702342 was obtained from the National Collection of Industrial Food and Marine Bacteria (Aberdeen, Scotland). The strain was surface plated on de Man Rogosa Sharpe (MRS, Becton Dickenson, Canada) agar plates and grown anaerobically for 24 h at 37°C. A single, well-isolated colony was picked and grown in MRS broth for 10 h and serially passaged following 10 and 8 h using a 1% inoculum. The above growth conditions were implemented unless otherwise stated.

Metabolic profiling

API Zym and API 50 CHL strips (bioMérieux, Canada) were used according to the manufacturer’s instructions for semiquantification of *L. fermentum* NCIMB 702342’s enzymatic activity and its carbohydrate metabolism profiles, respectively.

Genomic bacterial identification

Species level confirmation of *L. fermentum* NCIMB 702342 was performed using Sanger sequencing of the 16S rRNA gene. Briefly, 50 mL of *L. fermentum* NCIMB 702342 cell culture was grown 24 h and centrifuged at 3000 rpm for 20 min at 4°C. The resultant pellet was resuspended in 1 mL of lysis buffer (2% CTAB, 100mM Tris-HCl pH8, 20mM EDTA, 1.4M NaCl, 0.2% β-Mercaptoethanol, 1 mg/mL proteinase K, 0.05 mg/mL lysozyme and 0.5% SDS). The resuspension was serially incubated for 30 min at 37°C and 60°C. A basis phenol:chloroform extraction was performed, and the DNA was precipitated at -80°C using ethanol. Subsequently, the DNA was washed 2X in 70% ethanol, dried, resuspended in 200 µL TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8), and stored at -80°C until further use. PCR was used to amplify a <650bp segment encoding the 16S rRNA gene and subsequently Sanger Sequenced (Genome Quebec, Canada). The resultant sequence was compared to published 16s rRNA genes in GenBank using the BLAST (Basic Local Alignment Search Tool) algorithm (NCBI, MD).
Moreover, for comparison of RiboPrint patterns of *L. fermentum* NCIMB 7230 and *L. fermentum* NCIMB 702342, bacterial cultures were sent to Accugenix (DE, USA) for analysis.

**Antimicrobial resistance - MIC**

The minimum inhibitory concentration of a variety of antibiotics was determined for *L. fermentum* NCIMB 702342. Briefly, bacterial cultures were grown as previously outlined and centrifuged at 3000 rpm for 20 min at 37°C. The cell pellet was resuspended in sterile saline (0.85% w/v) to a turbidity of a McFarland standard #1 [169]. The solution was diluted 1000X in a LAB susceptibility test medium (90% ISO Sensitest + 10% MRS) and transferred to 96-well Vet MIC plates (Statens Veterinärmedicinska Anstalt, Sweden) with serially diluted concentrations of gentamicin, streptomycin, neomycin, tetracycline, erythromycin, clindamycin, chloramphenicol, and ampicillin. The 96-well Vet MIC plates were subsequently anaerobically incubated at 37°C for 48 h. The breakpoint values were recorded as an average of triplicate plates and as outlined by the ISO 10932 guidelines, *Lactobacillus paracasei* ATCC 334 was analysed as a control [170].

**Reuterin production**

Reuterin production was analyzed as previously described [171, 172]. Briefly, *L. fermentum* NCIMB 702342 and *L. reuteri* ATCC 23272 (ATCC, DC), a positive control for reuterin production, were passaged twice in MRS supplemented with 20 mM of filter-sterilized glycerol. Centrifugation of the cells was carried out at 4200 rpm for 20 min at 37°C. Using sterile 0.1 M potassium phosphate buffer, the pelleted cells were washed twice and resuspended in sterile 200 mM glycerol to a density of 0.1 g/ml (~10^10 CFU/mL). The resulting suspension was incubated for 2 h at 37°C. The cell suspension was centrifuged as above and the harvested supernatant was filtered with a 0.22µm syringe driven filter (Millex Millipore, MA). Supernatant was assayed to quantify reuterin production or stored at 4°C until further testing. Either 300 µL of standard solutions of acroleion or the sample was added to 225µL of fresh 10 mM tryptophan dissolved in 0.05M HCl. Upon subsequent addition of 900µL of concentrated (12M) HCl, the solution was incubated at 37°C for 20 min. The OD was measured at 560 nm using...
spectrophotometer (SpectraMax 250, Molecular Devices, PA). Using the calibration curve, reuterin concentrations were determined within the samples [173].

