

**A new probiotic approach for the prevention and/or treatment of dental caries, oral candidiasis and periodontal diseases**

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**To my parents, husband, brother and friends  
for their unconditional support through this expedition**

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## PREFACE

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This thesis was prepared in accordance with the McGill University Thesis Preparation guidelines. I selected a manuscript based thesis format and consist of one or more papers either accepted, submitted or to be submitted in peer reviewed journals. I am the first author along with co-authors, in these papers, which are included in chapter 3 to 8. Each chapter contains an abstract, introduction, materials and methods, results and discussion. The thesis also contains a common abstract, general introduction, literature review, general discussion of results, conclusion and a bibliography in accordance with the guidelines.

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## LIST OF ABBREVIATIONS

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AIDS: Acquired immunodeficiency syndrome

ALP: Alkaline phosphatase

ATCC: American Type Culture Collection

AUC: Area under the curve

BMD: Bone mineral density

BOP: Bleeding on probing

CFU: Colony forming unit

CMC: Carboxymethyl cellulose

CRP: C-reactive protein

DC: Dental caries

DMEM: Dulbecco's Modified Eagle Medium

DNS: Dinitrosalicylic

EFA: Ethyl ferulate

ELISA: Enzyme-linked immunosorbent assay

FA: Ferulic acid

FAE: Ferulic acid esterase

FAO: Food and Agriculture Organization of the United Nations

FBS: Fetal bovine serum

FDA: Food and Drug Administration

GBD: Glucan binding proteins

GCF: Gingival crevicular fluid

GI: Gingival Index

GIT: Gastro-intestinal tract

GRAS: Generally recognized as safe

GTFs: Glucosyltransferases

H<sub>2</sub>O<sub>2</sub>: Hydrogen peroxide

HIV: Human immunodeficiency virus

HOMD: The Human Oral Microbiome Database

HPLC: High-pressure liquid chromatography

IBD: Inflammatory bowel diseases

Ig: Immunoglobulins

IL: Interleukin

LPS: Lipopolysaccharide

LrrA: Leucine-rich repeat protein

LV: Loss of viability

MEM  $\alpha$ : Minimum Essential Medium alpha

MMP-3: Matrix metalloproteinase-3

MRS: De Man, Rogosa, Sharpe

MSB: Mitis-sucrose-bacitracin

Msp: Major sheath protein

NCIMB: National Collection of Industrial Food and Marine Bacteria

NED: Naphthylethylenediamine dihydrochloride

NIH: National Institutes of Health

NO: Nitric oxide

OC: Oral candidiasis



OD: Oral disease

OPG: Osteoprotegerin

OTF: Oral thin film

PBI: Papillary Bleeding Index

PBS: Phosphate buffered saline

PD: Periodontal disease

PDL: Periodontal ligaments

PI: Plaque Index

PMN: Polymorphonuclear

RANKL: Receptor activator of nuclear factor  $\kappa$ B ligand

ROS: Reactive oxygen species

SEM: Standard error of the mean

SSF: Simulated salivary fluid

TNF: Tumor necrosis factor

WHO: World Health Organization

## ABSTRACT

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The World Health Organization states that adults worldwide have a prevalence of almost 100% for dental caries and 15-20% for severe periodontal diseases. Astonishingly, the prevalence of oral candidiasis is high in HIV patients which stand at 40-50%. The main therapeutics targets for these diseases are pathogen inhibition, limiting oral inflammation and restoring lost oral tissue. Current treatments have limitations, as suggested by the high disease prevalence rates. More importantly, the chronicity of oral diseases requires a natural and safe long-term therapy. Probiotic bacteria, with recent works investigating the oral microbiome, have gained great interest for the development of biotherapeutics. The goals of this thesis includes screening *Lactobacillus* strains for the inhibition of *Streptococcus mutans*, and *Candida albicans*, dental caries and oral candidiasis causing microorganism, respectively. Following the selection of probiotic strains, probiotic characteristics such as probiotic bacteriocin activity, salivary pH modulation, probiotic nutrient (sucrose) competition, probiotic co-aggregation with *S. mutans*, bacterial attachment to oral epithelial keratinocytes, bacterial nitric oxide production and bacterial antioxidant activity were investigated. *Lactobacillus reuteri* and *Lactobacillus fermentum* strains demonstrated oral health promoting characteristics when compared to *S. mutans*. This was followed by investigations into probiotic hydrogen peroxide production, as hydrogen peroxide has important implications in oral health, including its antimicrobial and whitening properties. Indeed, it was demonstrated that the *L. reuteri* mechanism of *S. mutans* inhibition was directly correlated to peroxide production, as investigated using catalase inactivation of hydrogen peroxide. *L. reuteri* NCIMB 701359, the probiotic with the highest level of hydrogen peroxide production,

demonstrated significant reduction in *S. mutans* biofilm formation, as investigated in a developed model of biofilm under simulated oral conditions. Chronic inflammation and loss of oral tissue are characteristics of periodontal diseases. *L. fermentum* NCIMB 5221 treatment demonstrated significant reductions in pro-inflammatory cytokines secreted by inflamed osteoblast-like cells. Of potentially even greater interest for periodontitis, the probiotic *L. fermentum* NCIMB 5221 was also shown to promote the growth of osteoblast-like cells. *In vivo* studies shows that *L. fermentum* NCIMB 5221 modulated serum calcium levels demonstrating their potential role for bone remodeling. Also, a novel oral thin film (OTF) was developed using carboxymethyl cellulose (CMC), to improve the delivery and residence of probiotic bacterial cells into the oral cavity. Following film characterisation and optimization, *L. fermentum* NCIMB 5221 was incorporated in the films. The probiotic-CMC-OTFs when stored long-term at room temperature, did not change bacterial cell viability and anti-oxidant activity for 150 days. This novel work demonstrates the potential of probiotic bacterial formulations, as natural oral health bio-therapeutic for the prevention and treatment of oral diseases.

## RÉSUMÉ

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L'Organisation mondiale de la Santé estime que la prévalence des caries dentaires à travers le monde approche 100% et 15-20% pour les maladies parodontales sévères. De plus, la prévalence de la candidose buccale est élevée, approchant 40-50%, auprès des patients VIH. Les principales cibles des thérapeutiques pour ces maladies sont de contrôler la croissance de pathogènes, de limiter l'inflammation buccale et de restaurer des tissus buccaux perdus. Les traitements actuels ont des limitations importantes, comme le suggèrent les taux de prévalence élevés des maladies. Plus important encore, la chronicité des maladies bucco-dentaires nécessite une thérapie naturelle et sûre à long terme. Les bactéries probiotiques, avec les enquêtes récentes du microbiome oral, ont gagnées grand intérêt pour le développement de produits biothérapeutiques. Les objectifs de cette thèse incluent le dépistage de souches de *Lactobacillus* pour l'inhibition de *Streptococcus mutans*, et *Candida albicans*, respectivement. Des souches de *Lactobacillus* choisies ont été criblées pour l'inhibition de *Streptococcus mutans* et *Candida albicans*, les micro-organismes provoquant les caries dentaires et la candidose buccale, respectivement. Suite à la sélection de souches probiotiques, des caractéristiques probiotiques tels que l'activité de la bactériocine, modification du pH salivaire, compétition pour les nutriments (saccharose), co- agrégation probiotique-*S. mutans*, l'adhésion des bactéries aux kératinocytes épithéliales orales, production d'oxyde nitrique et antioxydant bactérien ont été étudiés. Des souches de *Lactobacillus reuteri* et *Lactobacillus fermentum* ont démontré des caractéristiques capable de promouvoir la santé bucco-dentaire. Cela a été suivi par des enquêtes sur la production de peroxyde d'hydrogène par les probiotiques. Le peroxyde d'hydrogène a des implications

importantes en matière de santé bucco-dentaire, y compris ses propriétés antimicrobiennes et de blanchiment. En effet, il a été démontré que le mécanisme de *L. reuteri* contre *S. mutans* est directement corrélée à la production du peroxyde d'hydrogène. *L. reuteri* NCIMB 701359, le probiotique avec le plus haut niveau de production de peroxyde d'hydrogène, a démontré une réduction significative de la formation de biofilm de *S. mutans*, étudié dans un modèle développé de biofilm dans des conditions simulées de la voie orale. L'inflammation chronique et la perte de tissus buccaux sont caractéristiques des maladies parodontales. *L. fermentum* NCIMB 5221 a démontré des réductions significatives des cytokines pro-inflammatoires sécrétées par des cellules ostéoblastes enflammées. De même, potentiellement un plus grand intérêt pour la parodontite, le probiotique *L. fermentum* NCIMB 5221 a été montré pour promouvoir la croissance des cellules ostéoblaste. Des études *in vivo* ont démontré que *L. fermentum* NCIMB 5221 a modulé les niveaux de calcium sérique modulés, intéressant pour leur potentiel de remodelage osseux. Enfin, un nouveau film mince orale (FMO) a été développé en utilisant un polymère naturel, la carboxyméthylcellulose (CMC) pour améliorer la prestation et le séjour des cellules bactériennes probiotiques dans la cavité buccale. Suite à la caractérisation et l'optimisation de la formation du film, *L. fermentum* NCIMB 5221 a été incorporé. Le probiotique-CMC-FMO, lorsqu'il a été conservé à long terme à la température ambiante, n'a pas entravé la viabilité de la cellule bactérienne et la production d'anti-oxydant au cours de 150 jours. Ce travail démontre le potentiel de formulations bactériennes probiotiques pour promouvoir la santé bio- thérapeutique orale spécifiquement pour la prévention et le traitement des maladies bucco-dentaires. Les études futures devraient inclure des recherches mécanistes et essais cliniques

supplémentaires pour explorer le plein potentiel des bio- thérapeutiques probiotiques pour la gestion des maladies bucco-dentaires.

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

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### 1.1 General introduction

Oral diseases, particularly dental caries and periodontal diseases, affect a large portion of the worldwide population. The World Health Organisation considers oral disease an economic burden with 5-10 % of overall public health expenditures spent for oral diseases [1,2]. The diseases prevalence is almost 100 % for dental caries and 15-20 % for periodontal diseases for adults [2]. Apart from disease prevalence, they also possess other very important health risks such as tooth loss, cellulitis, osteomyelitis, infective endocarditis, atherosclerosis and preterm babies that have potential to be fatal [3-11]. In terms of oral candidiasis, the prevalence is 31% in acquired immunodeficiency syndrome and almost 20% in cancer patients and can prove fatal [12,13]. Oral diseases have a multifactorial etiology and important factors such as bacterial overgrowth, oral biofilm formation and inflammation contributes to disease progression [14-16]. Current treatment modalities focus mainly on disease prevention. Following, disease onset treatment focus on limiting disease progression and restoring lost tissue [3-5,17]. However, current statistics clearly suggest that these modalities have important limitations.

The oral cavity is challenged by a wide range of bacteria and hence, is extremely susceptible to bacterial infections. *Streptococcus mutans* is the primary causative organism and the most abundantly found pathogen in dental caries. Likewise, oral candidiasis is an opportunistic infection caused due to *Candida albicans*, an oral opportunistic pathogen. Similarly, periodontal diseases are associated with microorganisms

such as *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* to name a few. Most important is the role of oral biofilms in disease progression which provides a niche for the growth of these pathogens [18]. Apart from bacterial overgrowth, an elevated host immune reaction to combat such infection, in turn leads to a state of chronic inflammation, an important factor that leads to connective tissue destruction in periodontal diseases [19]. Therefore, long-term therapeutic is needed for the prevention and treatment of chronic oral diseases.

Probiotics are health promoting bacteria that offers natural and safe long-term therapeutic option for such chronic diseases [20]. They have been investigated for a number of diseases, and most importantly, have demonstrated potential to improve dental caries, oral candidiasis and periodontal diseases [21-24]. To develop an effective probiotic bio-therapeutic for oral diseases, research has yet to elucidate the mechanisms that are involved in probiotic mediated oral pathogen inhibition. In addition, recent research has demonstrated that probiotic bacteria have immunomodulatory effects [25]. These effects can be useful in the management of periodontal diseases, characterised by chronic inflammation due to a hyperactive host immune system [26].

Another important aspect for developing a successful probiotic oral bio-therapeutics is an appropriate delivery system. Current delivery systems consist of mouth rinses, probiotic drops, chocolate, and food products such as milk, cheese and yoghurt [27-30]. However, they are rapidly swallowed which reduces probiotic therapeutic effectiveness and hence, a therapeutic that would increase the residence time of the therapeutic agent in the mouth would be beneficial. A film made of natural polymers can be used as a vehicle for probiotic bacteria delivery to the oral cavity and increase their



residence time in the oral cavity. This research was undertaken to address these limitations with the hypothesis that probiotic bacteria can be used to prevent and treat oral diseases.

## **1.2 Research hypothesis**

Probiotic bacteria can prevent and/or treat dental caries, oral candidiasis and periodontal disease and a novel dissolvable oral thin film formulation can be designed to deliver them to the oral cavity.

## **1.3 Research objectives**

The specific objectives are:

1. To screen probiotic bacteria for their potential to inhibit *S. mutans* and *C. albicans* responsible for dental caries and oral candidiasis.
2. To investigate features of probiotic bacteria that would provide benefits for prevention and treatment of dental caries and periodontal diseases.
3. To evaluate whether probiotic bacteria inhibits *S. mutans* via hydrogen peroxide production.
4. To study probiotic *Lactobacillus reuteri* NCIMB 701359 as an inhibitor of *S. mutans* biofilm for their potential to prevent and treat dental caries.
5. To investigate probiotic *Lactobacillus fermentum* NCIMB 5221 as a periodontal disease bio-therapeutic *in vitro* using osteoblast cells and *in vivo* using F344 rats.
6. To develop, characterise and investigate dissolvable carboxymethyl cellulose films for the oral delivery of probiotic bacteria, for their potential as dental caries, oral candidiasis and periodontal diseases therapeutic.

## **1.4 Thesis outline**

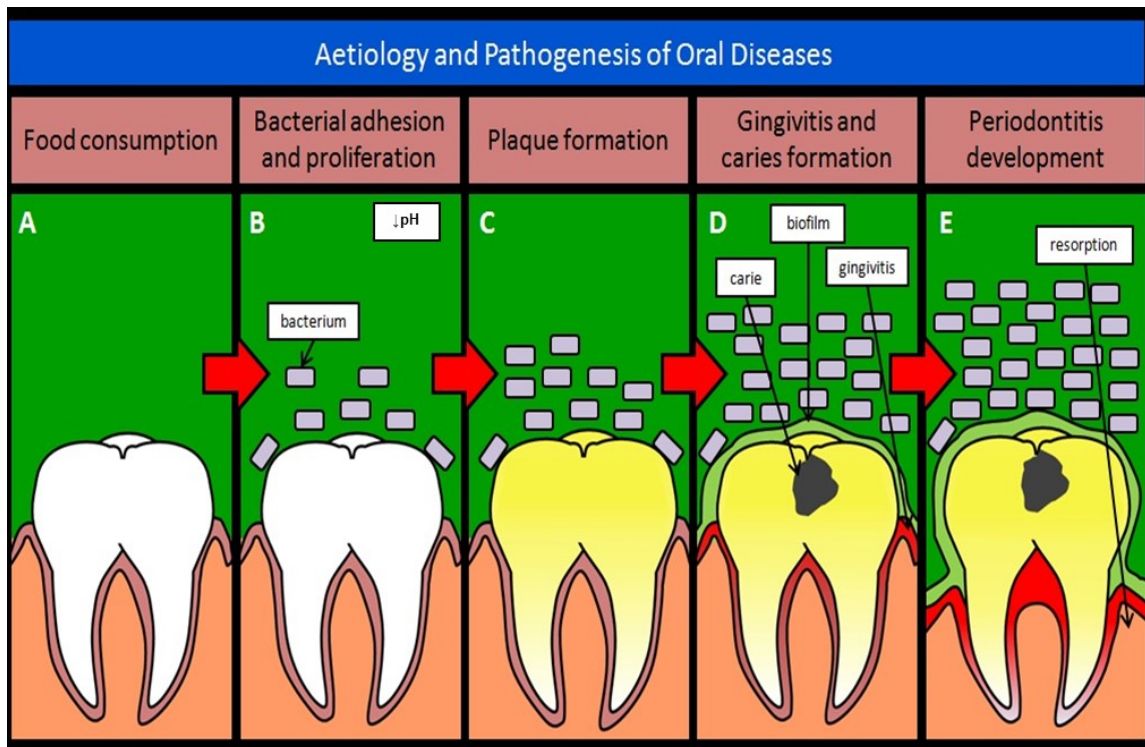
This thesis consists of 11 chapters. The first chapter presents a general introduction, research hypothesis, research goals and thesis outline. Chapter 2 is a comprehensive literature review. Chapters 3-8 are original research contributions that are either published or submitted in peer-reviewed journals. All of the chapters focus on each aforementioned research objectives. Chapter 9 provides a general discussion that summarizes the main observations of the thesis. Chapter 10 details summary and discusses the original research contributions and the conclusion. Chapter 11 details recommendations and future perspectives.

### 2.1 Introduction to oral disease

Oral disease is a global health concern despite the measures taken to prevent it [31]. It is an important economic burden with most industrialized nations spending 5-10% of their public health expenditure [2]. Of more importance is the limited access to adequate and timely diagnosis and treatment of oral diseases for people with low socio-economic status [32]. Dental caries and periodontal diseases, the most prevalent oral diseases, are the main contributors to the global oral health burden. Dental caries, also termed dental cavities, is one of the most common chronic diseases of childhood, five times more prevalent than asthma [33]. The World Health Organization (WHO) reports that almost 100% of adults and 60-90% of school children worldwide are affected by dental caries [2]. The worldwide prevalence of severe periodontal disease is 15-20 % in middle aged adults. There are very few studies that report on gingivitis prevalence. Therefore, although outdated, one study reports a 50-100% worldwide prevalence of gingivitis [2,34]. Apart from these aforementioned oral diseases, oral candidiasis is a major concern in immunocompromised patients, with a potential to be life threatening for these patients [13]. Oral candidiasis, also termed oral thrush, is a yeast infection of the oral cavity due to the overgrowth of *Candida albicans*. The incidence of oral candidiasis is not common in the general population. However, it is important to note that the current prevalence of oral candidiasis is 9-31% in acquired immunodeficiency syndrome (AIDS) patients and almost 20% in cancer patients [12].

Oral health is governed by the microbial community of the oral cavity. The resident oral microorganisms are typically in homeostasis with the host and a disturbance

in this balance can initiate and potentiate oral diseases [35,36]. The resulting shift in the oral ecosystem promotes the proliferation of opportunistic pathogens on the surfaces of the mouth [35,36]. Briefly, following food consumption, oral bacteria metabolize carbohydrates, leading to acid production. This facilitates the adhesion and proliferation of opportunistic pathogens and increased oral biofilm production. These events ultimately lead to the initiation and progression of several oral diseases (**Figure 2.1**).



**Figure 2.1:** Oral disease development pathways: (A) Consumption of food containing carbohydrates. (B) Fermentation of dietary carbohydrates and development of an acidic pH enabling bacterial adhesion and proliferation. (C) Formation of an oral biofilm known as dental plaque which facilitates the survival and proliferation of pathogens residing in it. (D) Development of gingivitis and dental caries due to established plaque. Gingivitis is recognised by red swollen gingiva and caries is recognised by tooth decay. (E) Development of periodontitis due to a disseminated infection into the supporting structures of the tooth.