**Bacteriocin production analysis**

Bacteriocin production by *L. fermentum* NCIMB 702342 was quantified by its capacity to inhibit growth of indicator strains (*L. delbrueckii bulgaricus* ATCC 11842, *P. aeruginosa* ATCC 10145, and *S. aureus* ATCC 43300), as previously described [172, 174]. Briefly, *L. fermentum* NCIMB 702342 was triple passaged overnight at 37°C and centrifuged at 2400 rpm for 20 min. at 4°C. The supernatant from each passage was filter sterilized using a 0.22 µm syringe driven filter (Millipore, MA). The pH of the sterilized supernatants were adjusted to pH 6.5 and heated to 37°C for 10 min. The indicator strains, *L. delbrueckii bulgaricus* ATCC 11842, *P. aeruginosa* ATCC 10145, and *S. aureus* ATCC 43300, were inoculated in MRS, tryptic soy, and nutrient agar plates, respectively, and grown at 37°C overnight. A single well-isolated colony was used to inoculate 5mL of the corresponding media. For bacteriocin quantification, a 1.5% (w/v) agar media was autoclaved and allowed to adjust to 50°C in a warm water-bath. The agar media was then inoculated with the indicator bacteria and 20 mL of the resulting suspension was poured into 10 cm petri dishes to make bacterial lawns, while retaining 2 mL of the agar solution in the 50°C water bath. Once the plates had set, 5 holes were bore into the agar and sealed with a drop of the appropriate molten agar. Starting with undiluted supernatant, 50 µL aliquots of two-fold serially diluted *L. fermentum* NCIMB 702342 supernatant was added to each hole. The plates containing *L. delbrueckii bulgaricus* ATCC 11842 were incubated at 4°C for 3 h under aerobic conditions followed by an anaerobic overnight incubation at 37°C. The plates containing the other indicator bacteria were incubated aerobically at 37°C overnight. Bacteriocin production was determined by visualization of inhibition halos around the holes in the indicator bacterial lawn. The method established by Cherif *et al.* was used to quantify the activity units per mL of culture (AU ml⁻¹) as the reciprocal of the highest dilution yielding a visible halo of inhibition [175]. The experiments were performed in triplicates per indicator organism in two independent experiments.
Hydrogen peroxide production

*L. fermentum* NCIMB 702342 and *L. acidophilus* ATCC 4356, a positive control for H₂O₂ release, were plated on MRS agar plates to obtain well-isolated colonies following overnight incubation at 37°C under anaerobic conditions. A single colony of each strain was picked and inoculated in MRS growth media, which was allowed to grow anaerobically at 37°C overnight. The resulting cultures were diluted appropriately and plated on MRS agar plates supplemented with tetramethylbenzidine (TMB) (0.25 mg/mL) and horseradish peroxidase (0.01 mg/mL) for anaerobic growth at 37°C for 48 h [176]. Hydrogen peroxide production was determined by colony coloration: white colony indicated a lack of production (-), pale blue colony indicated poor production (+), and dark blue colony indicated high production (++) [176].

Biogenic amine production

Biogenic amine production was assessed as previously described [172]. Briefly, five subcultures of *L. fermentum* NCIMB 702342 and *Lactobacillus reuteri* ATCC 23272, a positive control for biogenic amine production, were propagated as above in MRS supplemented with pyrridoxal-5-phosphate (0.005% m/v) and histidine (0.1% m/v) or tyrosine (0.1% m/v). As previously outline, decarboxylase indicator media plates used for cell plating and biogenic amine production was observed due to color development following 48 h of growth [177].

To quantify biogenic amine production, *L. fermentum* NCIMB 702342 was grown overnight, and the culture tubes were centrifuged at 4200 rpm for 20 min at 4°C. The supernatant was aspirated and the cell pellet resuspended in a 1:1 ratio of distilled water, which was subsequently added to an inulin solution (2.5% w/v final concentration). The resuspended pellets were frozen overnight at -80°C and lyophilized. Samples were sent to ETS Laboratories (CA, USA) for further analysis of biogenic amines by HPLC. Briefly, rehydrated lyophilized cells were filtered and quantified using a Dionex ICS-2000 ion chromatograph with an IonPac CS18 column and methanesulfonic acid as an eluent. Putrescine, histamine, and cadaverine were quantified using suppressed conductivity detection, whereas tyramine detection was achieved by UV at 225 nm. All
experiments were performed in triplicate and spermine (4 ppm) was used as an internal standard.
Results

Metabolic profile

Assessment of the fermentation profile of lactobacillus fermentum NCIMB 702342 using API 50 CHL indicated that it was a strain of lactobacillus fermentum as indicated by the APIweb database search (APIweb, bioMérieux, Canada) (Table 5.1). The fermentation profile of L. fermentum NCIMB 702342, as observed by the API 50 CHL, correlated with the results of the API Zym assay (Table 5.2). For instance, fermentation of D-galactose in the API 50 CHL was supported by the presence of both α and β-galactosidase in the API Zym assay whereas the absence of D and L-fucose fermentation was supported by the absence of enzymatic activity by α-fucosidase.

Genomic sequencing

Using the BLAST algorithm, the 16S rRNA gene sequence of L. fermentum NCIMB 702342 was compared to the NCBI nucleotide collection (nr/nt) database. The results indicate homology to previously published L. fermentum sequences. This further supports this strain as a L. fermentum. Moreover, comparison of the RiboPrint patterns of L. fermentum NCIMB 7230 and L. fermentum NCIMB 702342 indicate that the strains can be distinguished (Figure 5.1).

Antimicrobial resistance

EFSA guidelines were used to establish the MIC breakpoint values for L. fermentum NCIMB 702342 and Lactobacillus paracasei ATCC 334 (control). L. fermentum NCIMB 702342 was shown to be susceptible to gentamicin, streptomycin, neomycin, tetracycline, erythromycin, chloramphenicol, and ampicillin; however, it was resistance to clindamycin (Table 5.3).

Reuterin production

L. fermentum NCIMB 702342 reuterin production was quantified in conjunction with those of L. reuteri ATCC 23272 and glycerol used as positive and negative controls, respectively. L. reuteri ATCC 23272 demonstrated reuterin production, whereas both the glycerol control and L. fermentum NCIMB 702342 lacked detectable quantities of reuterin (Table 5.4).
**Bacteriocin production**

Following co-incubation of *L. fermentum* NCIMB 702342 with three indicator species (*L. delbrueckii bulgaricus* ATCC 11842, *P. aeruginosa* ATCC 10145, and *S. aureus* ATCC 43300), the indicator species demonstrated normal growth, thereby indicating *L. fermentum* NCIMB 702342 does not produce bacteriocin.

**Hydrogen peroxide production**

Hydrogen peroxide production was absent in *L. fermentum* NCIMB 702342 whereas *L. acidophilus* ATCC 4356, a positive control, exhibited production as indicated by the dark blue plates.

**Biogenic amine production**

Biogenic amine production was detected for the positive control, *Lactobacillus reuteri* ATCC 23272, in the region of the plate with the greatest concentration of bacterial colonies. However, histamine or tyrosine production were absent in *L. fermentum* NCIMB 702342. Moreover, quantification of biogenic amine production by *L. fermentum* NCIMB 702342 did not result in measurable quantities of putrescine, cadaverine, histamine, or tyramine (Table 5.5).
Discussion

Due to the great diversity and prevalence of unique bacterial strains, whenever the use of a strain for either human consumption or within an industrial setting is considered, considerations have to be made regarding strain safety. Although the genus *Lactobacillus* has several members that have been considered safe for use, general assumptions regarding safety cannot be made on the whole species. Hence, a polyphasic approach was applied to *L. fermentum* NCIMB 702342 to confirm its identity, antibiotic resistance profile, and metabolic by-product and antimicrobial production.

In accordance with guidelines established by a committee of experts from the FAO and WHO, internationally accepted phenotypic and genotypic characterization techniques were used for confirmation of a novel *L. fermentum* strain [178]. Initially, species level resolution, achieved by the API 50 CHL and API Zym commercial identification systems, indicated that the species was a *Lactobacillus fermentum*. Furthermore, 16S rRNA gene sequence and RiboPrint pattern of *L. fermentum* NCIMB 702342 validated that it is, in fact, a *L. fermentum* strain. Although the 16S rRNA sequencing approach offered species level subtyping, the RiboPrint patterns allowed for greater identification of strain level subtyping [167].

Upon correct assessment of taxonomic identity, a battery of tests, consistent with internationally recognized and standardized methods, were run to validate strain safety [178, 179]. The premier experiment was to establish the susceptibility of *L. fermentum* NCIMB 702342 to a range of antibiotics by phenotypic assessment of the minimum inhibitory concentration. *L. fermentum* NCIMB 702342 was shown to be at or below the breakpoint values established by the EFSA guidelines for all of the antibiotics except clindamycin. To ensure that clindamycin resistance genes is not transferred to commensal microbiota primarily through conjugation [180], whole genome sequencing is required to validate the absence of mobile elements.