Oral biofilm, an important factor for oral disease progression, provides a niche for the oral pathogen and shields it from the host's defense system [35,36]. An interesting

phenomenon termed quorum sensing further facilitates the development of oral biofilm [37]. According to quorum sensing, bacterial counts, upon reaching certain density, modulate their behaviour to adapt to the new environment. The bacteria secrete substances like exopolysaccharide via quorum sensing-mediated signal transduction, enhancing biofilm formation. Most importantly, the biofilm acts as a diffusion barrier protecting the innermost bacterial layer confined in the biofilm from antimicrobials. The exact signalling pathways in quorum sensing, that lead to altered bacterial gene expression and expansion and maintenance of the biofilm is complex, are discussed in detail by Sandheera *et al.* [37]. Of more importance is the characterization of the oral microbiome for better understanding oral disease pathogenesis and the ongoing field of research to enumerate the oral microbiota in oral diseases, discussed in the following sections.

## **2.2 The oral microbiota and their role in oral diseases**

As aforementioned, the normal microbiome has important roles such as the ability to synthesize vitamins and compete for nutrient with opportunistic pathogens [38]. There is a vast amount of emerging works on the normal oral microbiota, however, the role of each bacterial population in the oral cavity requires further understanding. Oral commensals demonstrate beneficial effects by secreting bacteriocins and antibacterial compounds such as hydrogen peroxide and competing for nutrients consumed by pathogens [39-41].

The human mouth is sterile at birth and is colonized by bacteria during and following birth [42]. The normal human oral microbiome is extremely diverse between individuals. For example, Holgerson *et al.* demonstrated that the oral microbiota of

infants was influenced by the mode of delivery [42]. The infants born via caesarian delivery had a distinctly different oral microbiota when compared to those born by normal delivery [42]. The Human Oral Microbiome Database (HOMD) (<http://www.homd.org/>) provides in depth information about the bacterial populations and reports that 17 % of the oral microbiome is yet to be named and 34 % still remains uncultivable [43]. Moreover, the exact level of the mouth's microbial diversity is contentious, cited differently by research groups, potentially due to variations in detection methods and immense diversity among the human population [44,45]. 1179 taxa in the human oral flora were identified by Dewhirst *et al.* (2010) [46]. Keijser *et al.* (2008) reported an estimated 19 000-26 000 species in the mouth [47]. Jorn *et al.* (2005) identified 141 predominant species and Paster *et al.* (2005) detected 415 species in the subgingival flora [48,49]. Historically, the analysis of a bacterial population was performed by growth on defined and selective media. Unfortunately, a large proportion of bacteria remain uncultivated and hence, research has turned to molecular techniques. Qualitative techniques used by researchers to characterize the oral microbiota involve: fingerprinting, terminal restriction fragment length polymorphism, ribosomal intergenic spacer analysis, 16S ribosomal RNA sequencing, microarrays and high-throughput genome sequencing technologies [50]. Quantitative methods of characterization may include fluorescence in situ hybridization, catalyzed reporter deposition-fluorescence in situ hybridization, quantitative polymerase chain reaction and scanning electron microscopy in situ hybridization [50]. In general, metagenomics is an approach used to analyze the genomic content of microbes living in oral ecosystem. These techniques all vary in their sensitivity and specificity.

Regardless of the exact species number, the prominent genera that inhabit the oral cavity are *Streptococcus*, *Veillonellaceae*, *Neisseria*, *Haemophilus*, *Corynebacterium*, *Rothia*, *Actinomyces*, *Prevotella*, *Capnocytophaga*, *Granulicatella*, *Porphyromonas* and *Fusobacterium* [45,51]. Bacterial species demonstrate predilection for different sites in the mouth. For example, *Streptococcus mitis* was found in almost all the oral surfaces and subjects [52]. Similar observations were made by Kononen *et al.* in young children [53]. However, certain bacteria such as *S. mitis* bv. 2 had a predilection for the lateral surface of the tongue and *S. parasanguinis* strain 85-81 were prominent in the dorsum of the tongue [52]. The observed differences were likely because of the anatomical dissimilarities between these surfaces which influences their ecology. The microbial diversity differs widely between individuals with one individual having 34 bacterial types and one having 72 bacterial types, in a recent investigation using subjects with healthy oral microbiota [52].

As aforementioned, oral dysbiosis encourages elevated growth of certain bacterial types, termed opportunistic pathogens. Microorganisms such as *S. mutans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, generally associated with oral disease, were not detected in any of the oral sites of subjects with healthy teeth [52]. Other research groups have reported low levels of these microorganisms as a part of the normal oral microbiome, suggesting these as opportunistic pathogens [54-56]. Other factors, such as long term antibiotic use can lead to overgrowth of pathogens such as *Clostridium difficile* in the gut or *Candida* in the oral cavity. Moreover, an immunosuppressed state of the host can lead to the overgrowth of opportunistic pathogens causing oral disease. The pathogenesis of dental caries,

periodontal disease and oral candidiasis is linked to specific oral microbes, as discussed below.

### **2.2.1 Role of oral microbiota in dental caries**

Multiple bacterial types are associated with dental caries, specifically *Streptococcus*, *Veillonella* and *Actinomyces* are the most frequently found genera [56]. *Streptococcus*, *Lactobacillus* and *Neisseria* are also associated with dental caries, suggesting a global microbiota approach (**Table 2.1**) [35,57]. It is important to note that not all *Streptococcus* species or *Lactobacillus* species are cariogenic. For example, *S. mitis* and *S. oralis* are not associated with dental caries while *S. mutans* are present abundantly in early carious lesions, typically known as white spot lesions [55,56]. *S. mutans* is linked with the initiation of dental caries. On the other hand, *Lactobacillus* species, although present, are only detected in late caries and root caries [56,58]. The most frequent caries-associated *Lactobacillus* species are *L. gasseri*, *L. casei*, *L. salivarius*, *L. ultunensis* and *L. crispatus* [59]. On the contrary, certain *Lactobacillus* species have demonstrated potential to significantly reduce caries risk [60,61]. More importantly, caries progression is also associated with a decrease in microbial diversity [55].



**Table 2.1:** The oral microbiota and its association with dental caries and periodontal disease.

Oral diseases	Pathogens	Ref(s)
Dental caries	<i>Streptococcus mutans</i>	[62-64]
	<i>Streptococcus sobrinus</i>	[65]
	<i>Streptococcus sanguinis</i>	[66]
	<i>Enterococci</i>	[62]
	<i>Actinomyces</i>	[62,67]
	<i>Veillonella atypia dispar parvula</i>	[66]
	<i>Lactobacillus gasseri johnsonii</i>	[66]
	<i>Lactobacillus casei paracasei</i>	[66]
	<i>Selemonas</i>	[66]
	<i>Neisseria</i>	[66]
	<i>Propionibacterium</i>	[66]
Periodontal Disease	<i>Fusobacterium nucleatum</i>	[68]
	<i>Porphyromonas gingivalis</i>	[69,70]
	<i>Bacteroides forsythus</i>	[69-71]
	<i>Treponema denticola</i>	[69-71]
	<i>Actinobacillus actinomycetemcomitans</i>	[69,71,72]
	<i>Prevotella intermedia</i>	[69]
	<i>Prevotella nigrescens</i>	[69]
	<i>Peptostreptococcus micros</i>	[73]
	<i>Proteus mirabilis</i>	[74]
	<i>Pseudomonas aeruginosa</i>	[74]
	<i>Pseudomonas maltophilia</i>	[74]
	<i>Pseudomonas</i>	[74]
	<i>Enterobacter cloacae</i>	[74]
	<i>Enterobacter agglomerans</i>	[74]
	<i>Escherichia coli</i>	[74]
	<i>Klebsiella pneumonia</i>	[74]
	<i>Klebsiella oxyloca</i>	[74]
	<i>Serratia marcescens</i>	[74]
	<i>Campylobacter rectus</i>	[69]
	<i>Eikenella corrodens</i>	[69]
	<i>Porphyromonas endodontalis</i>	[102]
	<i>Candida albicans</i>	[74,75]
Oral Candidiasis	<i>Candida albicans</i>	[17]

Note: *Enterococci* have been associated with causing caries in animal models [62]

Disease progression and virulence of cariogenic bacteria are mainly attributed to bacterial adhesion. Bacterial fimbriae, fibrils and pili are the most common mechanisms for adhesion of cariogenic bacteria [76]. Apart from dental plaque formation via quorum sensing, there are other *S. mutans* virulence attributes. The two most important mechanisms via which *S. mutans* attach to oral surfaces and cause disease are sucrose-dependant and sucrose-independent adhesion [77]. *S. mutans* have demonstrated the

ability to produce adhesive glucans via glucosyltransferases (GTFs) [77]. GTFs such as GtfB, GtfC, and GtfD, enable sucrose breakdown into glucose and fructose via sucrase-like activity, allowing glucan growth [77]. Glucans bind to salivary pellicle, a protein film formed over oral surfaces, and oral bacteria via hydrogen bonds. Another interesting discovery is that of the glucan binding proteins (GBP) on GTFs [77]. The role of GBPs such as GbpA, GbpC and GbpD is not well understood, however, it has been shown to be important for cell growth, attachment and biofilm formation [77-79]. As for sucrose-independent adhesion, *S. mutans* expresses surface proteins such as antigen I/II, P1, SpaP, SR, PAc, and B [77]. These proteins enable the attachment of bacteria to salivary elements via alanine and proline rich domains [77]. Apart from initiating dental caries, *S. mutans* can lead to endocarditis via Lipoprotein receptor antigen expressed by *S. mutans* [80]. Research is still ongoing to elucidate the pathogenic properties of cariogenic bacteria and a proper understanding will enable the development of appropriate caries therapeutics.

### **2.2.2 Role of oral microbiota in periodontal diseases**

Similar to dental caries, periodontal disease demonstrates a predilection for particular microbial populations. The most frequently detected periodontal pathogens are *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia*, *Capnocytophaga spp*, *Eikenella corrodens* and the red complex consisting of *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* (**Table 2.1**) [81-86]. Moreover, *Aggregatibacter actinomycetemcomitans*, formerly known as *Actinobacillus actinomycetemcomitans* and the red complex is found in gingival sulci of subjects with

periodontal disease [82,83,86,87]. These pathogens lead to a cascade of events such as increased inflammation and destruction of connective tissue.

The virulent characteristics of periodontal pathogens are complicated and studies are still ongoing to fully understand these characteristics. The attachment of these microorganisms is important for their virulent attributes. Bacterial cell surface components, such as adhesins and fimbriae help in the attachment of pathogens to oral surfaces [88,89]. Apart from bacterial adhesion, *A. actinomycetemcomitans* has demonstrated leukotoxic activity and the ability to impair neutrophil chemotaxis that interferes with the host's defense mechanism by affecting phagocytosis [89]. Moreover, *A. actinomycetemcomitans* demonstrates immunomodulatory effects by interfering with host B-cell function, T-cell function and complement activation. These effects are due to lipopolysaccharides (LPS) secretion, leading to macrophage activation and inflammatory cytokine secretion such as Interleukin (IL)-1 $\alpha$ , IL-1 $\beta$  and TNF [89]. *A. actinomycetemcomitans* further contributes to tissue destruction with collagenase-like activity [89].

The red complex microorganisms namely, *P. gingivalis*, *T. denticola* and *T. forsythia* also use adhesins and fimbriae to attach to oral surfaces and secrete LPS to cause disease [88]. They secrete proteases such as caspases responsible for apoptosis and tissue destruction [88]. Vesicle formation in the outer membrane of *P. gingivalis* is an important immunogen that destroys periodontal integrity. Moreover, proteins such as hemagglutinin, specifically, HagA, HagB, HagC, and HagE HagD and protease secretion by *P. gingivalis* play a role in attachment to host tissue receptors [88]. Subsequent to bacterial attachment to host tissue, these bacteria gain entry into cells where they multiply

[88]. An important protease secreted by *P. gingivalis* is gingipain responsible for increasing vascular permeability and interference with blood clotting fibrinogen, fibrin and the degrading factor X, thus explaining bleeding associated with periodontal diseases [88]. They have the potential to degrade immunoglobulins such as IgA, IgG and IgM, complement proteins and pro-inflammatory cytokines causing interference with the host's defense mechanisms [88]. They also demonstrate collagenase activity leading to the degradation of collagen type IV, laminin and fibronectin, contributing to impaired healing [88].

Similarly, *T. denticola* forms vesicles in outer membrane, enabling them to be immunogenic and enabling bacterial attachment to oral tissues [88]. Moreover, leucine-rich repeat protein (LrrA) and major sheath (gold surface) protein (Msp) expressed by *T. denticola* play a role in bacterial co-aggregation and biofilm formation [88]. Dentilisin, another protease secreted by *T. denticola* degrades IgA, IgG and IgM, complement proteins and pro-inflammatory cytokines [88]. In addition, *T. denticola*-mediated collagenase activity leads to periodontal destruction [88].

An important characteristic of periodontal disease is soft tissue destruction and bone resorption. The red complex secretes prostaglandin E<sub>2</sub>, a potent bone resorption factor and expresses matrix metalloproteinase that causes tissue and bone destruction [88]. Although there is considerable literature to understand the virulence of these pathogens, these mechanisms are not yet fully understood and a full understanding would prove advantageous.

### 2.2.3 Role of oral microbiota in oral candidiasis

As opposed to dental caries and periodontal disease, both caused by a group of microbes, the microbiology of oral candidiasis is straight forward, caused by elevated *C. albicans* growth (**Table 2.1**). *C. albicans* exists in two forms, the yeast and the hyphae form, termed dimorphism [90]. The virulence of *C. albicans* is largely due to its hyphae form, responsible for attachment of *C. albicans* and cell invasion [90]. Several genes are responsible for changes in *Candida* morphology. Interestingly, some of these genes have multiple roles, such as cell penetration, protease secretion and tissue breakdown [90]. For example, Hypha-specific G1 cyclin (HGC1) is responsible for *Candida* hyphae morphology [90]. Other *C. albicans* express adhesin proteins such as Als3p and Hwp1p are suggested to play important roles in the attachment of hyphae to oral tissues [90]. Interestingly, SAP4, SAP5 and SAP6 are genes responsible for both the secretion of aspartic proteases and hyphae morphology. Aspartic protease destroys host proteins and extracellular matrix causing tissue destruction [90].

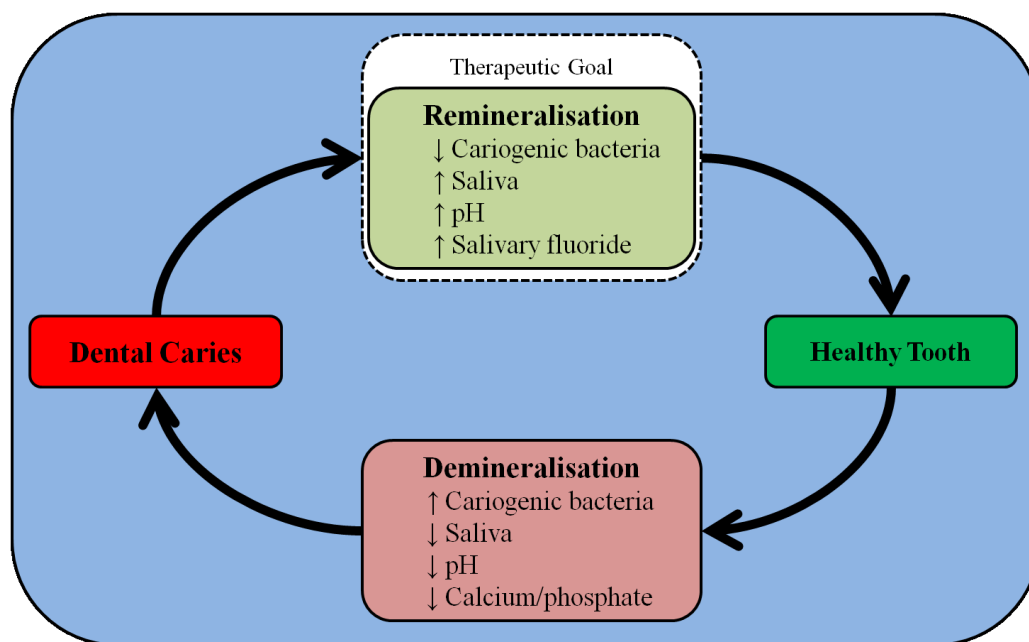
Two important characteristics of *Candida* in hyphae form are invasion of epithelial cells and endothelial cells via mechanical forces [90]. The adhesin, HWP1, is suggested to have a role in epithelial invasion permitting the internalisation of *Candida* inside host epithelial cells [90]. Tight adherence of *C. albicans* to host tissue is important for such invasion and the gene Efg1p aids in force application by hyphae [90]. As for endothelial cell invasion, *C. albicans* enter endothelial cells via endocytosis and damaged endothelial cells promoting the entry of other *C. albicans* cells [90]. This internalisation also plays a role in damaging phagocytes, thus interfering with host defense mechanisms [90]. Lastly, *C. albicans* is very efficient in adapting to diverse environmental conditions

and genes such as PHR1 and PHR2 have been suggested to play a part [91]. Understanding these characteristics can aid in developing effective therapeutics capable of bypassing the bacterial machinery contributing to disease.

## **2.3 Oral disease etiology, complications and current treatment options**

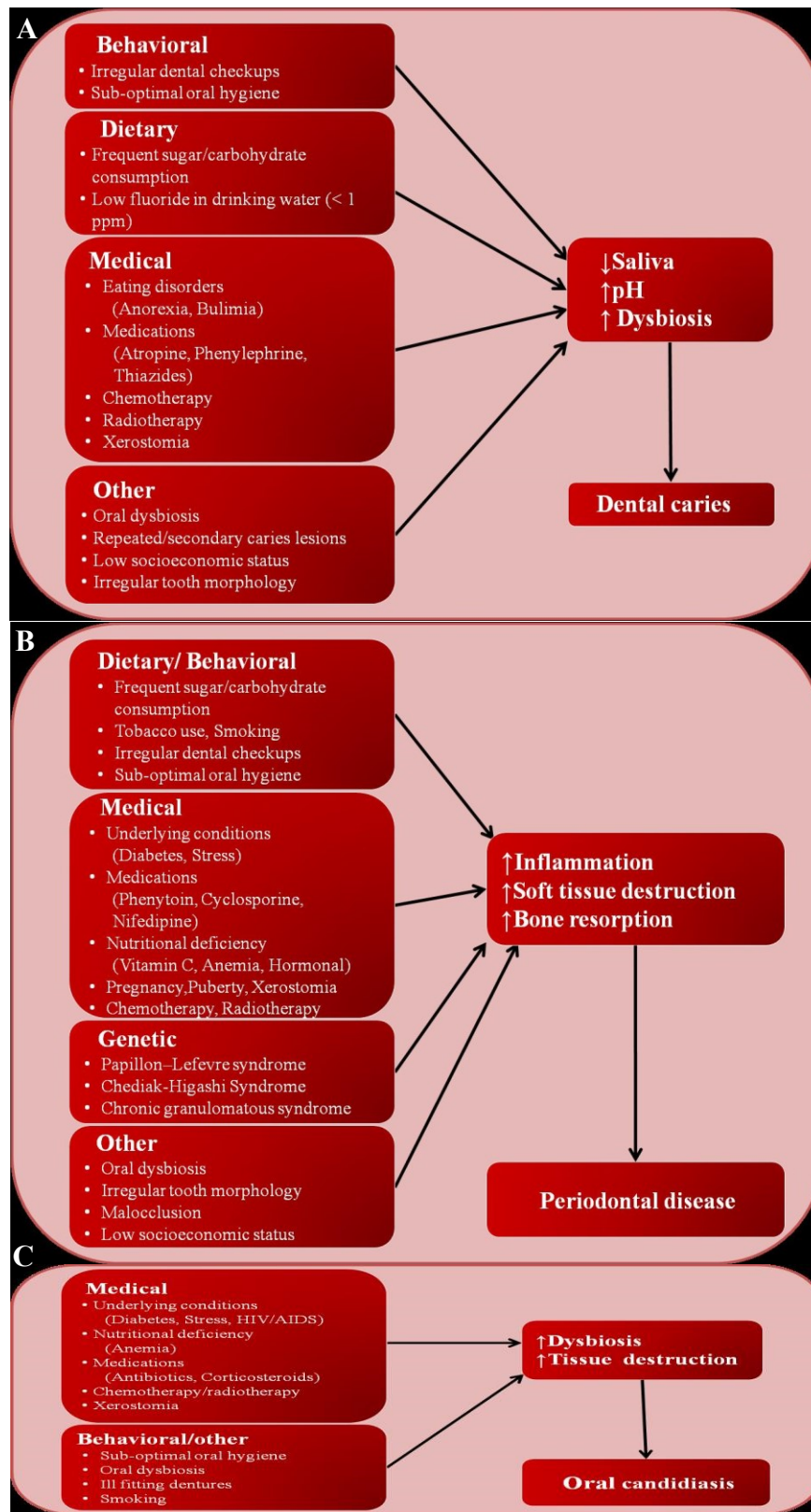
### **2.3.1 Dental caries**

Dental caries cause significant destruction to the hard structures of the tooth and is associated with dental plaque [92]. As aforementioned, certain commensal bacteria, including *Streptococci*, *Lactobacilli* and *Actinomyces*, thrive in dental plaque. Furthermore, when dietary carbohydrates are not eliminated from the oral cavity following food intake, due to poor oral hygiene practises or xerostomia (dry mouth), they are metabolized by plaque bacteria, leading to acid production [62,93,94]. This acid production results in the demineralisation of the tooth's outer layers (enamel and dentin), due to a leaching out of ions, promoting tooth decay. Saliva normally acts as a buffer, neutralising the acidic pH and providing ions such as calcium, fluoride and phosphorus which promote tooth remineralisation [94]. When demineralisation prevails over remineralisation, due to a combination of factors, tooth decay, caries incidence and caries progression are enhanced (**Figure 2.2**).