Production of metabolic by-products and antimicrobial agents have been attributed to *lactobacillus* strains and overproduction of certain agents have been linked with sever health consequences relating to the disruption of the gut microflora [173, 181].
Analysis of *L. fermentum* NCIMB 702342 did not reveal reutein, bacteriocin, hydrogen peroxide, or biogenic amine production.

These preliminary results suggest that *L. fermentum* NCIMB 702342 may be safe for use around humans and animals; however, for absolute confirmation, mucin degradation studies, whole genome sequencing, and further *in vivo* safety analysis must be performed.
### Table 5.1: Carbohydrate metabolism profile results of *L. fermentum* NCIMB 702342 using the API 50 CHL test.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Result</th>
<th>Substrate</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>-</td>
<td>Esculin ferric citrate</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol</td>
<td>-</td>
<td>Salicin</td>
<td>-</td>
</tr>
<tr>
<td>Erythritol</td>
<td>-</td>
<td>D-cellobiose</td>
<td>-</td>
</tr>
<tr>
<td>D-arabinose</td>
<td>+</td>
<td>D-maltose</td>
<td>+</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>+</td>
<td>D-lactose</td>
<td>+</td>
</tr>
<tr>
<td>D-ribose</td>
<td>+</td>
<td>D-melibose</td>
<td>+</td>
</tr>
<tr>
<td>D-xylose</td>
<td>-</td>
<td>D-saccharose</td>
<td>+</td>
</tr>
<tr>
<td>L-xylose</td>
<td>-</td>
<td>D-trehalose</td>
<td>+</td>
</tr>
<tr>
<td>D-adonitol</td>
<td>-</td>
<td>Inulin</td>
<td>-</td>
</tr>
<tr>
<td>methyl-βD-xylopyranoside</td>
<td>-</td>
<td>D-melezitose</td>
<td>-</td>
</tr>
<tr>
<td>D-galactose</td>
<td>+</td>
<td>D-raffinose</td>
<td>+</td>
</tr>
<tr>
<td>D-glucose</td>
<td>+</td>
<td>Amidon</td>
<td>-</td>
</tr>
<tr>
<td>D-fructose</td>
<td>+</td>
<td>Glycogen</td>
<td>-</td>
</tr>
<tr>
<td>D-mannose</td>
<td>+</td>
<td>Xylitol</td>
<td>-</td>
</tr>
<tr>
<td>L-sorbose</td>
<td>-</td>
<td>Gentibiose</td>
<td>-</td>
</tr>
<tr>
<td>L-rhamnose</td>
<td>-</td>
<td>D-turanose</td>
<td>-</td>
</tr>
<tr>
<td>Dulcitol</td>
<td>-</td>
<td>D-lyxose</td>
<td>-</td>
</tr>
<tr>
<td>Inositol</td>
<td>-</td>
<td>D-tagatose</td>
<td>-</td>
</tr>
<tr>
<td>D-mannitol</td>
<td>-</td>
<td>D-fucose</td>
<td>-</td>
</tr>
<tr>
<td>D-sorbitol</td>
<td>-</td>
<td>L-fucose</td>
<td>-</td>
</tr>
<tr>
<td>methyl-αD-mannopyranoside</td>
<td>-</td>
<td>D-arabitol</td>
<td>-</td>
</tr>
<tr>
<td>Methyl-αD-glucopyranoside</td>
<td>-</td>
<td>L-arabitol</td>
<td>-</td>
</tr>
<tr>
<td>N-acetylglucosamine</td>
<td>-</td>
<td>Potassium gluconate</td>
<td>±</td>
</tr>
<tr>
<td>Amygdalin</td>
<td>-</td>
<td>Potassium 2-ketogluconate</td>
<td>-</td>
</tr>
<tr>
<td>Arbutin</td>
<td>-</td>
<td>Potassium 5-ketogluconate</td>
<td>-</td>
</tr>
</tbody>
</table>

(-), negative; (+), positive; (±) unclear.
Table 5.2: Semi-quantification of the *L. fermentum* NCIMB 702342 enzymatic activity results using the Api Zym test.

<table>
<thead>
<tr>
<th>Enzyme tested</th>
<th>Result</th>
<th>Enzyme tested</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>-</td>
<td>Naphthol-AS-BI-phosphohydrolase</td>
<td>±</td>
</tr>
<tr>
<td>Esterase (C4)</td>
<td>±</td>
<td>α-galactosidase</td>
<td>+</td>
</tr>
<tr>
<td>Esterase lipase (C8)</td>
<td>+</td>
<td>β-galactosidase</td>
<td>+</td>
</tr>
<tr>
<td>Lipase (C14)</td>
<td>-</td>
<td>β-glucuronidase</td>
<td>-</td>
</tr>
<tr>
<td>Leucine arylamidase</td>
<td>+</td>
<td>α-glucosidase</td>
<td>+</td>
</tr>
<tr>
<td>Valine arylamidase</td>
<td>-</td>
<td>β-glucosidase</td>
<td>-</td>
</tr>
<tr>
<td>Cystine arylamidase</td>
<td>-</td>
<td>N-acetyl-β-glucosaminidase</td>
<td>-</td>
</tr>
<tr>
<td>Trypsin</td>
<td>-</td>
<td>α-mannosidase</td>
<td>-</td>
</tr>
<tr>
<td>α-chymotrypsin</td>
<td>-</td>
<td>α-fucosidase</td>
<td>-</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(-), negative; (+), positive; (±) unclear.
Figure 5.1: RiboPrint patterns of *L. fermentum* NCIMB 702342 (top) and *L. fermentum* NCIMB 7230 (bottom) using EcoRI (a) and PvuII (b) restriction enzymes.
Table 5.3: The minimal inhibitory concentrations (MICs) of antibiotics necessary for inhibition of *L. fermentum* NCIMB 702342 growth.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (µg/ml)</th>
<th>EFSA breakpoint value (µg/ml)</th>
<th>Resistant or Sensitive R / S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin</td>
<td>1</td>
<td>8</td>
<td>S</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>8</td>
<td>64</td>
<td>S</td>
</tr>
<tr>
<td>Neomycin</td>
<td>2</td>
<td>8</td>
<td>S</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>4</td>
<td>16</td>
<td>S</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.125</td>
<td>1</td>
<td>S</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>16</td>
<td>1</td>
<td>R</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>2</td>
<td>4</td>
<td>S</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0.125</td>
<td>2</td>
<td>S</td>
</tr>
</tbody>
</table>

R, resistance; S, sensitivity.
Table 5.4: Comparison of reuterin production by *L. fermentum* NCIMB 702342 and control, *L. reuteri* ATCC 23272, as measured at OD_{560}.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Reuterin concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (200 mM Glycerol)</td>
<td>0</td>
</tr>
<tr>
<td><em>L. fermentum</em> NCIMB 702342</td>
<td>0</td>
</tr>
<tr>
<td><em>L. reuteri</em> ATCC 23272</td>
<td>115 ± 12</td>
</tr>
</tbody>
</table>
Table 5.5: Quantification of biogenic amines in independent lyophilized culture samples of *L. fermentum* NCIMB 702342 as detected by HPLC.

<table>
<thead>
<tr>
<th>Amine</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putrescine</td>
<td>&lt;1 mg/L</td>
<td>&lt;1 mg/L</td>
<td>&lt;1 mg/L</td>
</tr>
<tr>
<td>Cadaverine</td>
<td>&lt;1 mg/L</td>
<td>&lt;1 mg/L</td>
<td>&lt;1 mg/L</td>
</tr>
<tr>
<td>Histamine</td>
<td>&lt;1 mg/L</td>
<td>&lt;1 mg/L</td>
<td>&lt;1 mg/L</td>
</tr>
<tr>
<td>Tyramine</td>
<td>&lt;1 mg/L</td>
<td>&lt;1 mg/L</td>
<td>&lt;1 mg/L</td>
</tr>
</tbody>
</table>
CHAPTER 6: GENERAL DISCUSSION

Nitrite has been a controversial compound over the past 50 years, where its consumption has been linked to both health benefits and risks [4, 5]. Although it has been considered a water contaminant due to its production as a result of air pollution, run-off of agricultural fertilizer use, oxidation of nitrogenous human and animal waste, and reduction of nitrate-rich, oxygen-poor drinking water in galvanized steel pipes by *Nitrosomonas* bacteria [5], it is constitutively present within an individual either through exogenous consumption or endogenous production. Both of these pathways converge at the enterosalivary recirculation where consumed nitrate or secreted nitrated, by the salivary glands, are reduced to nitrite by the commensal facultative anaerobic bacteria present on the posterior dorsal surface of the tongue [35]. The resultant nitrate is subsequently ingested and further reduced to nitric oxide, a potent small molecule that elicits a variety of functions endogenously.

There has been concern that nitrate consumption can potentially cause gastric or bladder cancer and methemoglobinemia in infants [5]. Although bladder and gastric cancer have been observed in cell culture and animal studies, they have not been observed in humans potentially due to concurrent vitamin C ingestion with nitrate rich foods [36, 60, 63-66]. However, nitrate consumption has been linked to “blue baby syndrome” as nitrite adducts to hemoglobin, resulting in tight oxygen binding that prevents oxygen release and its subsequent reduced availability [5, 64].