**Figure 2.2:** The balance between demineralisation and remineralisation of tooth leading to either dental caries or maintenance of healthy tooth.

It is clear from the dental caries etiology that sticky foods, drinks containing high sucrose, frequent consumption of carbohydrates, poor oral hygiene, teeth location in the oral cavity that provides bacterial stagnation, sub-optimal levels of fluoride in drinking water and dehydration are important risk factors of dental caries. In addition, eating disorders such as anorexia, bulimia and gastrointestinal diseases such as reflux and inflammatory bowel disease are important dental caries risk factors (**Figure 2.3**). Moreover, disease progression is promoted when the causative organisms reach the deeper layers of the teeth, areas below and surrounding the tooth surfaces, such as the periapical area and the gingival sulcus. These events lead to complications such as severe tooth pain, tooth loss, facial cellulitis, osteomyelitis and in some cases can even prove fatal, with the spread of infection to the systemic circulation. In addition, *Streptococcal* species have been associated with infective endocarditis with potential to be fatal [8,92].



**Figure 2.3:** The risk factors leading to (A) dental caries (B) periodontal disease and (C) oral candidiasis



The primary prevention and treatment strategies for dental caries involve proper oral hygiene practices, the addition of fluoride ions in certain countries' drinking water and the use of other fluoride substitutes/supplements such as toothpastes, varnishes, gels, tablets, lozenges and chewing gums to promote remineralisation [95-97]. The application of dental sealants is a method currently used to inhibit bacterial attachment and propagation responsible for caries progression [98,99]. A fluoride prevention regime for remineralisation of tooth may lead to fluorosis causing staining, pitting of the teeth and enamel damage, while receiving dental sealants depends highly on the socioeconomic status of the patient [32]. Following disease onset, treatment methods focus on arresting caries progression and restoring the lost tooth structure (**Table 2.2**) [100].

**Table 2.2:** Current dental caries therapeutic modalities

<b>Dental caries treatment modalities</b>	
<b>Prevention</b>	<ol style="list-style-type: none"> <li>1. Life style changes</li> <li>2. Oral hygiene <ul style="list-style-type: none"> <li>• Tooth brushing</li> <li>• Mouth washing</li> <li>• Dental flossing</li> </ul> </li> <li>3. Remineralisation <ul style="list-style-type: none"> <li>- Fluoridated water</li> <li>- Fluoride tooth paste</li> <li>- Fluoride gels</li> </ul> </li> <li>4. Reducing areas for bacterial stagnation <ul style="list-style-type: none"> <li>- Dental sealants</li> </ul> </li> </ol>
<b>Treatment</b>	<ol style="list-style-type: none"> <li>1. Restoration <ul style="list-style-type: none"> <li>- Tooth fillings <ul style="list-style-type: none"> <li>• Glass ionomer cement</li> <li>• Composites</li> <li>• Amalgam</li> </ul> </li> </ul> </li> <li>2. Endodontic procedures <ul style="list-style-type: none"> <li>• Pulpotomy</li> <li>• Pulpectomy</li> <li>• Root filling</li> </ul> </li> </ol>

Dental restorations are performed to replace the lost tooth structure and, with extensive loss, prosthetics may be necessary [5]. Moreover, extensive tooth structure removal can

expose the inner pulp cavity, probing further endodontic treatment [101]. Endodontic procedures involve the removal of pulp tissue, the disinfection of root canal and reshaping the root canal, which present additional challenges. Anatomical differences in each root canal make these procedures difficult with respect to identification of the number of canals, determining canal shapes and navigating through torturous canals [102]. Technical difficulties such as canal perforations, sub-optimal canal disinfection and allergic reactions to irrigants, over or under-obturation of the canal and variable biocompatibility of filling materials are important factors for a successful prognosis [102-104]. Moreover, disease progression to periapical areas requires surgical interventions [105]. Success of restorative procedures depends on many factors as mentioned above, rendering treatment success variable.

### **2.3.2 Periodontal diseases**

Periodontal diseases affect the supporting structures of the tooth and include both gingivitis and periodontitis. Gingivitis is characterised by inflammation of the gingiva and bleeding of the gums which, upon progression, may result in periodontitis [3,106,107]. As aforementioned, the latter involves the additional inflammation and infection of the supporting structures of the teeth, such as the periodontal ligaments and the alveolar bone. Gingivitis is reversible when efficiently treated early; however, upon progression to periodontitis the disease is irreversible. The irreversible nature of periodontitis is due to the extensive loss of the supporting structures of the teeth such as periodontal ligaments and alveolar bone, intensified further by the host's inability to restore these lost structures [108]. Periodontitis can ultimately result in complications

such as tooth loss, tooth shifting, abscesses causing facial cellulitis, osteomyelitis and trench mouth [3,109].

Similar to dental caries etiology, dental plaque contributes to the pathogenesis of periodontal disease [63]. Indexes such as Plaque Index (PI), Papillary Bleeding Index (PBI) and Gingival Index (GI) are approaches used to evaluate the capability of a therapeutic to arrest disease progression. PI, introduced matrix consists of bacteria and bacterial exopolysaccharides, high-molecular weight polymers made up of sugar residues [110]. Environmental changes around the gingiva during periodontal disease include an increased amount of gingival crevicular fluid (GCF), an inflammatory exudate, and a rise in pH, further encouraging the growth of periodontopathogens [63,111]. The progression of gingivitis and periodontitis is linked to a hyperactive host immune response, demonstrated by increased levels of inflammatory markers in diseased individuals [112]. There are methods used to qualify/quantify the capability of probiotic bacteria to inhibit the formation of dental plaque, to regulate the production of host inflammatory markers and to reduce the prevalence of periodontal pathogens [113-116]. Indexes such as Plaque Index (PI), Papillary Bleeding Index (PBI) and Gingival Index (GI) are approaches used by Silness and Loe in 1964, to evaluate the state of oral hygiene by recording the amount of soft and mineralised deposits on tooth surfaces (levels of plaque). PBI is a scoring system for scoring the inflammation of the interdental papillae by evaluating bleeding on probing. GI is an index that evaluates the gingival status by specifically investigating swelling and redness. Using these established methods, researchers and clinicians can assess overall oral health [117].

The activated host immune responses, to combat periodontal disease are directly correlated with periodontal disease progression and severity [19]. Receptor activator of nuclear factor  $\kappa$ B ligand (RANKL), a member of the tumor necrosis factor (TNF) cytokine family, is an important player in the activation of the immune-mediated disease progression. RANKL is an osteoclast differentiation factor expressed on osteoblasts, T cells and B cells, important in bone resorption [118]. Unlike RANKL, Osteoprotegerin (OPG), from the same TNF cytokine family, blocks RANK binding to RANKL, limiting bone resorption. RANKL/OPG determines the fate of bone metabolism [119]. In short, factors such as an altered subgingival microbiota, consisting of increased amounts of periodontopathogens, and an amplified inflammation along with a hyperactive host immune system all contribute, directly or indirectly, to the destruction of the supporting structures of the tooth [63]. The risk factors for periodontal diseases are hence factors that can inflict an inflammatory reaction in the host. Smoking, stress, certain medications such as antidepressants, pregnancy, diseases that interfere with patient's healing process such as diabetes are just a few factors (**Figure 2.3**) [120-123].

The primary therapeutic strategies of periodontal disease lie in their prevention, entailing good oral hygiene practises such as tooth brushing, flossing and the use of antibacterial rinses (**Table 2.3**).

**Table 2.3:** Current treatment modalities for periodontal diseases

Periodontal disease treatment modalities	
<b>Prevention</b>	<ol style="list-style-type: none"> <li>1. Life style changes</li> <li>2. Oral hygiene <ul style="list-style-type: none"> <li>• Tooth brushing</li> <li>• Mouth washing</li> <li>• Dental flossing</li> </ul> </li> <li>3. Treating underlying health conditions <ul style="list-style-type: none"> <li>• Diabetes</li> <li>• Vitamin deficiency</li> <li>• Anemia</li> </ul> </li> </ol>
<b>Treatment</b>	<ol style="list-style-type: none"> <li>1. Drugs used <ol style="list-style-type: none"> <li>1. Tetracycline</li> <li>2. Amoxicillin</li> <li>3. Metronidazole</li> </ol> </li> <li>2. Non-surgical periodontal therapy <ul style="list-style-type: none"> <li>• Dental scaling</li> <li>• Tooth root planning</li> </ul> </li> <li>3. Surgical periodontal <ul style="list-style-type: none"> <li>• Periodontal pocket reduction</li> <li>• Regenerative procedures</li> </ul> </li> </ol>

Following disease onset, treatment practises include scaling (removal of plaque and tartar) and root planing (smoothing of rough surfaces on the root) to impede any additional bacterial adhesion and growth [3,106,107]. Ultimately, surgical procedures are required to eliminate pockets, to support loosened teeth and to extract teeth to stop the disease from spreading to the adjacent areas [3]. Antiseptics such as chlorhexidine, povidone-iodine, stannous fluoride and antibiotics, such as minocycline, doxycycline, metronidazole and tetracycline have also been used as therapies [124,125]. However, there are several adverse reactions of antibiotic therapy including hypersensitivity, GIT disorders and antibiotic resistance [126,127]. The susceptibility to periodontal diseases can be further influenced by smoking and other underlying conditions such as diabetes, viral infections and stress [128-131]. Diabetes (Type 1 and Type 2) influences the immune mechanisms of the body, increases the incidence of inflammation and tissue breakdown and impairs tissue regenerative capacity [130]. Periodontal diseases have

been associated with atherosclerosis and preterm babies, substantiating their importance [9,10]. With shortcomings in terms of the development of a therapeutic, an alternative strategy is required.

### **2.3.3 Oral candidiasis**

*C. albicans* are oral commensals that cause oral candidiasis with elevated proliferation. Their increased proliferation is attributed to dry mouth, in elderly patients, to patients with ill-fitting dentures, to patients receiving broad spectrum antibiotics and to patients with a weakened immune system such as those undergoing cancer therapies, using corticosteroids, diabetics and patients with human immunodeficiency virus (HIV). Similar to dental caries and periodontal disease, *C. albicans* also finds shelter in oral biofilm and facilitates disease progression [17]. Disease progression, particularly in HIV-infected patients can prove to be fatal [13]. Manifestation of oral candidiasis may vary from painless white patches, that can be wiped easily, to red, painful and bleeding ulcers on the oral mucosa, throughout the mouth [17]. Poor oral hygiene, diabetes mellitus, immune deficiency, cancer treatments, corticosteroid use, broad spectrum antibiotics and denture use are a few of the risk factors of oral candidiasis (**Figure 2.3**) [17].

The maintenance of good oral hygiene is pivotal to the prevention of oral candidiasis in susceptible patients. For example, patients on corticosteroid inhalation therapy are advised to rinse their mouth after every inhalation, to limit the growth of *Candida*. With increases in *C. albicans* counts in the mouth, the first line of treatment is to rinse the mouth with a saline solution. In addition, a physical removal of white patches using swabs or tongue scrapers is encouraged. With further disease progression, antifungals are prescribed. Polyenes such as amphotericin B and nystatin are common

antifungals used for candidiasis treatment [17]. Candidiasis treatment via antifungal therapy is also challenged by acquired resistance, which is constantly on the rise [132]. Moreover, it is important to note that *C. albicans* is usually susceptible to most anti-fungals, but when established as a biofilm, mechanical disruption of a biofilm prior to anti-fungal application is required. **Table 2.4** entails the current modalities used to treat oral candidiasis.

**Table 2.4:** Current treatment modalities for oral candidiasis

Oral candidiasis treatment modalities	
<b>Prevention</b>	<ol style="list-style-type: none"> <li>1. Oral hygiene <ul style="list-style-type: none"> <li>• Tooth brushing</li> <li>• Mouth washing</li> <li>• Dental flossing</li> </ul> </li> <li>2. Physical removal of dental plaque <ul style="list-style-type: none"> <li>- Tooth brushing</li> <li>• Tongue scrapping</li> </ul> </li> </ol>
<b>Treatment</b>	<ol style="list-style-type: none"> <li>1. Correcting underlying conditions <ul style="list-style-type: none"> <li>• Denture readjustment</li> <li>• Control diabetes</li> <li>• Mouth wash after corticosteroid inhalation</li> </ul> </li> <li>2. Drugs used <ul style="list-style-type: none"> <li>• Nystatin</li> <li>• Clotrimazole</li> <li>• Fluconazole</li> <li>• Itraconazole</li> </ul> </li> </ol>

## 2.4. Oral microbiota modulation and introduction to probiotic bacteria

The modulation of the oral microbiota in oral disease is an important aspect for the development of a successful oral health bio-therapeutic. As aforementioned, oral disease is initiated and maintained by an overgrowth of opportunistic pathogens. Hence, the use of antibiotics and antimicrobials are obvious choices to reduce the counts of these pathogens. Penicillin, tetracycline and metronidazole are commonly used antibiotics for limiting periodontal diseases. The oral cavity is challenged by bacterial invasion throughout the patient's life and hence, oral diseases such as dental caries and periodontal

diseases are chronic diseases. Therefore, lifelong administration of antibiotics would be required to treat oral diseases, which can lead to resistance, predisposing patients to other opportunistic infections [126,133]. Three main mechanisms by which bacteria become resistant to these drugs are the inability of the drug to reach the target, target alteration and inactivation of the drug itself, discussed in detail by Soares *et al.* [134]. Indeed, a sharp rise in nosocomial *Clostridium* and Methicillin-resistant *Staphylococcus aureus* infections have been reported [135,136]. In addition, antibiotic-associated GIT disorders and hypersensitivity reactions, limit their use in oral diseases [126,133].

The modulation of oral microbiota via probiotic bacteria is a safe and long-term therapeutic approach for such chronic conditions. Probiotic bacteria are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host”, as defined by the FAO and WHO [137]. Probiotic microorganisms include bacteria but also certain fungi. *Lactobacillus* and *Bifidobacterium* are the most commonly investigated probiotic bacteria, as they are naturally occurring organisms found in foods such as milk and yoghurt. Probiotics have yet to present serious side effects associated with currently available antimicrobials [138]. A number of probiotic strains have been investigated for their overall safety, including their potential for transmissible antibiotic resistance [138,139]. They have shown promising potential to prevent and treat several diseases, such as allergies, inflammatory bowel disease, irritable bowel syndrome, colorectal cancer, metabolic syndrome and oral disorders [140-147]. Although probiotic research in the field of dental sciences is relatively new, preliminary studies suggest their beneficial influence on maintaining oral health. Several probiotic bacteria have



demonstrated an ability to inhibit oral pathogens. However, the efficacy of probiotic therapeutics has proven limited, requiring further investigations.

The exact modes of action responsible for the observed effects are not well-understood. Production of antibacterial substances such as bacteriocins, H<sub>2</sub>O<sub>2</sub> and nisin, anti-inflammatory substances such as ferulic acid and nitric oxide are just a few molecules that are believed to be responsible for the effects observed. Interestingly, there has been considerable research into probiotic co-administration with antibiotics to modulate the intestinal microbiota for promoting health [148,149]. The co-administration of probiotic bacteria demonstrated improvement in patients with *C. difficile* nosocomial infections and *Helicobacter pylori* infections. Nevertheless, there is limited research for such combination therapy to combat oral diseases. Hence, it would be worthy to investigate the effect of such combination therapy for oral diseases such as periodontitis.

As a side-note, a prebiotics is a “non-viable food component that confers a health benefit on the host, associated with a modulation of the microbiota”, defined by the Food and Agriculture Organization of the United Nations (FAO) and WHO [137]. Some examples of prebiotics are inulin, fructooligosaccharides, lactulose and trans-galactooligosaccharides, found in many foods such as asparagus, garlic, leek, onion, cereals, breads, yoghurt and drinks. Although there is no literature on their effects on oral microbiota modulation, there is considerable research that investigates the use of prebiotics to modulate intestinal microbiota, with promising outcomes for the prevention and treatment of GIT disorders [150]. The increase in beneficial gut bacterial counts and activity, such as probiotic bacteria is an interesting attribute of prebiotics. However, their mode of action on microbial populations remains non-specific and requires further

investigations. A symbiotic, a combinatory formulation that includes both a prebiotic and a probiotic, could act in synergy to improve health and has demonstrated potential as a cancer therapy, reducing infection risks and influencing bone metabolism [151-153]. Indeed, colonization of probiotic bacteria could also be aided with prebiotic therapy by providing a substrate for probiotic attachment to the oral tissues.

## **2.5 Introduction to probiotic bio-therapeutics for oral diseases**

Keeping in mind the pathogenesis of oral diseases, a probiotic bio-therapeutic for the prevention and treatment of oral diseases should fulfill certain requirements. A successful therapeutic should target the etiologic factors discussed previously. For a dental caries therapeutic, the focus should lie in the reduction of the causative organisms such as *S. mutans* and the reduction of dental plaque. For gingivitis and periodontitis, the mechanism of action of the therapeutic should focus on the diminution of dental plaque, the reduction of the number of periodontal pathogens such as the red complex and the modulation of the host immune response by controlling the release of inflammatory cytokines which may slow the destruction of supporting tissues [154]. As for oral candidiasis, the main focus should lie in the reduction of *Candida* counts in the mouth as the primary etiology is dysbiosis. A number of human clinical trials have reiterated the potential of probiotics as biotherapeutics for the prevention and treatment of oral diseases (**Table 2.5**). The main probiotic therapeutics goals would be pathogen inhibition, reduction in biofilm formation and reduction in inflammation (**Table 2.6**).

**Table 2.5:** List of probiotic bacteria used in clinical trials for the prevention and treatment of oral diseases

Probiotic strain	Oral disease	Reference(s)
<i>L. reuteri</i> ATCC 55730	Dental caries Periodontal disease	[155,156]
<i>L. reuteri</i> ATCC 5289	Periodontal disease	[156-158]
<i>L. reuteri</i> Lru-1038	Gingivitis	[159]
<i>L. reuteri</i> DSM 17938	Dental caries Periodontal disease	[158]
<i>L. reuteri</i> DSM 17938	Periodontal disease	[157]
<i>L. rhamnosus</i> GG	Dental caries	[160]
<i>L. casei</i> strain Shirota	Gingivitis	[115]
<i>L. acidophilus</i> La-14	Gingivitis	[159]
<i>L. brevis</i> CD2	Periodontal disease	[161]
<i>L. paracasei</i> Lpc-37	Gingivitis	[159]
<i>L. plantarum</i> Lp2001	Gingivitis	[159]
<i>L. salivarius</i> WB21	Periodontal disease	[162]
<i>L. salivarius</i> Ls-33	Gingivitis	[159]
<i>Streptococcus salivarius</i> K12	Gingivitis	[159]
<i>Bifidobacterium animalis</i> ssp. lactis DN- 1173010	Dental caries	[163]
<i>Bifidobacterium lactis</i> Bb-12	Dental caries	[164]
<i>B. lactis</i> BB12	Dental caries	[165]

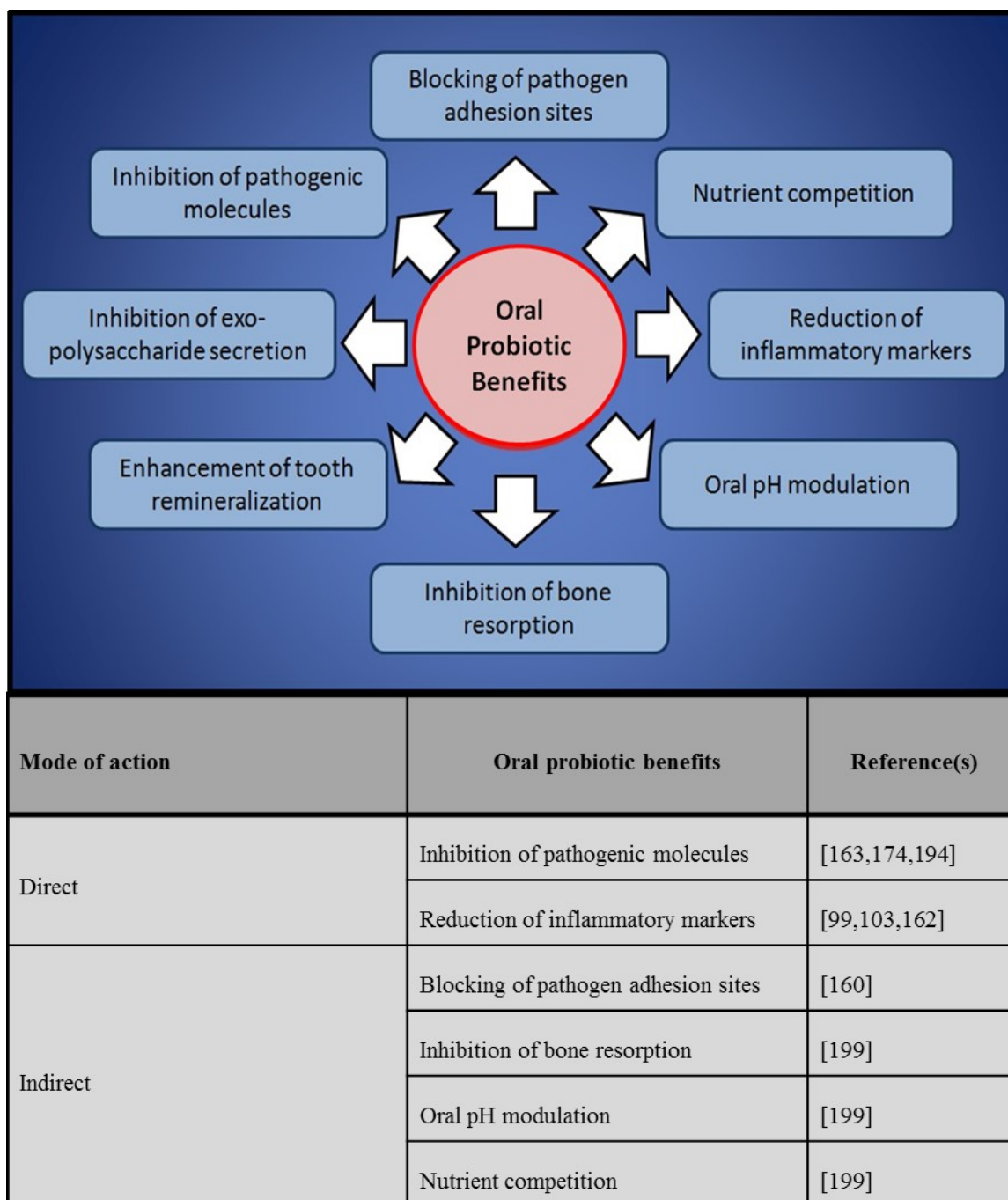
**Table 2.6:** Probiotics and oral disease prevention/therapeutics with respect to mode of action: 1) Modulation of inflammatory responses 2) Inhibition of biofilm formation and 3) Inhibition of disease-causing organisms.

Probiotic	Modes of Action	Experimental design	Ref(s)
<i>L. casei</i> strain Shirota	Modulation of inflammatory responses	<i>In vivo</i> (human)	[115]
<i>L. reuteri</i> ATCC 55730	Pathogen inhibition	<i>In vitro</i>	[166]
		<i>In vivo</i> (human)	[155]
		<i>In vitro</i>	[22]
	Modulation of inflammatory responses	<i>In vivo</i> (human)	[156]
<i>L. reuteri</i> ATCC 5289	Inhibition of biofilm formation	<i>In vitro</i>	[22]
	Modulation of inflammatory responses	<i>In vivo</i> (human)	[156]
		<i>In vivo</i> (human)	[157]
	Pathogen inhibition	<i>In vitro</i>	[166]
		<i>In vitro</i>	[22]
	Inhibition of biofilm formation	<i>In vitro</i>	[22]
		<i>In vivo</i> (human)	[157]
<i>L. lactis</i> byproduct Nisin	Inhibition of biofilm formation	<i>In vivo</i> (human)	[113]
		<i>In vivo</i> (Dog)	[167]
	Inhibition of disease-causing organisms	<i>In vitro</i>	[168]
<i>L. rhamnosus</i> GG	Inhibition of biofilm formation	<i>In vitro</i>	[22]
	Inhibition of disease-causing organisms	<i>In vitro</i>	[22]
		<i>In vivo</i> (human)	[160]
<i>L. plantarum</i> DSM 9843	Inhibition of biofilm formation	<i>In vitro</i>	[22]
	Pathogen inhibition	<i>In vitro</i>	[22]
<i>L. paracasei</i> DSMZ 16671	Inhibition of biofilm formation	<i>In vivo</i> (rats)	[169]
<i>Bifidobacterium lactis</i> Bb-12	Pathogen inhibition	<i>In vivo</i> (human)	[170]
<i>L. reuteri</i> DSM 17938	Inhibition of biofilm formation	<i>In vivo</i> (human)	[157]
<i>W. salivarius</i> WB21	Pathogen inhibition	<i>In vivo</i> (human)	[162]
<i>L. acidophilus</i> La5	Pathogen inhibition	<i>In vitro</i>	[166]
<i>L. plantarum</i> 299v	Pathogen inhibition	<i>In vitro</i>	[166]
<i>L. rhamnosus</i> LB21	Pathogen inhibition	<i>In vitro</i>	[166]
<i>L. paracasei</i> F19	Pathogen inhibition	<i>In vitro</i>	[166]
<i>L. rhamnosus</i> LC 705	Pathogen inhibition	<i>In vivo</i> (human)	[160]
<i>Bifidobacterium</i> DN-173010	Pathogen inhibition	<i>In vivo</i> (human)	[171]
<i>S. salivarius</i> K12	Pathogen inhibition	<i>In vivo</i> (human)	[157]

Research has initiated investigations into probiotic bacteria with the potential to inhibit oral pathogens. For example, Mayanagi *et al.* demonstrated a significant inhibition

of the pathogens: *A. actinomycetemcomitans*, *P. intermedia*, *P. gingivalis*, *T. denticola* and *T. forsythia* with *Lactobacillus salivarius* WB21 treatment [172]. Moreover, research has shown that probiotic bacteria are capable of producing antibacterial substances such as bacteriocins. Nisin is one such bacteriocin, produced by lactic acid bacteria and extracted from the probiotic *Lactococcus lactis*. It is a lantibiotic, a class of bacteriocin obtained from gram-positive bacteria and considered safe by the American Food and Drug Administration (FDA) [173]. In 2007, Noordin *et al.* demonstrated significant decreases in the PI and the GI in patients, when treated with mouth rinse containing nisin, indicating its role in both the reduction of plaque accumulation and gingivitis [113]. Elevated levels of inflammatory cytokines including polymorphonuclear (PMN) elastase and matrix metalloproteinase-3 (MMP-3) are present in the GCF in periodontal disease, in order to restrict disease progression [111].

Probiotic bacteria have not only been shown to compete with pathogenic species but have also shown remarkable anti-inflammatory properties, useful for limiting elevated levels of inflammatory cytokines [174]. **Figure 2.4** provides a schematic representation of suggested modes of action that could be responsible for observed probiotic effects.



**Figure 2.4:** (A) Potential oral probiotic targets and mechanisms to enhance oral health. (B) The direct and indirect modes of action to be addressed by a probiotic therapeutic

One such mode of action is competition for nutrients such as sucrose, a preferred nutrient source for the dental caries-causing pathogen *S. mutans*. Keeping this in mind, probiotic

bacteria could potentially compete for sucrose, limiting its availability for *S. mutans*. The ability of probiotic bacteria to aggregate with pathogens, termed co-aggregation, is another interesting phenomenon. Probiotic bacteria in *in vivo* conditions could reduce the load of oral pathogens in the mouth via co-aggregation. Moreover, low concentrations of anti-bacterial substances such as  $H_2O_2$  and NO have demonstrated anti-inflammatory properties [175]. Additionally, low levels of  $H_2O_2$  have also demonstrated potential to enhance axon regeneration and prevent cytotoxicity. Similar regenerative effects could be observed with osteoblast cells and would be beneficial for bone regeneration for periodontitis therapy [176,177]. Probiotic bacteria such as *L. acidophilus* have been shown to be  $H_2O_2$  producers [139], suggesting that probiotic bacteria should be screened for the production of  $H_2O_2$  and NO, which can be both antibacterial and anti-inflammatory.

Several probiotic strains have demonstrated the ability to produce antioxidant compounds such as ferulic acid (FA) [178]. Antioxidants have the ability to scavenge free radicals such as reactive oxygen species, found in increased levels in inflammation. Tomaro-Duchesneau *et al.* demonstrated the probiotic production of FA [178]. FA is a phenolic acid derivative and is a potent antioxidant. The effect of probiotic bacteria that produce such molecules should be studied for the reduction of oral inflammation associated with oral diseases. Lastly, oral pathogens thrive in oral biofilms on oral surfaces such as oral mucosa and teeth. Apart from antibacterial and anti-inflammatory effects, probiotic bacteria could also out-compete the attachment of pathogens to oral tissues.

### 2.5.1 Current status of probiotic biotherapeutics for dental caries

As aforementioned, dental caries is a chronic disease initiated by the accumulation of dental plaque on the oral surfaces [99]. Recent studies investigated the use of probiotics for the prevention of dental caries. The goals for the successful prevention of dental caries by a therapeutic are to inhibit the proliferation of pathogens such as *S. mutans*, to inhibit its adherence to the oral surfaces and to inhibit oral biofilm formation. The first study investigating probiotics for the inhibition of oral streptococci was performed by Meurman *et al.*, in 1995 [179]. They performed an *in vitro* study and demonstrated the ability of LGG to inhibit oral *Streptococcus sobrinus* [179]. In a more recent *in vitro* study, the inhibition of *S. mutans* proliferation and plaque formation by the probiotic strains: LGG, *L. plantarum* DSM 9843, *L. reuteri* ATCC PTA 5289 and *L. reuteri* ATCC 55730 was investigated [180]. In terms of biofilm formation, all of the above probiotic strains demonstrated some inhibition, however, *L. reuteri* ATCC PTA 5289 and LGG demonstrated the highest inhibitory activities, as measured by spectrophotometry and plate counts [180]. Probiotic strains also proved effective at inhibiting the growth of *S. mutans* in a pre-formed biofilm, with LGG and *L. plantarum* DSM 9843 demonstrating complete inhibition of *S. mutans* proliferation [180]. These results suggest that probiotic strains capable of inhibiting *S. mutans* should be investigated further, as potential dental caries therapeutics.

A number of probiotic strains were investigated, for their *in vitro* inhibition of *S. mutans* [181]. The following Lactobacilli were investigated: *L. plantarum* 299v, *L. plantarum* 931, LGG ATCC 53103, *L. rhamnosus* LB21, *L. paracasei* F19, *L. reuteri* PTA 5289, *L. reuteri* ATCC 55730 and *L. acidophilus* La5 [181]. Five streptococci



strains: *S. mutans* NCTC 10449, *S. mutans* Ingbritt, *S. sobrinus* OMZ 176 and clinical isolates *S. mutans* P1:27 and *S. mutans* P2:29 [181] were investigated. Using an agar overlay interference test, all of the probiotic strains were shown to inhibit *S. mutans*, although the capacity for inhibition varied between each probiotic strain [181]. *L. plantarum* 299v and *L. plantarum* 931 were the most potent inhibitors, demonstrating complete inhibition of *S. mutans* at the lowest concentration of probiotic [181]. However, the inhibition for each of the *S. mutans* strains was variable [181]. Taking these results into consideration, this study suggests that selected probiotic strains have the ability to prevent dental caries by inhibiting the growth of *S. mutans*, but the extent of inhibition remains strain specific for the probiotic and pathogenic strain.

In an *in vivo* study, *L. paracasei* DSMZ16671 was investigated for its potential to inhibit the development of dental caries [169]. Wistar rats were administered heat-killed *L. paracasei*, at a concentration of  $5 \times 10^8$  cfu/g incorporated in a high-sucrose caries-inducing diet inoculated with *S. mutans* [169]. The administration of the probiotics resulted in the inhibition of colonization by *S. mutans* 10449S [169]. In another human trial, there was a significant decrease in *S. mutans* counts following the consumption of probiotic ice cream containing *Bifidobacterium lactis* Bb-12 at a concentration of  $10^7$  cfu/g of ice cream [170]. Caglar *et al.* demonstrated that the consumption of a probiotic chewing gum containing *L. reuteri* ATCC 55730 and *L. reuteri* ATCC PTA 5289, at a concentration of  $10^8$  cells per gum, reduced the levels of *S. mutans* in saliva when compared to placebo [182]. In addition, Turner *et al.* investigated the effects of nisin on *E. faecalis* and *S. gordonii* in the root canal system, relevant to the prevention of root caries [183]. Nisin, at a concentration of 100 mg/ml, eliminated root caries-associated

*Actinomyces* species, *Enterococcus faecalis* and *Streptococcus gordonii*, in pure culture [168]. In another clinical trial, Caglar *et al.* investigated the inhibitory potential of the probiotic strain *L. reuteri* ATCC 55730 on *S. mutans* proliferation [184]. Probiotic administration was achieved using straws and tablets containing  $10^8$  probiotic cells, administered once daily for 3 weeks. *S. mutans* and *L. reuteri* were quantified using Dentocult® SM and Dentocult® LB chair-side kits and cfu counts. This group demonstrated a reduction of *S. mutans* following probiotic consumption, highlighting the potential of *L. reuteri* ATCC 55730 for dental caries prevention [184]. Another clinical trial demonstrated that LGG, incorporated in milk, reduced dental caries in a population of 3-4 year old children [185]. The treatment group received the probiotic formulation at  $5-10 \times 10^5$  cfu/ml for 7 months (5 days a week). The results demonstrated that the LGG treatment group had a lower incidence of dental caries associated with reduced *S. mutans* counts when compared to the control group [185]. This further confirmed LGG's ability to prevent childhood caries, proposing additional potential as a dental caries therapeutic [185].

### **2.5.2 Current status of probiotic biotherapeutics for periodontal diseases**

Probiotic formulations have shown great potential as periodontal disease therapeutics. Grudianov *et al.* were one of the first who reported the beneficial effects of probiotics on periodontal disease [186]. They demonstrated that probiotic bacteria, as a biofilm, reduced the counts of pathogenic bacteria in humans [186]. A synopsis of other relevant research on probiotic bacteria follows. Slawik *et al.* conducted a clinical study to evaluate the effects of probiotics on gingivitis [187]. Probiotic milk containing *L. casei* strain Shirota demonstrated a significant reduction of bleeding on probing (BOP) and GCF

volume in the test group compared to the control group and when compared to baseline, at day 14 [187]. In related research, Adam *et al.* performed an *in vitro* study demonstrating that *Streptococcus salivarius* K12 and M18 can reduce inflammatory cytokines associated with periodontal disease [188]. Similarly, Staab *et al.* conducted a study investigating the effects of *L. casei* strain Shirota on gingivitis by evaluating its ability to modulate the host immune responses [115]. Significant decreases in GCF PMN elastase and MMP-3 levels in the test group patients were noted [115]. These results suggest that *L. casei* strain Shirota has the capacity to modulate the plaque-induced inflammatory immune responses by reducing cytokine production.

In another study, Twetman *et al.* investigated the effects of probiotics on gingival inflammation [116]. *L. reuteri* ATCC 55730 and *L. reuteri* ATCC PTA 5289, incorporated in chewing gums, significantly decreased BOP and GCF volumes in the patients when compared to those consuming placebo, following a treatment period of 4 weeks. In addition, a significant decrease was observed in the levels of TNF- $\alpha$  and IL-9 [116]. This study suggests that a cocktail formulation of probiotics is able to modulate immune responses linked to periodontal disease, by reducing BOP, GCF volume and inflammatory cytokines.

With respect to probiotic reduction of dental plaque and inhibition of periodontal pathogens, Vivekananda *et al.* demonstrated the effect of *L. reuteri* (Prodentis) lozenges on plaque accumulation and inflammation in a clinical trial [157]. The lozenges contained *L. reuteri* DSM 17938 and *L. reuteri* ATCC PTA 5289. A substantial decrease was observed in PI, GI and gingival bleeding in patients treated with the Prodentis lozenges compared to those administered placebo. In addition, the group also demonstrated that the

probiotic formulation inhibited the proliferation of the periodontal pathogens: *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* [157]. This study demonstrates the ability of Prodentis to reduce dental plaque and gingival inflammation, suggesting its role as a potential oral therapeutic [157]. In addition, the bacteriocin, nisin was investigated in beagle dogs, where it reduced plaque and gingivitis when applied in the premolar area twice daily for 88 days [167]. With this data, nisin may also prove beneficial as a therapeutic formulation against gingivitis and periodontitis.