Aside from the potentially risks nitrate consumption may pose infants, it has repeatedly demonstrated a variety of health benefits ranging from cardiac health to increased mucosal secretion and inhibition of platelet adhesion [4]. In fact, consumption of 500 mL of beetroot juice, has been linked to sustained attenuation of systolic and diastolic blood pressure by 19 and 8 mmHg, respectively [91]. Moreover, in mice it has been shown to reduce myocardial infarct size by 67% [134].

Due to the potential benefits and risks associated with nitrate consumptions, it is imperative that this molecule and its derivatives are studied and the benefits of nitrate consumption used within the medical health setting. Herein, the enzymatic activity of
Lactobacillus fermentum NCIMB 7230 and Lactobacillus fermentum NCIMB 72023 were assayed in a nitrate-rich beet root juice assay media under anaerobic conditions. Subsequently, the strains were evaluated in an animal model to determine their efficacy to reduce dietary nitrate in vivo and counteract the effects of L-NAME induced hypertension. Moreover, preliminary in vitro safety validation was performed on L. fermentum NCIMB 72023.

A library of lactobacillus strains was screened because this genus has demonstrated nitrate reductase activity [7]. Moreover, lactobacillus strains have historically been used in a variety of fermented food [103] and several strains have been recognized under the generally-recognised-as-safe (GRAS) status [8]. L. fermentum NCIMB 702342 was serially screened from a library of lactobacillus strains using a MRS media supplemented with nitrate and a natural nitrate-rich beet root juice assay media. To determine the ideal assay conditions, it was shown that the strain entered post-exponential phase following 8 h of incubation, which corresponded to maximum nitric oxide production when a final 5% (v/v) cell suspension was assayed using a 5% (w/v) beet root assay media. Moreover, an anaerobic environment was necessary because, as previously outlined, the nitrate reductase enzyme are sensitive to oxygen [59].

Upon development of the assay for quantification of nitrate reductase activity by L. fermentum NCIMB 7230 and L. fermentum NCIMB 702342, an animal study was performed to determine the in vivo efficacy of the two strains to reduced dietary nitrate. A disease state was induced by chronic administration of L-NAME to result in hypertension via the inhibition of the endogenous NOS pathway [13, 127]. The results indicated that although attenuation of hypertension could not be achieved through the administration of the probiotics, two hours following treatment at two time points, plasma nitrite concentrations of treated animals were significantly elevated relative to control animals. These may suggest that the therapy is capable of reducing nitrate to nitrite in vivo. This phenomenon can manifest itself in other clinical effects such as increasing gastric mucosal blood flow and mucosal secretion, which will result in gastroprotective effects [11, 84, 85], or inhibiting platelet aggregation [86, 87]. Further, a potential explanation for the lack of efficacy in the animal model may be due to the reduced survivability of the
probiotic in the gastrointestinal tract of the animal model. Hence, a substantial reduction in bacterial viability in transit can account for a reduction in probiotic bioavailability.

Finally, a preliminary *in vitro* safety validation of *L. fermentum* NCIMB 702342 was performed. Although strains of the *lactobacillus* genus have been assigned the GRAS status, assumptions on the whole genus cannot be made. Metabolic and genetic techniques confirmed the species as a *lactobacillus fermentum*. Moreover, although *L. fermentum* NCIMB 702342 was shown to be resistant to clindamycin, it was susceptible to seven other antibiotics. Since *lactobacillus* strains are known to be resistant to a range of antibiotics, whole genome sequencing is required to identify whether the clindamycin resistance is on a mobile element, and therefore potentially transferable to pathogenic strains. Finally, production of potential metabolic by-products and antimicrobial agents was not detected in *L. fermentum* NCIMB 702342.

In conclusion, this study demonstrates the development of an enzymatic assay for determination of the enzymatic activity of nitrate reductase active bacteria. *L. fermentum* NCIMB 7230 and *L. fermentum* NCIMB 702342 were identified as potent nitrate reductase active strains. These strains were assayed in an animal model to determine their capacity to reduce nitrate *in vivo*. Furthermore, a preliminary safety validation of *L. fermentum* NCIMB 702342 was conducted; however, further studies must be performed to validate *L. fermentum* NCIMB 702342 as a bacterial strain safe for human consumption.
CHAPTER 7: SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

7.1 Summary of Results

1. A comprehensive literature review was conducted that provided a review of the following topics: background information regarding nitrate, nitrite, and nitric oxide; means of endogenous and exogenous production of nitrate, nitrite, and nitric oxide; health effects associated with consumption of nitrate, nitrite, and nitric oxide; *Lactobacillus* use in food and assays for safety validation; and the use of animal models for metabolic syndrome.