Similarly, Tsubura *et al.* demonstrated the effect of *Bacillus subtilis* E-300 on periodontal pathogens [189]. The red complex consisting *P. gingivalis*, *T. denticola* and *T. forsythia* are associated with periodontal disease [190]. These periodontal pathogens have the ability to hydrolyze trypsin substrates such as N-benzoyl-DL-arginine-2-naphthylamide to form a colour reaction, used for quantification in the BANA test [190]. Following 30 days of probiotic treatment, a significant decrease in the counts of the red complex organisms was noted in the treated patients, using the BANA test [189]. This research further suggests the role of probiotics, like *Bacillus subtilis* E-300, to inhibit periodontal disease-associated pathogens.

### **2.5.3 Current status of probiotic biotherapeutics for oral candidiasis**

As aforementioned, the etiology of oral candidiasis is associated with an imbalance in the oral ecosystem which amplifies the growth of *C. albicans*, an otherwise harmless oral commensal [191]. Moreover, inflammation is an important aspect that accompanies disease progression. To recapitulate, the three main aspects of *Candida* infection are their attachment to host tissue, evasion of the host immune system and destruction of host tissue mediated by the secretion of proteolytic enzymes [191].

It has been demonstrated that probiotic bacteria can enhance host mucosal immunity mediated by stimulating the secretion of mucosal antibody (IgA) and cytokines such as IL-2, IL-4 and IL-10, as a response to gut viral and bacterial infections [192,193]. Interestingly Elahi *et al.* observed that infection-resistant BALB/c mice had an increase in NO and a decrease in IL-4 production with onset of disease [194]. In addition, when these mice were treated with a NO inhibitor, they observed a reduction in NO production with inverse co-relation of *Candida* growth. An increase in cytokine IL-4 levels was also observed in these mice. Moreover, a reduction in IL-4 levels was accompanied by increased levels of IFN- $\gamma$  in infection-prone DBA/2 mice [194]. In a similar study they observed that following 2 weeks of probiotic *L. acidophilus* administration, the treated DBA/2 mice had increased clearance of *C. albicans* with increased levels of NO and IFN- $\gamma$  as compared to controls [195]. In addition, a significant increase in IL-4 levels was observed in mice administered probiotic treatment [195]. They concluded that NO was a host defense mechanism to counteract *Candida* infection and probiotic treatment enhanced the host defense mechanism by increasing the levels of NO, IL-4 and IFN- $\gamma$  [195].

Positive results were also observed by Wagner *et al.* when immunodeficient mice were administered the probiotic *Bifidobacterium animalis*. They observed increased survival, reduction in systemic dissemination of *C. albicans*, reduction in *C. albicans* counts in the alimentary canal and stimulation of antibody and cell-mediated immunity [196]. Similar observations were also made when immunodeficient gnotobiotic mice were fed heat-killed probiotic *L. acidophilus* and *L. casei* for two weeks. Although longevity of the mice could not be prolonged with onset of *Candida* infection, the

probiotic treated mice demonstrated a reduction in the systemic dissemination of *C. albicans* and reduction in *C. albicans* counts in the alimentary canal [21].

An increase in the incidence of oral candidiasis is observed in older populations. Hataka *et al.* demonstrated that a 16 week treatment period with probiotic *Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705 and *Propionibacterium freudenreichii* led to a reduction in salivary *Candida* counts and the prevalence of hyposalivation, when compared to the control group [197]. Similarly, other *in vitro* studies demonstrated that probiotic bacteria can inhibit the growth of *C. albicans* [24,166]. Although the exact mechanisms responsible for these observed effects are not fully elucidated, probiotic bacteria present promising potential for the prevention and treatment of oral candidiasis.

## **2.6 Shortcomings of current oral health probiotic biotherapeutics**

Probiotics, unlike antibiotics can be used to treat and prevent oral diseases as a natural and long-term approach. Results from current research appear optimistic that probiotic bacteria can be successful oral health bio-therapeutics. However, the current research has several limitations and shortcomings that need to be addressed. One important aspect is the large variety of probiotic bacteria that can prove beneficial to prevent and treat oral diseases. Current studies have demonstrated the potential of probiotic bacteria, but primarily with a focus on *L. rhamnosus* ATCC 53103 (GG) and a few *L. reuteri* strains. However, more probiotic bacteria should be screened for the inhibition of oral disease causing organisms. Meticulous effort is needed to screen a variety of probiotic strains for limiting oral pathogens. However, these studies are time consuming and costly. Initial screening of these bacteria should be followed by

mechanistic studies that explore the reasoning for the observed effects of those probiotic bacteria.

In addition, several clinical trials suggest that probiotic bacteria can reduce the numbers of oral pathogens, reduce clinical markers of periodontal disease such as reduction in GCF levels, GI, PI and PBI. However, basic research investigating the underlying events responsible for health benefits is lacking. Well-planned studies should focus on the different aspects of oral disease and investigate each factor with probiotic treatment. For example, nutrient competition by probiotic bacteria, probiotic bacteriocin production for the inhibition of oral pathogens, co-aggregation of probiotic bacteria with oral pathogens and probiotic attachment to oral tissue should be investigated in details.

Moreover, probiotic bacteria have been shown to have the potential to secrete antibacterial substances. Further screening should be based on the probiotic release of such substances. Hydrogen peroxide and nitric oxide are examples of such antibacterial compounds secreted by probiotic bacteria. The inhibition of oral pathogens via such probiotic produced antibacterial compounds should be investigated. To confirm the inhibitory activity of probiotic bacteria due to the release of antibacterial compounds, the release of probiotic antibacterial substances should be blocked or inactivated. Many studies have not performed this additional and conclusive investigation step. Similar studies can also be performed to elucidate the potential of probiotic bacteria to release antioxidant substances and their potential to alleviate oral diseases. This type of investigation in the field probiotic bacteria for oral disease bio-therapeutics is limited. More important is the lack of established methods for such mechanistic investigations.

The formation of oral biofilm is a mechanism by which oral pathogens establish themselves in the oral cavity. The inhibition of the screened probiotic bacteria to be able to limit biofilm formation and not only the planktonic cultures is essential. Moreover, most studies attempting to elucidate probiotic inhibition of oral biofilm; have done so in mixed cultures. For a clear understanding of probiotic specific inhibition, pathogenic biofilms should be kept separate from the probiotic treatment. Again, a lack of established protocols limits such studies. Recent *in vitro* studies have used transwell systems to separate the two bacteria [198]. Such studies are needed to unravel an important mechanism of probiotic mediated oral biofilm inhibition and can explain the antimicrobial effects of probiotic bacteria further. As previously discussed, inflammation is an important aspect of periodontal disease, contributing to soft tissue and alveolar bone destruction. Current research has demonstrated that clinical markers of periodontitis can be improved with probiotic treatment [115,116,187]. However, there are very few studies highlighting the reasoning for these effects. Studies demonstrating probiotic modulation of pro-inflammatory and anti-inflammatory markers should be investigated in bone and soft tissue important in limiting both gingivitis and periodontitis. Although, there are few studies that attempt to elucidate such reasoning by looking at cytokine levels in GCF, there is a large variety of inflammatory cytokines that should be tested. Protocols should be established *in vitro*, for studies that will prompt appropriate animal and human clinical trials.

Equally important is the need for the development of a delivery system that allows for a prolonged residence time of the delivered probiotic in the oral cavity. Previous and current research has focused on the use of chewing gums, mouth rinses,



probiotic drops, chocolate, and food products such as milk, cheese and yoghurt for probiotic delivery [27-30]. **Table 2.7** provides a brief overview of the carrier systems previously investigated for probiotic delivery.

**Table 2.7:** Formulations used for probiotic deliveries for oral diseases

Formulations	Probiotic bacterial strain	Probiotic dose	Duration	Ref(s)
Milk	<i>L. casei strain Shirota</i>	$10^9$ cfu/ml	8 weeks	[115]
Chewing Gum	<i>L. reuteri ATCC 55730</i>	$10^8$ cfu/gum	4 weeks	[156]
Straws/ tablets		$10^8$ cfu once daily	3 weeks	[184]
Chewing Gum	<i>L. reuteri ATCC 5289</i>	$10^8$ cfu/gum	4 weeks	[156]
Lozenge		$10^8$ cfu/day	3 weeks	[157]
Milk	<i>L. rhamnosus GG</i>	$5 \times 10^5$ cfu/ml 5 days/week	7 months	[60]
Cheese		$1.9 \times 10^7$ cfu/g; 5x 15 g/day	3 weeks	[160]
Diet	<i>L. paracasei DSMZ16671</i>	$5 \times 10^8$ cfu/ g	-	[169]
Ice-cream	<i>Bifidobacterium lactis Bb-12</i>	$10^7$ cfu/g	1-2 weeks, Cross-over study	[164]
Lozenge	<i>L. reuteri DSM17938</i>	$10^8$ cfu/day	3 weeks	[157]
Tablet	<i>W. salivarius WB21</i>	$6.7 \times 10^8$ cfu 3times/day	8 weeks	[162]
Cheese	<i>L. rhamnosus LC 705</i>	$1.2 \times 10^7$ cfu/g; 5x 15 g/day	3 weeks	[160]
Yoghurt	<i>Bifidobacterium DN-173</i>	$7 \times 10^7$ cfu/g; 200 g/day	4 weeks	[171]
Lozenge	<i>S. salivarius K12</i>	$10^9$ cfu once daily	3 weeks	[157]

Although these delivery systems have shown promising results, there remain a number of limitations to be addressed. For example, probiotics incorporated in food products, upon swallowing, result in rapid loss from the oral cavity. A loss of probiotics from the oral cavity means a loss of their efficiency in preventing and treating oral diseases. Developed delivery systems should, hence, focus at increasing the retention time of the probiotics in the oral cavity, increasing the efficiency of the probiotic therapeutic. Carrier systems, such as chewing gums may increase the retention time of probiotics in the oral cavity, however the retention time may need to be increased further for an optimal effect. Factors that need to be considered while developing a delivery system include the probiotic's targeted delivery while maintaining the viability and metabolic activity of the probiotic

cells. Lastly, the shelf-life of a carrier system and the probiotic incorporated in the system should be investigated, as bacterial viability is directly correlated with the efficacy of any developed therapeutic.

## PREFACE TO CHAPTERS 3 - 8

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Chapter 3 to 8 consists of investigations that were performed to establish the hypothesis of this thesis and to achieve the stated research objectives.

**Chapter 3:** Earlier studies have demonstrated that probiotic bacteria have the potential to reduce the counts of oral pathogens. The goal of this study was to screen *Lactobacillus* probiotic bacteria for the inhibition of *Streptococcus mutans* and *Candida albicans*, the causative organisms of dental caries and oral candidiasis. Effects of both cell-free supernatant and live bacteria were tested. Analysis was performed using MRS agar plates containing the pathogen, with probiotic bacteria in pre-formed wells in these plates. Clearance zone indicating pathogen inhibition, was measured to estimate the potential of probiotic effects.

**Chapter 4:** With the intention of testing thesis hypothesis *Lactobacillus* strains were selected for quantitative assessment of *S. mutans* inhibition in simulated salivary conditions. Our aim was to understand the features of the tested probiotic bacterial strains with a goal to develop a bio-therapeutic for dental caries and periodontal diseases. Probiotic bacteriocin activity, salivary pH modulation, probiotic nutrient (sucrose) competition, probiotic co-aggregation with *S. mutans*, bacterial attachment to oral epithelial keratinocytes, bacterial nitric oxide production and bacterial antioxidant activity were evaluated in simulated oral conditions.

**Chapter 5:** In previous chapters we established that *L. reuteri* bacteria are superior *S. mutans* inhibitors. Additionally, H<sub>2</sub>O<sub>2</sub> is a well-known anti-bacterial compound. The aim of this study was to screen the selected *Lactobacillus* strains for hydrogen peroxide production. Both qualitative and quantitative colorimetric analyses were performed using

trimethyl benzidine and horseradish peroxide that determines H<sub>2</sub>O<sub>2</sub> production by the probiotic bacteria. Catalase that can destroy the H<sub>2</sub>O<sub>2</sub> produced was added with probiotic to prove that probiotic H<sub>2</sub>O<sub>2</sub> production inhibits *S. mutans*.

**Chapter 6:** As established in the previous chapters, *Lactobacillus reuteri* NCIMB 701359 was a potent H<sub>2</sub>O<sub>2</sub> producing probiotic and inhibited planktonic *S. mutans* cultures. The goal of this study was to evaluate *Lactobacillus reuteri* NCIMB 701359 for *S. mutans* biofilm inhibition. *S. mutans* biofilms were formed in 24 well plates and were treated with probiotic bacteria using transwell systems. The study was performed for 5 days. Selective agar was used for bacterial enumeration. Biofilm mass, *S. mutans* aggregates in the biofilms and *S. mutans* viability in the biofilms were evaluated to determine probiotic mediated biofilm inhibition.

**Chapter 7:** As studied in previous chapters and earlier studies, *Lactobacillus fermentum* NCIMB 5221 have demonstrated the potential to inhibit oral pathogens and host immunomodulation. Moreover, periodontal disease is characterised by chronic inflammation and loss of connective tissue such as alveolar bone. The aim of the present investigation was to evaluate *L. fermentum* NCIMB 5221 for antioxidant activity, ferulic acid and nitric oxide production that would prove beneficial for reducing inflammation. *L. fermentum* NCIMB 5221 was then evaluated for its potential in modulating inflammatory cytokine production by inflamed pre-osteoblasts. Potential of probiotic bacteria to enhance bone growth was also evaluated. We investigated the probiotic effects on F344 rats for serum C-reactive protein, serum alkaline phosphatase and serum calcium levels using Hitachi 911 automated clinical chemistry autoanalyzer. Femur and

mandibular bone mineral density and alveolar bone support using radiography and image j analysis.

**Chapter 8:** Based on previous findings that demonstrated the beneficial effects of probiotic bacteria, the goal of this study was to develop oral thin films (OTF) efficient at delivering probiotic bacteria to the mouth. Carboxymethyl cellulose (CMC) was used to develop the OTFs. Films were optimized and characterised for CMC concentration, weight, thickness, hygroscopicity and dissolving time. Following optimization CMC-OTF of wet film weight 10 g and CMC concentration 5 mg/mL were used for *L. fermentum* NCIMB 5221 incorporation, which were then stored in non-controlled conditions. The probiotic CMC-OTFs were evaluated for bacterial viability and antioxidant activity following long-term storage at room temperature.

During this project and thesis period, I contributed to 25 scientific papers published or to be published, in peer reviewed journals. 7 of these articles are as first author, 18 are either published or in press. I also contributed to 33 research abstracts or proceeding and have presented 10 of them as presenting author. I have selected 6 articles to use in this thesis where I am the first author.

**Original research articles included in the thesis:**

1. **S Saha**, C Tomaro-Duchesneau, L Rodes, M Malhotra, M Tabrizian, S Prakash. (2014) Investigation of probiotic bacteria as dental caries and periodontal disease biotherapeutics. *Beneficial Microbes*. 5 (4), 447-460.
2. **S Saha**, C. Tomaro-Duchesneau, L. Rodes, M.Tabrizian and S. Prakash. (2014). Probiotic hydrogen peroxide production inhibits *Streptococcus mutans*: potential dental caries therapeutic.(submitted)
3. **S Saha**, C Tomaro-Duchesneau, M. Tabrizian and S. Prakash. (2014). Inhibition of dental caries causing *Streptococcus mutans* biofilm by probiotic *Lactobacillus reuteri* NCIMB 701359. (To be submitted)
4. **S Saha**, C. Tomaro-Duchesneau, M. Malhotra, K. Sabally, L. Rodes, M. Tabrizian and S. Prakash. (2014). *Lactobacillus fermentum* NCIMB 5221 as a periodontal disease bio-therapeutic: *in vitro* and *in vivo* investigations using F344 rats. (To be submitted).
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**Original articles not included in the thesis (published/accepted/submitted):**

7. **S Saha**, C Tomaro-Duchesneau, M Tabrizian, S Prakash. (2012) Probiotics as oral health biotherapeutics. *Expert Opinion on Biological Therapy*. 12(9), 1207-1220.
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10. C Tomaro-Duchesneau, **S Saha**, M Malhotra, ML Jones, L Rodes, S Prakash. (2014) Ferulic acid producing *L. fermentum* as cholesterol-lowering probiotic biotherapeutics. (submitted)
11. C Tomaro-Duchesneau, ML Jones, D Shah, P Jain, **S Saha**, S Prakash (2014) Cholesterol assimilation by *Lactobacillus* probiotic bacteria: an *in vitro* investigation. (submitted)
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1. **S Saha**, C Tomaro-Duchesneau, M Malhotra, M Tabrizian, S Prakash (2014). A novel probiotic oral disease bio-therapeutic. McGill University-Faculty of Dentistry–9<sup>th</sup> Annual Research Day, Montreal, QC, Canada. March 26
2. **S Saha** and C Tomaro-Duchesneau (2014). Introduction to the field of microbiome modulation and delivery systems for live probiotic bacteria. *Lecture in Cell and Tissue Engineering course (BMDE 505)*, McGill University. Feb 5. Montreal, QC, Canada
3. **S Saha**, C Tomaro-Duchesneau, M Malhotra, M Tabrizian, S Prakash (2011). Role of Probiotics in Dental Caries Causing Organism *Streptococcus mutans* and Other Bacterial Systems: In-Vitro Analysis. McGill University-Faculty of Dentistry–6<sup>th</sup> Annual Research Day, Montreal, QC, Canada. February 21.
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7. **S Saha**, C Tomaro-Duchesneau, M Malhotra, M Tabrizian, S Prakash. (2014). Novel probiotic dental caries and periodontal disease bio-therapeutic. “Canadian Society for Pharmaceutical Sciences”. June 10-13. Montreal, QC, Canada.
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11. **S. Saha**, C. Tomaro-Duchesneau, L. Rodes, M. Malhotra, M. Tabrizian, S. Prakash. **(2013)** Probiotic bio-therapeutic for oral diseases: in vitro screening and characterisation. *McGill University-Faculty of Dentistry – 8th Annual Research Day*, March 28, Montreal, QC, Canada.
12. **S Saha**, C Tomaro Duchesneau, M Malhotra, I Kahouli, M Tabrizian, S Prakash. **(2012)** Potential role of probiotics for the inhibition of *S. mutans* and *C. albicans*. “Reflections” *9th Annual NHP Conference and Tradeshow*. May 22-25, Kelowna, BC, Canada.
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## CONTRIBUTIONS OF CO-AUTHORS

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The works presented in this thesis are original research articles (published/unpublished). All the experiments and data analysis were performed by me. The data analysis was also performed by me. Dr. Satya Prakash, my thesis supervisor is the last and corresponding author for each research article. Contributions all by co-authors for each study are as follows:

**Chapter 3:** Dr. Catherine Tomaro-Duchesneau provided me with intellectual and technical help with cell culture, experiment methodology and manuscript preparation. Dr. Meenakshi Malhotra contributed with proof reading of the manuscript. Dr. Maryam Tabrizian provided experimental ideas and manuscript editing.

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**Chapter 5:** Dr. Catherine Tomaro-Duchesneau provided help with intellectual ideas, experimental designing and manuscript preparation. Ms. Laetitia Rodes provided technical support with cell culture and proof reading of the manuscript. Dr. Maryam Tabrizian contributed with editing and proof reading of the manuscript.

**Chapter 6:** Dr. Catherine Tomaro-Duchesneau provided me help with experiment methodology and manuscript preparation. Dr. Maryam Tabrizian contributed with editing and proof reading of the manuscript.