2. A novel *in vitro* assay to quantify the bacterial nitrate reductase enzymatic activity was developed using both synthetic assay media (MRS media supplemented with 30 mM nitrate) and a natural assay media (resuspended beet root powder).

3. Relative to the synthetic assay, the natural assay media resulted in elevated nitric oxide (NO) production by the nitrate reductase active bacteria.

4. *Lactobacillus fermentum* NCIMB 702342 demonstrating maximum nitrate reduction using both the synthetic and natural assay media.

5. *Lactobacillus fermentum* NCIMB 702342 entered post-exponential phase 8 h following incubation, which correlated with maximum nitrate reductase activity of 1.9 U.

6. A 5% (w/v) solution of beet root juice assay media inoculated with a final 5% (v/v) of *Lactobacillus fermentum* NCIMB 702342 cell culture resulted in maximum nitrate reductase enzymatic activity.

7. Hypertension was induced in male Wistar-Kyoto rats using 0.6 mg/mL of chronic N\(^G\)-nitro-L-arginine methyl ester (L-NAME) administration in the water.

8. Administration of substrate (beet root juice) with control or nitrate reductase active bacteria (*Lactobacillus fermentum* NCIMB 7230 or *Lactobacillus fermentum* NCIMB 72023) to hypertension-induced rats indicated that the optimal time point for evaluating nitrite and nitrate concentrations in blood plasma was two hours following treatment but that this lasted for up to 6 hours.
9. Evaluation of *Lactobacillus fermentum* NCIMB 7230 or *Lactobacillus fermentum* NCIMB 72023 and substrate requirements indicated that *Lactobacillus fermentum* NCIMB 72023 produced the maximal increase in plasma nitrite with a corresponding decrease in nitrate at the two hour time point.

10. Evaluation of the hypotensive efficacy of the treatment indicated that the treatment did not result in attenuation of blood pressure following induced hypertension using L-NAME.

11. Chronic administration of L-NAME potentially led to liver damage as was evident by the increase in ALP, ALT, and AST concentrations in all L-NAME treated animals.

12. Both metabolic (API Zym and API 50 CHL) and genetic (16S rRNA and RiboPrinting) approaches were applied to verify *L. fermentum* NCIMB 702342 identity at the strain level.

13. MIC breakpoint values for *Lactobacillus fermentum* NCIMB 702342 indicated strain susceptibility to gentamicin, streptomycin, neomycin, tetracycline, erythromycin, chloramphenicol, and ampicillin; however, the strain was resistance to clindamycin.

14. *Lactobacillus fermentum* NCIMB 702342 did not produce reuterin, bacteriocin, hydrogen peroxide, or biogenic amines.
7.2 Conclusions and future directions

The present study was based on the hypothesis that an *in vitro* chemiluminescence assay can be developed to screen a library of candidate *lactobacillus* strains to isolate a nitrate reductase active strain. Moreover, an animal study can be conducted to determine the selected strain’s *in vivo* potential to reduce dietary nitrates, followed by an *in vitro* strain safety validation. Both the use of a synthetic and natural assay media were investigated. The natural assay media consisting of reconstituted beet root powder yielded elevated nitric oxide production as the nitrate within the media was eventually converted to nitric oxide within the acidic environment of the bacterial assay media. This assay led to the identification of *L. fermentum* NCIMB 7230 and *L. fermentum* NCIMB 702342 as an efficient nitrate reductase active strains that resulted in the high nitric oxide production relative to the other candidate strains in the library. Using a hypertension induced model using L-NAME, the two stains were tested *in vivo*. This approach implemented blood pressure measurements as well as determination of plasma nitrate and nitrite concentrations. Moreover, serum biochemistry of the animals was determined to identify potential end organ damage. Furthermore, the safety profile of *L. fermentum* NCIMB 702342 was examined. Metabolic and molecular techniques confirmed the strain identity. Although the strain did not produce metabolic by-products or antimicrobial compounds, its antibiotic resistance profile indicated that it was susceptible to all of the antibiotics tested except clindamycin. This study establishes the fundamental upon which further studies such as whole-genome sequencing and *in vivo* animal haematology studies are necessary to confirm strain safety. The findings of these studies provide the framework for the use of a highly nitrate reductase active strain to potentially treat metabolic syndrome.

Based on the experimental results, the following conclusions can be made:

1. A novel, reproducible assay was developed that allows for quantification of the enzymatic activity of nitrate reductase bacterial strain
2. Although attenuation of blood pressure was not achieved in hypertension induced animals, increases in plasma nitrite concentrations were observed and following two hours post-gavage and were maintained for 6 hours
3. *L. fermentum* NCIMB 702342 is a potentially safe strain for public use; however, further *in vitro* and *in vivo* studies are necessary.

Furthermore, the following are some future directions of study for the results in this thesis:

1. To potentially develop a probiotic using a nitrate reductase active bacteria, further considerations have to be make:
   
   a. SHR Study using Telometry: The experiments can be repeated in spontaneously hypertensive rats (SHR) which had been operated to implant telemetric probes (DSI, St Paul, MN, USA) in the femoral or carotid artery to monitor blood pressure and heart rates during the treatment. The treatment will be the same as described in the methods section of chapter 4 but with the exclusion of L-NAME administration and chow containing nitrate. Moreover, there will be four groups with different treatments: two control groups (saline and nitrate-rich-juice), as well as two treated groups (probiotic alone, and nitrate rich juice in combination with a selected probiotic treated). Additionally, liver, kidney, adrenal glands, stomach, aortic trees and hearts will be collected to look for hypertension related pathologies. We do not expect to see a reduction in blood pressure for the saline-treated control group, whereas nitrate-rich-juice-treated control animals should demonstrate some decrease in blood pressure. A positive result in the nitrate-rich-juice-treated group will mean that the animals host a microorganism capable of reducing nitrate to nitrite. A positive result in the probiotic only treated group will mean that the probiotic is capable of lowering blood pressure by a nitrate independent mechanism. Furthermore, we expect the group treated with nitrate rich juice in combination with a probiotic to demonstrate greater attenuation in blood pressure relative to the nitrate-rich-juice treated control group.

   b. Encapsulation of Probiotics: The alginate/poly-L-lysine microencapsulation of probiotics is a technology used to immobilize and protect bacteria from harsh conditions while allowing the free transport of
nutrients and waste products with the milieu. Such technology is suitable to deliver probiotics through the acidic stomach environment, preserving viabilities and allowing further colonization of the lower gastrointestinal tract. The administration of nitrate-reducing free or microencapsulated probiotics, in combination with a nitrate rich diet, presents a potential opportunity to generate physiologically relevant amounts of NO in the upper and lower gastrointestinal tract. Hence, animals will be gavaged with free and microencapsulated probiotics to determine whether encapsulation improves nitrate reductase activity in vivo as determined by an increase in nitrite levels in plasma. The bacteria will be delivered either free or encapsulated, with adjustment made for the amount of delivered APA probiotic relative to the CFU of free bacteria. Blood will be collected before gavage, and 2 and 3 hours post gavage for the maximum nitrate/nitrite levels (250 ul). Blood pressure will be measured 4-6 times a day. Twice before gavage, and 2-4 times after gavage every hour. The experiment will be repeated twice, a week apart.

c. Optimal ratio of probiotic to nitrate-rich source: The goal of this study is to maximize the nitrite levels in blood by nitrate reductase active probiotics. As mentioned in the chapter 3, in vitro studies demonstrated a substantial level of nitric oxide production with probiotic: beetroot juice when the concentrations of the two components were 5% (w/v). However, deviation of both decreases and increases of these values led to decreased nitric oxide production. Here, we proposed the following concentrations: 5%, 0.5%, 0.1%, and 0.05%. Blood will be collected before gavage, and 2 and 3 hours following gavage for determination the maximum change in nitrate/nitrite levels (250 µl). Furthermore, blood pressure will be measured 4-6 times a day. Twice before gavage, and 2-4 times after gavage every hour. The experiment will be repeated twice, a week apart.

2. To completely validate *L. fermentum* NCIMB 702342 as a safe strain acceptable for potential consumption by humans, further *in vitro* and *in vivo* studies are necessary:
a. Whole genome sequencing is necessary to ensure that the clindamycin resistance is not encoded on a mobile element within the bacterial genome and hence transferable to pathogenic strains.

b. The mucin degradation and translocation ability of the bacteria must be examined to ensure that the bacteria strain does not degrade the protective mucosal barrier within the intestinal wall, thereby allowing translocation of deleterious strain through the barrier and subsequent invasion of the epithelial cells.

c. An animal study must be conducted to validate the in vitro safety results and ensure that ill-effects are not observed in the animals’ health due to administration of the bacteria.
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