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**Chapter 8:** Dr. Catherine Tomaro-Duchesneau contributed on all aspects of this study including intellectual, experimental contributions and manuscript preparation. Dr. Jamal T. Daoud provided technical help with dielectric spectrophotometry and methodology. Dr. Maryam Tabrizian provided intellectual help and contributed in editing and proof reading of the manuscript.

**CHAPTER 3: SUPPRESSION OF *STREPTOCOCCUS MUTANS* AND *CANDIDA ALBICANS* BY PROBIOTICS: AN *IN VITRO* STUDY**

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**Preface:** Earlier studies have demonstrated that probiotic bacteria have the potential to reduce the counts of oral pathogens. The goal of this study was to screen *Lactobacillus* probiotic bacteria for the inhibition of *Streptococcus mutans* and *Candida albicans*, the causative organisms of dental caries and oral candidiasis. Effects of both cell-free supernatant and live bacteria were tested. Analysis was performed using MRS agar plates containing the pathogen, with probiotic bacteria in pre-formed wells in these plates. Clearance zone indicating pathogen inhibition, was measured to estimate the potential of probiotic effects.

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### 3.1 Abstract

Oral infections caused by microorganisms have led to increased risk of oral health problems such as dental caries (DC), periodontitis and oral candidiasis (OC). *Streptococcus mutans* and *Candida albicans* are the primary organisms responsible for DC and OC, respectively. The goal of the presented study was to investigate the potential of probiotics to prevent and treat DC and OC. An *in vitro* assay was developed to investigate several probiotic strains for their ability to inhibit the aforementioned oral pathogens. Probiotic by-products present in probiotic supernatant and live probiotic cells were both investigated for their ability to inhibit the growth of *S. mutans* and *C. albicans*. The probiotic strains investigated were *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 11951, *L. reuteri* NCIMB 702656, *L. reuteri* NCIMB 702655, *L. fermentum* NCIMB 5221, *L. fermentum* NCIMB 2797, *L. fermentum* NCIMB 8829, *L. acidophilus* ATCC 314, *L. plantarum* ATCC 14917 and *L. rhamnosus* ATCC 53103. The presented research demonstrates that live probiotic cells are needed to inhibit oral pathogens, as cell-free supernatant could not inhibit the pathogens. Further experiments were performed to investigate and optimize the dose-dependent inhibition of the pathogens by live probiotic cells. As desired, an increased inhibition was observed with an increase in dose, as demonstrated by the increasing size of the zones of clearance. In addition, the observed inhibition was dependent on the strain of the probiotic used. This research implies that probiotic bacteria are capable of inhibiting the selected oral pathogens, *S. mutans* and *C. albicans*, holding promise for the future development of a probiotic therapeutic to treat and prevent oral/dental diseases. Furthermore, the research



proposes further investigations into the probiotic mechanism(s) of action and efficacy for the development of an optimal therapy.

### **3.2 Introduction**

Dental caries (DC), periodontitis and oral candidiasis (OC) are widespread disorders. According to the World Health Organization, 60-90% of worldwide school-children [2] and 95% of adults in the United States have DC [199]. Furthermore, 5-10% of public health expenditures relate to oral health - a significant economic burden [1]. It has been demonstrated that the initiation and progression of oral disease is primarily due to the increased proliferation of opportunistic microorganisms [200]. A large variety of microorganisms are associated with oral disease, however, the increased proliferation of *S. mutans*, present in carious lesions, is the primary cause for the initiation and the progression of DC, and hence is of primary interest in the presented research [16,77]. The relationship between this causative organism and pathogenesis is not clear, although it has been suggested that *S. mutans* proliferation leads to acid production, promoting tooth decay and DC [201]. OC, also termed thrush, is a yeast infection of the genus *Candida*, most often caused by *C. albicans*, the second pathogenic microorganism of focus in this research [16,35,202].

The current methods for minimizing the incidence of DC focus on prevention techniques such as proper oral hygiene, drinking water fluoridation and application of dental sealants [5]. Unfortunately, as the statistics suggest, these prevention methods have shown only limited success. OC is typically controlled by anti-fungal drugs such as amphotericin B [203]. If not established as a biofilm, *C. albicans* is usually susceptible to most anti-fungals, but mechanical disruption of a biofilm prior to anti-fungal application

may be required. A well-known problem is that long-term exposure to anti-fungal agents promotes acquired resistance [132]. The documented levels of resistance in oral candida are indeed on the rise [132]. To address these shortcomings, an alternative therapeutic is necessary for controlling the incidence of DC and OC, without the need for antimicrobials. Recent research has elucidated the role and importance of the oral microflora in pathogenesis [35,55]. With this in mind, investigators have proposed probiotics, specifically Lactobacilli, for maintaining and regaining oral health [204]. Lactobacilli have been used as probiotics for a number of disease applications, and are generally regarded as safe for human use, making them an ideal candidate for the development of a therapeutic [140,174].

This work investigates probiotic Lactobacillus strains that have potential of inhibiting oral pathogens, specifically *S. mutans* NCIMB 702062 and *C. albicans* ATCC 11006. The probiotic strains investigated were *L. rhamnosus* ATCC 53103, *L. fermentum* NCIMB 5221, *L. fermentum* NCIMB 2797, *L. fermentum* NCIMB 8829, *L. reuteri* NCIMB 11951, *L. reuteri* NCIMB 702656, *L. reuteri* NCIMB 702655, *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 701089, *L. acidophilus* ATCC 314 and *L. plantarum* ATCC 14917.

### **3.3 Materials and Methods**

#### **3.3.1 Bacterial growth media and chemicals**

De Man, Rogosa, Sharpe (MRS) broth was purchased from Fisher Scientific (Ottawa, ON, Canada). Water was purified with an EasyPure reverse osmosis system and a NanoPure Diamond Life Science (UV/UF) ultrapure water system from Barnstead

(Dubuque, IA, USA). All other chemicals used were of HPLC or standard analytical grade purchased from Sigma-Aldrich (Oakville, ON, Canada).

### **3.3.2 Bacterial strains and culture conditions**

*L. rhamnosus* ATCC 53103, *L. acidophilus* ATCC 314, *L. plantarum* ATCC 14917 and *Escherichia coli* ATCC 8739 were purchased from Cedarlane Laboratories (Burlington, ON, Canada). *L. fermentum* NCIMB 5221, *L. fermentum* NCIMB 2797, *L. fermentum* NCIMB 8829, *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 702655, *L. reuteri* NCIMB 702656 and *L. reuteri* NCIMB 11951 were purchased from NCIMB (Aberdeen, Scotland, UK). *S. mutans* NCIMB 702062, isolated from carious dentine, was purchased from NCIMB (Aberdeen, Scotland, UK) and *C. albicans* ATCC 11006 was generously provided by Micropharma Limited (Montreal, Canada). All of the bacterial strains were maintained at -80°C as 20% (v/v) glycerol stocks. A MRS agar plate was streaked and incubated at 37°C for 24 hours with 5% CO<sub>2</sub> to ensure the purity of the culture. One colony from the MRS agar plate was inoculated in MRS broth for 24 hours at 37°C. A 1% (v/v) inoculum was subcultured into MRS broth and incubated at 37°C for 24 hours for the assays.

### **3.3.3 Inhibition of *S. mutans* by probiotic supernatant and live cells**

*S. mutans* was tested for inhibition by the probiotic supernatants and live cultures of the following probiotic strains: 1) *L. rhamnosus* ATCC 53103, 2) *L. acidophilus* ATCC 314, 3) *L. plantarum* ATCC 14917, 4) *L. fermentum* NCIMB 5221, 5) *L. fermentum* NCIMB 2797, 6) *L. fermentum* NCIMB 8829, 7) *L. reuteri* NCIMB 701089, 8) *L. reuteri* NCIMB 701359, 9) *L. reuteri* NCIMB 702655, 10) *L. reuteri* NCIMB 702656 and 11) *L. reuteri* NCIMB 11951. MRS agar (15% (w/v)) was prepared and

autoclaved, after which it was cooled to 55°C in a water bath. *S. mutans* was incorporated in the molten agar at a concentration of 0.5 % (v/v) overnight culture. *L. rhamnosus* ATCC 53103 was used as positive control, as it successfully inhibited *S. mutans* demonstrated by Hasslof *et al.* previously [166]. Agar plates were poured, each consisting of 30mL and cooled to room temperature. Four wells were formed in each plate. The supernatant was collected from each Lactobacillus strain by pelleting overnight and 48 hour probiotic cultures at 4000rpm for 30 minutes at 4°C (NAPCO 2028R centrifuge). To test the inhibition via probiotic by-product, the supernatant was used in two different ways. The supernatant was filtered using a 0.2µm filter and the pH was adjusted to 6.7 using 2M NaOH. The supernatants were prepared in 2-fold serial dilutions using sterile MRS broth: 1) 100% (v/v), 2) 50% (v/v) 3) 25% (v/v) and 4) 12.5% of the original supernatant concentration. Secondly, the supernatant was filtered using a 0.2µm filter and 2-fold serial dilutions were prepared using sterile MRS broth as aforementioned, without adjusting the pH.

Similarly, for tests with probiotic live cultures, 10-fold serial dilutions of the probiotic overnight cultures were prepared in sterile MRS broth: 1) 100% (v/v) 2) 10% (v/v) 3) 1% (v/v) and 4) 0.1% (v/v). The wells in each plate were filled with 100µl of the respective diluted probiotic overnight strain culture/ supernatant at desired concentration. Sterile MRS broth and *E. coli* ATCC 8739 were used as negative controls. The plates were incubated at 37°C and 5% CO<sub>2</sub> for 48 hrs. Each probiotic strain was tested in triplicate to ensure accuracy and reproducibility and controls were treated exactly the same way as test samples. Following 48 hours, the diameters of the inhibition zones were

measured for each well. Clearance zones were measured from the edge of the wells to the edge of the clearance zones.

### **3.3.4 Inhibition of *C. albicans* via live probiotics and probiotic by-products**

The probiotic by-product and live culture mediated inhibition of *C. albicans* was investigated using the methods described above for *S. mutans* with slight modifications. *C. albicans* was incorporated in the molten agar at a concentration of 1 % (v/v) of both overnight and 48 hour culture. The same probiotic strains, described above for *S. mutans*, were tested for the probiotic-mediated inhibition of *C. albicans*.

### **3.3.5 Bacterial viability**

The viability of the pathogenic and probiotic bacteria was measured using standard colony forming units (cfu). 0.85% (w/v) NaCl was used to achieve 10-fold serial dilutions of each strain, as required. Each strain was streaked on MRS-agar plates and incubated at 37°C and 5% CO<sub>2</sub> for 48 hours, prior to colony counting. Each strain was plated in triplicate to ensure accuracy and reproducibility.

### **3.3.6 Statistical analysis**

Experimental results are expressed as mean  $\pm$  standard error of the mean (SEM). Statistical analysis was carried out using SPSS Version 17.0 (Statistical Product and Service Solutions, IBM Corporation, New York, NY, USA). Non-linear regression was performed to correlate probiotic cell count and zone of clearance.

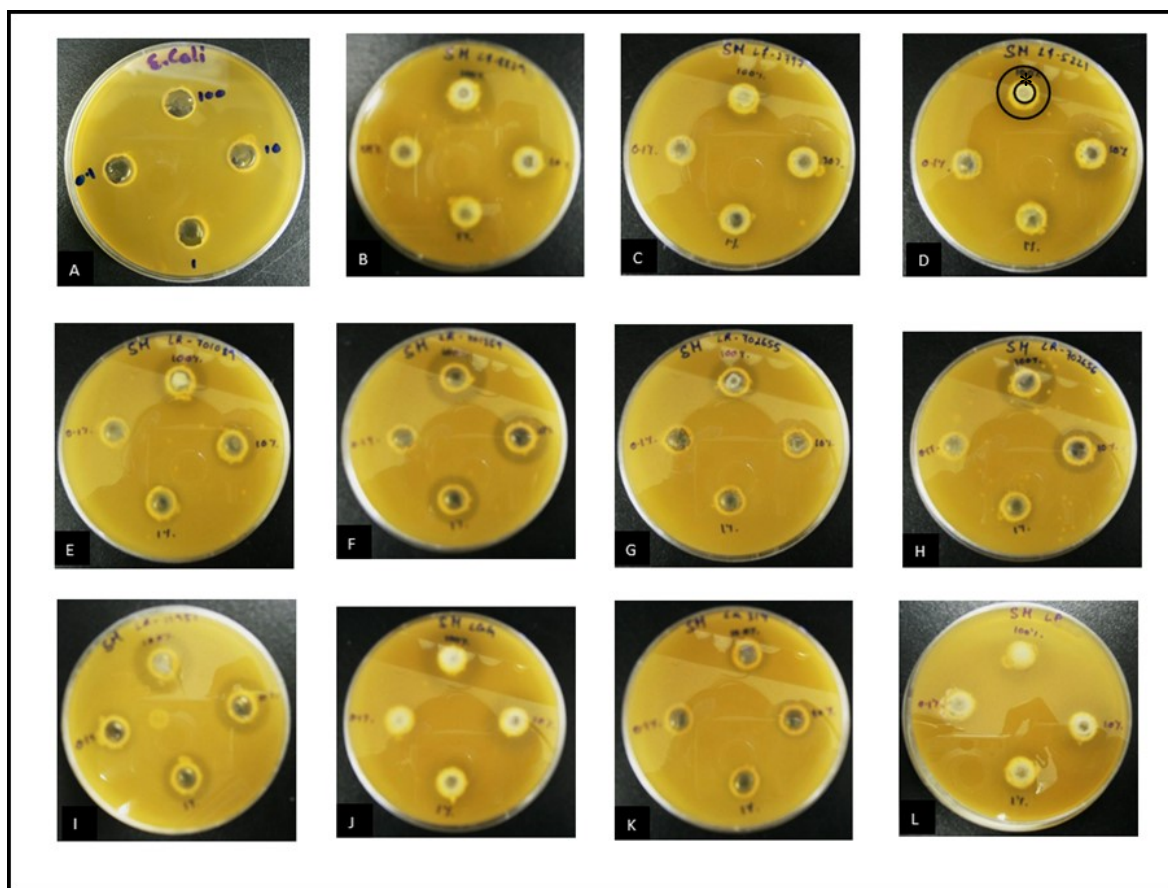
## **3.4 Results**

### **3.4.1 *S. mutans* and *C. albicans* inhibition via probiotic by-products**

There were no visible zones of inhibition of *S. mutans* ATCC 702062 and *C. albicans* ATCC 11006 for any of the supernatant of the tested probiotics and controls (results not shown).

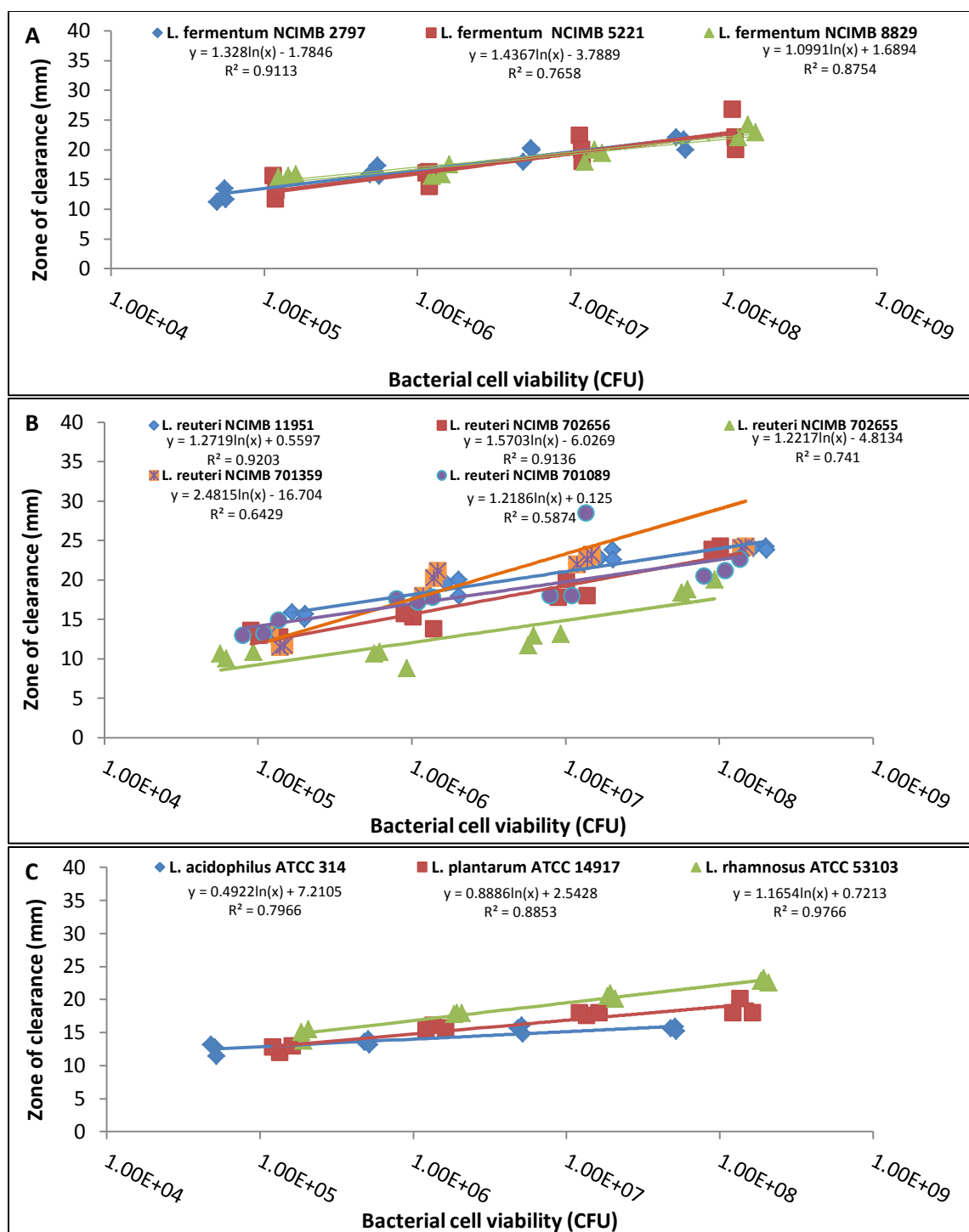
### 3.4.2 *S. mutans* inhibition using live probiotic cultures

The inhibition of *S. mutans* ATCC 702062 was visible by clearing zones in the MRS agar around the wells containing the probiotic strains. The viability of the incorporated *S. mutans* was  $1.04 \times 10^7$  cfu/ml of agar. The inhibition zones, produced by probiotic activity, were clearly visible, as seen in **Fig 3.1**. All the tested probiotic bacteria were all able to inhibit the growth of *S. mutans*, with varying inhibition activity for each strain. A positive correlation between the area of the zones of clearance and the concentration of probiotic cells was observed (**Fig.3.3**). Pathogenic microorganism clearance zones were quantified using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-2011). The negative controls (sterile MRS broth) and *E. coli* ATCC 8739, showed no inhibition, suggesting a probiotic-specific inhibition.



**Figure 3.1:** Inhibition assay for *S. mutans* ATCC 702062 demonstrating the zone of inhibition around the wells containing: (A) Negative Control – *E. coli* ATCC 8739; (B) Test strain *L. fermentum* NCIMB 8829; (C) Test strain *L. fermentum* NCIMB 2797; (D) Test strain *L. fermentum* NCIMB 5221; (E) Test strain *L. reuteri* NCIMB 701089; (F) Test strain *L. reuteri* NCIMB 701359; (G) Test strain *L. reuteri* NCIMB 702655; (H) Test strain *L. reuteri* NCIMB 702656; (I) Test strain *L. reuteri* NCIMB 11951; (J) Positive control- *L. rhamnosus* ATCC 53103; (K) Test strain *L. acidophilus* ATCC 314; (L) Test strain *L. plantarum* ATCC 14917 and \* shows the clearance zone. The four wells contained the probiotic overnight culture of desired concentration diluted using sterile MRS: 100% (v/v), 10% (v/v), 1% (v/v) and 0.1% (v/v).

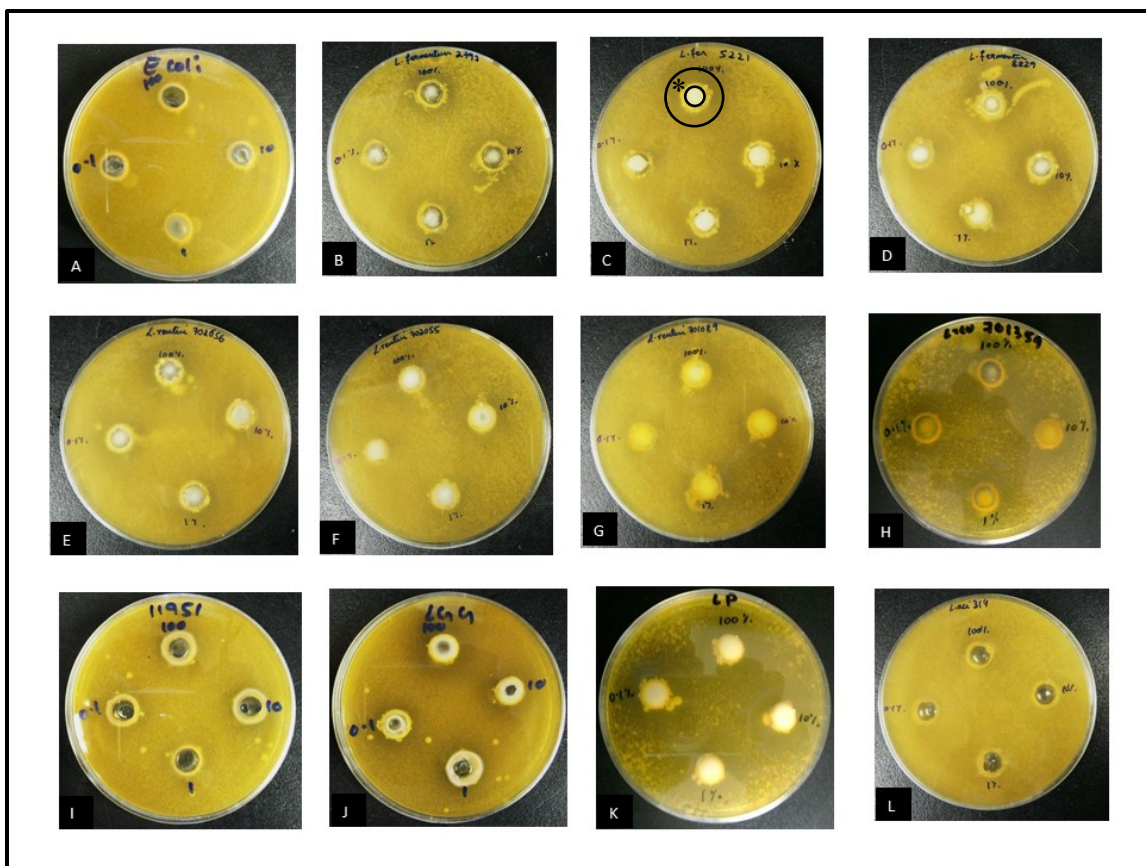




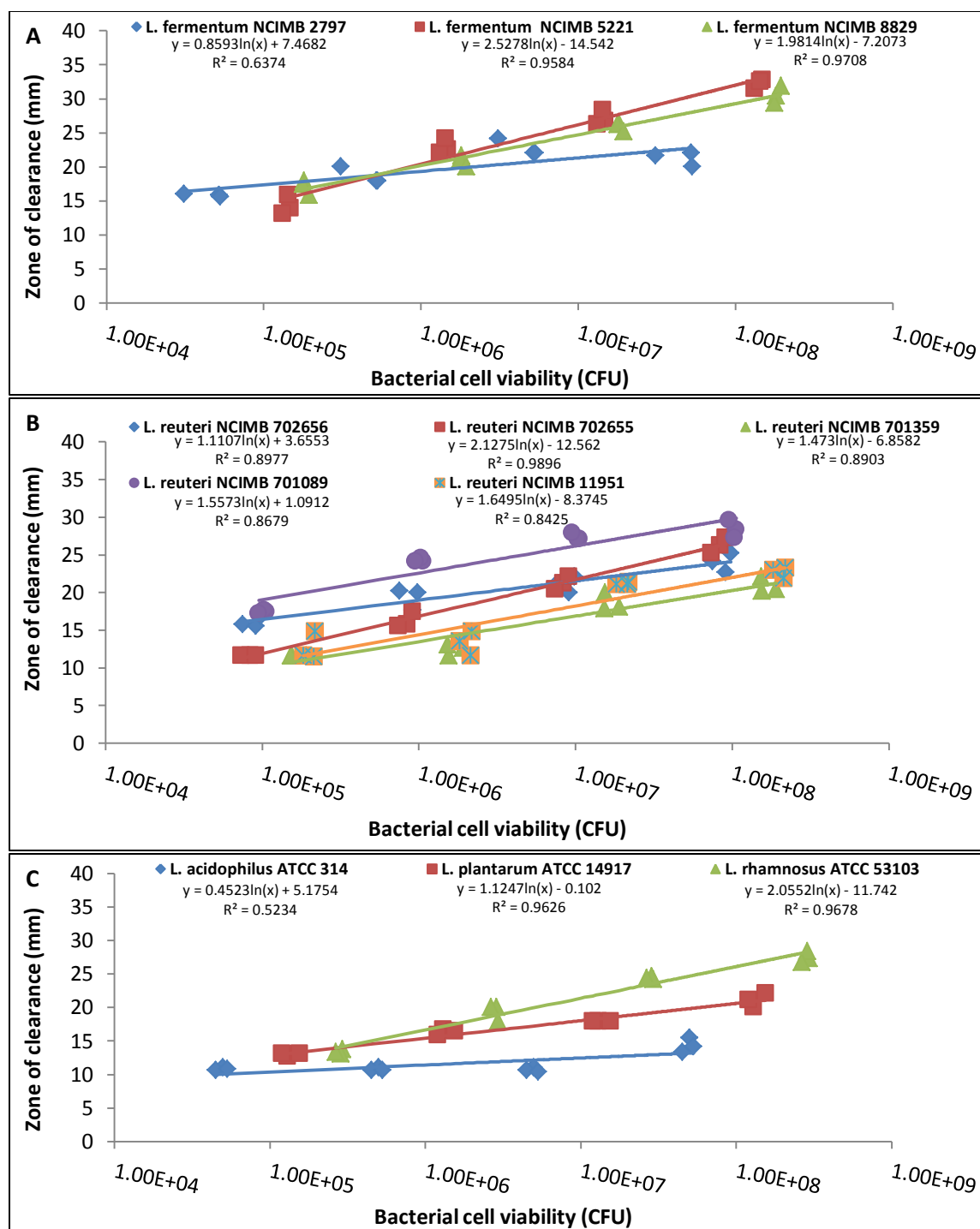
**Figure 3.3:** Inhibition of *S. mutans* ATCC 702062 by probiotic strains: A) *L. fermentum* NCIMB 2797, *L. fermentum* NCIMB 5221 and *L. fermentum* NCIMB 8829; B) *L. reuteri* NCIMB 11951, *L. reuteri* NCIMB 702655, *L. reuteri* NCIMB 702656, *L. reuteri* NCIMB 701359 and *L. reuteri* NCIMB 701089; C) *L. acidophilus* ATCC 314, *L. plantarum* ATCC 14917 and *L. rhamnosus* ATCC 53103. Non-linear regression was performed to correlate bacterial viability with zone of clearance (n=3).

### 3.4.3 *C. albicans* inhibition using live probiotic cultures

The inhibition of *C. albicans* ATCC 11006 was visible demonstrated by clearance zones in the MRS agar around the wells containing the probiotics. The viability of the incorporated *C. albicans* was  $3.67 \times 10^4$  cfu/ml of agar. The inhibition zones, due to probiotic activity, were visible, as seen in **Fig 3.2**. *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 11951, *L. reuteri* NCIMB 702656, *L. reuteri* NCIMB 702655, *L. fermentum* NCIMB 5221, *L. fermentum* NCIMB 2797, *L. fermentum* NCIMB 8829, *L. acidophilus* ATCC 314, *L. plantarum* ATCC 14917 and *L. rhamnosus* ATCC 53103 all inhibited the growth of *C. albicans*, with varying activity for each strain, as seen in **Fig 3.4**. Clearance zones were measured for all the samples. A positive correlation between the area of the zones of clearance and the concentration of probiotic cells was observed (**Fig.3.4**). The negative controls with sterile MRS and *E. coli* ATCC 8739 showed no zones of inhibition, demonstrating probiotic-specific inhibition of *C. albicans*.



**Figure 3.2:** Inhibition assay for *C. albicans* ATCC 11006 demonstrating zone of inhibition around wells containing: (A) Negative Control – *E. coli* ATCC 8739; (B) Test strain *L. fermentum* NCIMB 2797; (C) Test strain *L. fermentum* NCIMB 5221; (D) Test strain *L. fermentum* NCIMB 8829; (E) Test strain *L. reuteri* NCIMB 702656; (F) Test strain *L. reuteri* NCIMB 702655; (G) Test strain *L. reuteri* NCIMB 701089; (H) Test strain *L. reuteri* NCIMB 701359; (I) Test strain *L. reuteri* NCIMB 11951; (J) Positive control- *L. rhamnosus* ATCC 53103; (K) Test strain *L. plantarum* ATCC 14917; (L) Test strain *L. acidophilus* ATCC 314 and \*shows the clearance zone. The four wells contained the probiotic overnight culture of desired concentration diluted using sterile MRS: 100% (v/v), 10% (v/v), 1% (v/v) and 0.1% (v/v).



**Figure 3.4:** Inhibition of *C. albicans* ATCC 11006 by probiotic strains: A) *L. fermentum* NCIMB 2797, *L. fermentum* NCIMB 5221 and *L. fermentum* NCIMB 8829; B) *L. reuteri* NCIMB 11951, *L. reuteri* NCIMB 702655, *L. reuteri* NCIMB 702656, *L. reuteri* NCIMB 701359 and *L. reuteri* NCIMB 701089; C) *L. acidophilus* ATCC 314, *L. plantarum* ATCC 14917 and *L. rhamnosus* ATCC 53103. Non-linear regression was performed to correlate bacterial viability with zone of clearance (n=3).

### 3.5 Discussion

The aetiology of disorders such as OC and DC has been directly correlated with the presence and proliferation of pathogenic microorganisms. Various microorganisms are associated with the initiation and progression of dental caries, among which *S. mutans* and *S. sobrinus* are the most prominent caries causing agents [205]. However, a number of research groups have demonstrated that *S. mutans* is more frequently associated with DC than *S. sobrinus*, hence the focus is on the former in this work [205-208]. As for OC, *C. albicans* is the principle causative organism [76,208].

Probiotic bacteria have been used to modify microfloral ecosystems, and have already shown some success as a therapeutic for oral diseases, as described in a recent review [26,60]. For the development of a more efficacious therapeutic, optimization and selection of probiotic strains need to be undertaken. The goal of the presented research was to screen probiotic strains, chosen based on already demonstrated beneficial properties in other human disorders, to investigate their potential to inhibit the two prominent oral pathogens, *S. mutans* and *C. albicans* via probiotic by-products and live probiotic cultures.

Inhibition assays were performed using two probiotic formulations: 1) cell supernatant (by-products) and 2) live cells. An *in vitro* assay was designed to investigate the probiotic inhibition of *S. mutans* NCIMB 702062 and *C. albicans* ATCC 11006. The *S. mutans* used in this investigation was isolated from carious dentine and was used as a proof of concept for other pathogenic strains. *C. albicans* was used in its yeast form as opposed to hypha form as that is the phenotype involved in the clinical manifestation of oral candidiasis [209]. The inhibition assay consisted of MRS agar embedded with the

oral pathogens, with preformed wells in the agar containing the supernatant or live cells. Clearance zones demonstrated the ability of the supernatant or live probiotic formulation to inhibit the growth of the pathogens. Previous research has demonstrated that probiotic supernatant filtered of the cell contents showed inhibition of oral biofilm due to bacteriocin activity, such as reuterin, originating from *L. reuteri* [22]. Hence, the first experimental approach undertaken was to use probiotic supernatant to determine whether a secreted factor produced during culturing could inhibit the oral pathogens that contribute to biofilm formation. The results demonstrated no detectable inhibition for either *C. albicans* or *S. mutans*. The overnight probiotic cultures were further grown for an extended period of 48 hours to confirm that probiotic supernatants contained enough secreted products for optimum function. However, none of the probiotic supernatants were able to demonstrate any inhibition of *S. mutans* nor *C. albicans*. As aforementioned, the pH of the supernatant from all the tested probiotic bacterial strains was adjusted to 6.7 by the addition of NaOH. Hence, to rule out the effect of NaOH, both 24 and 48 hours of bacterial supernatants were also tested without the addition of NaOH. The rationale behind these two approaches was first to evaluate the effect of secreted products from the probiotics, by eliminating the possibility of an observed inhibition due to the effect of pH of the tested supernatants and second, to eliminate the possibility of inactivating/modifying the functionality of the probiotic supernatant by the addition of NaOH. An explanation for the lack of inhibition by probiotic supernatant may be due to a number of factors, for example, organic acids or bacteriocins produced by the probiotic may be unstable or degraded or are simply present in too low concentrations. However, when the live probiotic cell cultures were used, all of the tested strains proved capable of

inhibiting the proliferation of *S. mutans* and *C. albicans*, with the zones of inhibition variable for each probiotic strain. Further experiments were then performed to evaluate the capability and dose response of different live probiotic cell concentrations to inhibit *S. mutans* and *C. albicans*. As expected, a positive correlation between the area of the zones of clearance and the concentration of probiotic cells was observed.

*S. mutans* and *C. albicans* were incorporated at 0.5% (w/v) and 1% (w/v) respectively. This corresponded to bacterial concentrations of  $1.04 \times 10^7 \pm 7.04 \times 10^5$  cfu/ml for *S. mutans* and  $3.67 \times 10^4 \pm 1.67 \times 10^4$  cfu/ml for *C. albicans*. Due to the low bacterial count for *C. albicans* as compared to *S. mutans*, the former was incorporated in a higher concentration to yield enough growth for a visible inhibition by the probiotic strains. Zones of clearance were then evaluated and attempts were made to evaluate the cfu required for a 1mm inhibition by normalizing the results. However, when the zones of clearance were plotted against the cfu, a strong non-linear correlation was demonstrated by some of the probiotic strains (indicated by their respective  $R^2$  values), as observed in **Fig 3.3**. A logarithmic relationship was observed when the zone of clearance was plotted against the cfu count as observed in **Fig 3.3**. As observed in **Fig 3.3(b)**, *L. fermentum* NCIMB 5221 and *L. reuteri* NCIMB 701359 demonstrated the largest zones of clearance as compared to the other tested strains, suggesting them as the most efficient strains for the inhibition of *S. mutans* ATCC 702062. Similar results were observed for the inhibition assay designed for *C. albicans* where a non-linear correlation was also demonstrated.

As observed in **Fig 3.4(a)**, *L fermentum* NCIMB 5221 demonstrated the largest zone of clearance as compared to the other tested probiotic strains., Hence, *L. reuteri* NCIMB 701359 and *L fermentum* NCIMB 5221 appears to be the most effective probiotic amongst all the tested strains for the inhibition of *S. mutans* NCIMB 702062 and *C. albicans* ATCC 11006 respectively.

Apart from assessing clearance zones to investigate pathogen inhibition, other methods such as deferred antagonism method can also be used for such investigations [210]. In a similar study, Hasslof *et al.* have demonstrated the inhibitory action on oral pathogens via probiotics [166]. They investigated the ability of probiotic suspension but did not compare it to their cell free supernatants which could potentially have an effect on the aforementioned pathogens. As described, similar results were demonstrated in this study, moreover it could also be concluded that probiotic by-products were either inefficient at inhibiting *S. mutans* and *C. albicans* or were not stable or degraded to be able to functional optimally. In addition, this observation clues that if the inhibition of pathogens was not due to the substance releases by the probiotics, it could be due a competition of nutrients between the pathogens and the probiotics, for example for sucrose. In addition, ten different Lactobacillus strains were investigated for an efficient therapeutic for DC and OC were *L. rhamnosus* ATCC 53103 was used as a positive control from the Hasslof et al. study [166].

The mechanisms of action behind the observed inhibition need to be investigated further. *In vivo*, several mechanisms may be involved in a successful probiotic inhibition of pathogens, namely: 1) inhibition of pathogenic molecules 2) inhibition of exopolysaccharide (EPS) secretion, responsible for dental plaque formation which allows for



accelerated growth of opportunistic organisms 3) blocking of pathogen adhesion sites 4) oral pH modulation and 5) nutrient competition as discussed in another review [144]. For example, EPS is a component of oral biofilm that is essential for the growth of oral pathogens [200]. A secreted probiotic product that could decrease the production of EPS could be beneficial for the prevention of biofilm formation and dental caries pathogenesis. Methods such as Fourier transform infrared spectrophotometry and high performance liquid chromatography can be used to quantify EPS levels, and hence may prove beneficial for future such investigations [211].

Moreover, research groups such as Noordin *et al.* were successful in demonstrating that probiotics reduced the accumulation of dental plaque/oral biofilm [113]. However, modulation of composition of the oral biofilm with an increased amount of non-cariogenic organisms and a decrease of pathogenic organisms could prove beneficial. Therefore, another approach to prevent/treat oral diseases via probiotic therapeutic could also be a modulation of oral biofilm. Hence, further research that demonstrates a decrease in a bacterial count of pure pathogenic strains and pathogenic strain incorporated in biofilm could prove beneficial. One concern with the inhibition of *C. albicans* and *S. mutans* is the possibility that the observed inhibition was simply due to environmental changes by the probiotic bacteria, more specifically acid production. It is to be noted that most of the tested probiotic cultures, following incubation had a significantly higher pH than the tested pathogens (data not shown). Furthermore, *S. mutans* is an acidogenic/aciduric bacterium that thrives under acidic conditions. We, therefore, hypothesise that the observed inhibition is not due to acid production by the probiotic bacteria, although further investigations are required into the exact mechanism

of action for the inhibition [212]. This study is a semi-quantitative assay, which attempts to select probiotic bacteria capable of inhibiting two significant oral pathogens. It is clear, that further quantitative investigations are required for a better understanding of the kinetics of the observed inhibition. Future experiments must also focus on the inhibition of probiotics in simulated conditions, biofilms and broth cultures, a more quantitative approach. As required for the selection of the best probiotic strain among these and other strains, a more quantitative assay needs to be performed involving conditions resembling the oral ecosystem.

Although a starting point, this research establishes the fact that the formulation of a probiotic therapy has enormous potential to prevent or treat DC, OC and potentially other oral disorders. However, it is important to note that bacterial antagonism is not the only criteria for the development of a probiotic therapeutic, and so further investigations into the proposed mechanism(s) of actions are a must. Future work should involve the additional screening and characterisation of a final formulation for potential preclinical use, in terms of the mechanisms of action and safety of the probiotic strains with proper *in vitro* and *in vivo* studies. The successful delivery and residence time of probiotics in the oral cavity also remains an issue. Techniques such as the incorporation and immobilization of probiotics in chewing gums, food matrices and dissolvable films may prove useful [156,157]. There is also the need for further investigations into the probiotic mechanism(s) of action before any formulation optimization can be undertaken. Using this assay, future work may also investigate additional strains for the inhibition of all pathogens related to oral diseases, including those of the red complex involved in periodontal diseases [190].

In summary, the presented work successfully investigated probiotics capable of inhibiting *S. mutans* and *C. albicans*. The results demonstrate that live probiotics are required for this inhibition since cell-free probiotic supernatant was not able to inhibit the pathogens. This work opens up future potentials for the development of a probiotic replacement therapy for oral diseases.

### **3.6 Acknowledgements**

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### **3.7 Conflict of interest**

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

## CHAPTER 4: INVESTIGATION OF PROBIOTIC BACTERIA AS DENTAL CARIES AND PERIODONTAL DISEASE BIOTHERAPEUTICS

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**Preface:** With the intention of testing thesis hypothesis *Lactobacillus* strains were selected for quantitative assessment of *S. mutans* inhibition in simulated salivary conditions. Our aim was to understand the features of the tested probiotic bacterial strains with a goal to develop a bio-therapeutic for dental caries and periodontal diseases. Probiotic bacteriocin activity, salivary pH modulation, probiotic nutrient (sucrose) competition, probiotic co-aggregation with *S. mutans*, bacterial attachment to oral epithelial keratinocytes, bacterial nitric oxide production and bacterial antioxidant activity were evaluated in simulated oral conditions.

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<http://wageningenacademic.metapress.com/content/a13251r8028v3026/#.U9g4GfldV9A>

## 4.1 Abstract

Oral diseases, specifically dental caries and periodontal disease, are characterized by increases in pathogenic microorganisms, increased demineralization and increased inflammation and levels of inflammatory markers. Despite the therapeutic strategies, oral diseases have elevated prevalence rates. Recent work has demonstrated that probiotic bio-therapeutics can decrease oral pathogen counts, including caries-causing *Streptococcus mutans* and oral inflammation. The aim of this work was to investigate putative probiotic bacteria, selected for *S. mutans* inhibition and for their oral health-promoting characteristics. The probiotic bacteria were investigated for *S. mutans* inhibition, probiotic bacteriocin activity, salivary pH modulation, probiotic nutrient (sucrose) competition, probiotic co-aggregation with *S. mutans*, bacterial attachment to oral epithelial keratinocytes, bacterial nitric oxide production and bacterial antioxidant activity. The results indicate that *Lactobacillus reuteri* NCIMB 701359, *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 702655 and *L. reuteri* NCIMB 702656 inhibited *S. mutans* to non-detectable levels ( $< 10$  cfu/ml). *L. reuteri* strains also demonstrated the highest antioxidant capacity of the tested strains (7.73-13.99  $\mu$ M Trolox equivalents), suggesting their use as both caries and periodontal disease therapeutics. Although *L. fermentum* NCIMB 5221 inhibited *S. mutans* at lower levels, it significantly buffered the pH (4.18) of saliva containing *S. mutans*, it co-aggregated with *S. mutans* (10.09%), it demonstrated high levels of sucrose consumption (138.11 mM) and it successfully attached to gingival epithelial cells (11%). This research identified four *L. reuteri* strains and *L. fermentum* NCIMB 5221 to be further investigated as oral disease biotherapeutics.

## 4.2 Introduction

Oral health is governed by the extensive and diverse microbial population of the mouth [144]. Alterations in the composition of the oral microbiota are associated with the initiation and development of oral diseases such as dental caries and periodontal disease [76]. These diseases can lead to severe pain, impaired quality of life and infections such as cellulitis and osteomyelitis [3,213]. More importantly, they can predispose patients to potentially fatal diseases such as infective endocarditis [214,215]. Indeed, oral diseases impose significant economic burdens on society [1].

Despite the current prevention and treatment strategies, the worldwide prevalence of caries stands at 60-90% for school children, strikingly, almost 100% for the adult population [216]. Similarly, although outdated, studies have estimated that 50-100% of the adult population is affected by gingivitis, characteristic of early-stage periodontal disease and 15-20% of middle-aged adults are affected by severe periodontal diseases [34,216]. The etiologies of both diseases are multifactorial and associated with disturbances in the oral environment [217,218]. Disturbances can be due to factors such as poor oral hygiene and underlying medical conditions, including dry mouth, termed xerostomia, which accelerate the growth of microbial biofilms, termed dental plaque [36,76]. Clinical studies have correlated the increased proliferation of acidogenic bacteria, which metabolize dietary sugars producing acid and a local pH decrease, with the initiation and progression of caries [36]. A variety of microorganisms have been associated with caries pathogenesis, including *Streptococcus*, *Enterococcus*, *Actinomycetes* and *Selemonas* [200,219]. Besides the preference of these bacteria to propagate in different areas of the tooth structure/mouth, genetic predisposition is an

important factor linked to pathogenesis of oral diseases [220,221]. However, it has been demonstrated that *Streptococcus mutans* are the most cariogenic organisms and play a decisive role in dental plaque formation and caries development [200,219]. Similarly, periodontal disease is associated with increased counts of *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* [63,70,200] and is propagated by an over-active local immune response and chronic inflammation [70,76]. Moreover, an increase in inflammatory cytokines such as TNF- $\alpha$  and IL6, accompanied with an increased Receptor activator of nuclear factor kappa-B ligand to Osteoprotegerin (RANKL:OPG) ratio is associated with periodontal disease. The current therapeutic approaches for oral disease focus on prevention strategies, such as maintaining oral hygiene by flossing and brushing, dental sealants, fluoride supplements and antibacterial mouthwashes [144]. With the onset of caries, treatment strategies entail the removal of infected tissue and the restoration of lost tooth structure [5]. As for periodontal disease, scaling, root planing, antibiotics and surgery limit disease progression [3]. Despite these therapeutic measures, the prevalence of oral disease is alarming. A novel long-term biotherapeutic capable of limiting the growth of cariogenic organisms, as well as modulating periodontal disease-associated inflammation is required.

Probiotics are “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” [137]. Lactobacilli and Bifidobacteria, natural commensals of the human gastrointestinal tract (GIT), are the most common probiotic genera [137] and have the potential to prevent and treat various health conditions including hypercholesterolemia, liver diseases, metabolic syndrome, allergy

and colon cancer [140-143,145,146,222]. Several researchers have investigated the potential of probiotic bacteria for the prevention and treatment of oral diseases [144]. A number of putative probiotic *Lactobacillus* strains have been shown to reduce the prevalence of both caries-causing *S. mutans* and periodontal disease-causing periodontopathogens [144,223]. The *Lactobacillus* strains selected for this study were chosen based on our previous study, where the strains proved capable of inhibiting both *S. mutans* and *Candida albicans* [223].

There are number of probiotic characteristics that may promote oral health [144,204]. We hypothesize that probiotic bacteria may compete for nutrients and/or buffer the oral pH towards a more neutral pH, reducing the numbers of opportunistic pathogens, may limit the progression of dental plaque by bacterial co-aggregation and by the production of antibacterial substances, such as bacteriocins and nitric oxide [144,224]. They may also adhere to the oral surfaces, outcompeting pathogen adhesion and proliferation. In addition, probiotic bacteria can possess anti-inflammatory properties of interest for the modulation of periodontal disease-associated inflammation. This study investigated probiotic *Lactobacillus* strains for their ability to inhibit *S. mutans* and their pH modulation, bacteriocin activity, sucrose utilization, co-aggregation with *S. mutans*, adhesion to gingival epithelial cells, NO production and antioxidant activity.

### **4.3 Materials and Methods**

#### **4.3.1 Bacterial growth media and chemicals**

De Man, Rogosa, Sharpe (MRS) broth was purchased from Fisher Scientific (Ottawa, ON, Canada). Rogosa agar was purchased from Sigma-Aldrich (Oakville, ON, Canada). All other chemicals were of HPLC or analytical grade and purchased from



commercial sources. Water was purified with an EasyPure reverse osmosis system and a NanoPure Diamond Life Science (UV/UF) ultrapure water system from Barnstead (Dubuque, IA, USA).

#### **4.3.2 Bacterial strains and culture conditions**

*Lactobacillus fermentum* NCIMB 5221, *L. fermentum* NCIMB 2797, *L. fermentum* NCIMB 8829, *Lactobacillus reuteri* NCIMB 701089, *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 702655, *L. reuteri* NCIMB 702656, *L. reuteri* NCIMB 11951 and *S. mutans* NCIMB 702062 isolated from carious dentine, were purchased from the National Collection of Industrial Food and Marine Bacteria (NCIMB) (Aberdeen, Scotland, UK). *Lactobacillus acidophilus* ATCC 314 was purchased from Cedarlane Laboratories (Burlington, ON, Canada). All of the bacterial strains were kept at -80 °C as 20% (v/v) glycerol stocks. Prior to any experiment, a MRS agar plate was streaked and incubated at 37 °C for 24 h with 5% CO<sub>2</sub> to ensure the purity of the culture. One colony from the MRS agar plate was inoculated in MRS broth for 24 h at 37 °C. A 1% (v/v) inoculum was subcultured into MRS broth and incubated at 37 °C for 24 h immediately prior to any assay.

#### **4.3.3 Determination of bacterial viability**

Selective agar was used to differentiate bacteria in mixed cultures. Rogosa agar was used for probiotic selectivity whereas mitis-sucrose-bacitracin (MSB) agar was used for *S. mutans* selectivity. Rogosa agar was prepared using the manufacturer's instructions. MSB agar was prepared according to previous work by Gold *et al.* [225]. The cell counts of the pathogens and probiotic bacteria were measured using standard colony forming units (cfu). Briefly, 0.85% (w/v) NaCl was used for 10-fold serial

dilutions and all samples were plated in triplicate to ensure accuracy and reproducibility. The plates were incubated at 37 °C and 5% CO<sub>2</sub> for 48 h prior to colony counting. Agar plates with all the tested *L. reuteri strains* were incubated using AnaeroGen gas bags from Sigma-Aldrich (Oakville, ON, Canada) on Rogosa agar for optimal growth.

#### **4.3.4 Quantification of probiotic inhibition of *S. mutans* in MRS broth and in simulated salivary fluid**

All the aforementioned *Lactobacillus* strains were tested for their ability to inhibit the growth of *S. mutans* NCIMB 702062. 1% (v/v) *S. mutans* and 5% (v/v) probiotic overnight cultures, corresponding to 10<sup>7</sup> cfu/ml of each *S. mutans*/probiotic strain, were used to inoculate MRS broth. The controls were pure cultures of all the tested probiotic strains and *S. mutans* NCIMB 702062 alone. The test and control samples were placed on an orbital shaker (Napco, Fisher Scientific, Suwanee, GA, USA) set at 75 rpm and 37°C, to simulate oral conditions. Bacterial counts were determined by standard colony counting methods using selective agar following 0 and 24 h of incubation. The experiment was performed in triplicate.

Simulated salivary fluid (SSF) formulation was adapted from previous work by Wong and Sissions [226]. **Table 4.1** presents the specific composition of SSF. All the aforementioned *Lactobacillus* strains were tested for their ability to inhibit *S. mutans* NCIMB 702062. Moreover, to evaluate the growth of the tested *Lactobacillus* strains in SSF and to ensure that *S. mutans* do not reduce *Lactobacillus* counts, the mixed cultures were plated on Rogosa agar following 24 h of incubation. The probiotic-*S. mutans* co-cultures were treated as with the inhibition assay in MRS broth. The controls were pure

cultures of all the tested probiotic strains and *S. mutans* NCIMB 702062 alone. The experiment was performed in triplicate.

**Table 4.1:** Formulation of simulated salivary fluid adapted from Wong *et al* [226]. Composition and their corresponding concentrations.

Component	Concentration	Component	Concentration
Alanine	50.0 µM	Leucine	25.0 µM
Arginine	50.0 µM	Lysine	50.0 µM
Ascorbic acid (Vitamin C)	5.0 µM	Menadione (Vitamin K <sub>3</sub> )	5.0 µM
Asparagine	25.0 µM	Methionine	10.0 µM
Aspartic acid	25.0 µM	MgCl <sub>2</sub>	0.2 mM
Biotin (Vitamin B <sub>7</sub> )	0.1 µM	Mucin from porcine	2.5 g/L
CaCl <sub>2</sub>	1.0 mM	NaCl	10.0 mM
Casein	5.0 g/L	NH <sub>4</sub> Cl	2.0 mM
Choline chloride	100.0 µM	Niacin (Vitamin B <sub>3</sub> )	5.0 µM
Citrate	50.0 µM	<i>p</i> -Aminobenzoic acid	1.0 µM
Creatinine	1.0 µM	Pantothenic acid (Vitamin B <sub>5</sub> )	1.0 µM
Cyanocobalamin (Vitamin B <sub>12</sub> )	0.05 µM	Phenylalanine	25.0 µM
Cysteine	50.0 µM	Proline	100.0 µM
Folic acid (Vitamin B <sub>9</sub> )	0.025 µM	Pyridoxine (Vitamin B <sub>6</sub> )	4.0 µM
Glutamic acid	25.0 µM	Riboflavin (Vitamin B <sub>2</sub> )	0.3 µM
Glutamine	25.0 µM	Serine	25.0 µM
Glycine	100.0 µM	Taurine	75.0 µM
Haemin	10 µM	Thiamine (Vitamin B <sub>1</sub> )	1.0 µM
Histidine	10.0 µM	Threonine	25.0 µM
Inositol	10.0 µM	Tryptophan	10.0 µM
Isoleucine	25.0 µM	Tyrosine	15.0 µM
K <sub>2</sub> HPO <sub>4</sub>	1.5 mM	Urea	1.0 mM
KCl	15.0 mM	Uric acid	50.0 µM
KH <sub>2</sub> PO <sub>4</sub>	3.5 mM	Valine	25.0 µM

#### **4.3.5 pH measurement of *Lactobacillus-S. mutans* cultures**

We investigated the capability of *Lactobacillus* probiotic strains to buffer the pH of *S. mutans* in SSF. All the aforementioned *Lactobacillus* strains were cultured with *S. mutans* NCIMB 702062 for 24 h at 37 °C and 75 rpm in SSF. The initial SSF pH was adjusted to a physiological oral pH of 6.7 [217]. Thereafter, the pH was recorded following 0 and 24 h of incubation using an Oakton digital pH meter from Fisher Scientific (Suwanee, GA, US). The pH of a pure culture of *S. mutans* was recorded as a control. The experiment was performed in triplicate.

#### **4.3.6 Determining the bacteriocin activity of probiotic strains**

Bacteriocin activity of the probiotic strains was tested. All the aforementioned *Lactobacillus* strains and *S. mutans* NCIMB 702062 were grown in MRS broth for 24 and 48 h at 37 °C. Following incubation, the bacterial supernatants containing the bacteriocin were extracted with a protocol by Branton *et al.* [139] To test the bacteriocin activity of the bacteria, 5% (v/v) ( $\sim 10^8$  cfu/ml) of the resulting probiotic supernatants were incubated with 0.5 % (v/v) ( $2.82 \times 10^7 \pm 0.07 \times 10^5$  cfu/ml) *S. mutans* in MRS for 24 h at 37°C and 75 rpm. *S. mutans* viability, following 24 h of incubation, was determined using standard cfu methods and MRS agar plates. The experiment was performed in triplicate.

#### **4.3.7 Determining sucrose consumption of the *Lactobacilli* and *S. mutans***

Sucrose consumption by the probiotic *Lactobacilli* and the *S. mutans* incubated in SSF was quantified using a dinitrosalicylic (DNS) colorimetric method, with modifications from previous work by Miller *et al.* and Miloski *et al.* [227,228]. Both *S. mutans* and probiotic bacterial cells were added separately to SSF at a concentration of

1% (v/v) ( $\sim 10^7$  cfu/ml) of overnight cultures in MRS of each *S. mutans*/probiotic strain, and incubated at 37 °C and 75 rpm for 24 h. The SSF at 0 h contained 5% (w/v) sucrose. Following 24 h of incubation, the bacterial suspensions in SSF were centrifuged at 4,000 rpm for 30 min at 4 °C. The supernatant was collected and filtered using a 0.22  $\mu$ m to measure the sucrose remaining in the SSF.

The assay consisted of two steps. Firstly, 1 ml of the bacterial supernatant containing sucrose was treated with 20  $\mu$ l of HCl (37% (w/v)) and incubated at 90 °C for 5 min to hydrolyze sucrose. 1 ml of sample was kept un-hydrolyzed. Following hydrolysis, 50  $\mu$ l of KOH (5 N) were added. For the DNS test, 1 ml of sample (hydrolyzed and un-hydrolyzed) and 1% DNS acid reagent solution was incubated for 15 min at 90 °C. Following incubation, 333  $\mu$ l of 40% (w/v) KNa tartrate were added. The resulting solution was cooled to room temperature and was measured spectrophotometrically at 570 nm (PerkinElmer 1420 Multilabel Counter). A standard curve was generated using sucrose solutions treated exactly the same way as the samples. The concentrations of sucrose used were 0.002, 0.009, 0.036, 0.14, 0.58, 2.34 and 9.37 mM ( $R^2 = 0.971$ ). The sucrose consumption in the SSF was calculated using the equation below (n=3):

$$\text{Total Sucrose consumption} = \text{Initial sucrose concentration} - (\text{Sucrose}_h - \text{Sucrose}_{uh})$$

where sucrose<sub>h</sub> is the concentration of the hydrolyzed sample and sucrose<sub>uh</sub> is the concentration of the unhydrolyzed sample. Both hydrolyzed and unhydrolyzed samples were measured to remove any glucose and fructose molecules from the bacterial-SSF samples.

#### **4.3.8 Determining the co-aggregation of *S. mutans* with probiotic bacteria**

The ability of the *Lactobacillus* strains to co-aggregate with *S. mutans* NCIMB 702062 was determined using a method adapted from Collado *et al.* [229]. Co-cultures of all the aforementioned *Lactobacillus* strains were incubated with *S. mutans* NCIMB 702062 for 24 h at 37°C. Briefly, the overnight bacterial cultures were pelleted by centrifugation at 4,000 rpm for 5 min at 4 °C. The washed pellets were suspended in 0.85% (w/v) NaCl. The turbidity of the re-suspended cells was determined using a spectrophotometer set at 620 nm and was normalized to 0.7 to ensure equal bacterial densities at the start of the assay. Following dilutions, 1.5 ml of each probiotic suspension was added to 1.5 ml of *S. mutans* NCIMB 702062 and 3 ml of 0.85% (w/v) saline, for a final volume of 6 ml. The bacterial suspensions were incubated for 24 h at 37 °C without shaking and absorbance measured at 620 nm. Values for co-aggregation were calculated using the following formula (n=3):

$$\% \text{ co-aggregation} = [(OD_0 - OD_{24}) / OD_0] \times 100$$

where OD<sub>0</sub> is the absorbance of the mixed bacterial solution at 0 h and OD<sub>24</sub> represents the absorbance of the mixed bacterial solution at 24 h.

#### **4.3.9 Determining bacterial adhesion to human gingival epithelial cells**

Probiotic bacterial cells and *S. mutans* NCIMB 702062 attachment to human gingival keratinocytes (Tuija), generously gifted by Dr. Hannu Larjava (University of British Columbia, Canada). was quantified using a protocol adapted from work by Grootaert *et al.* [230]. Briefly, Tuija cells were seeded at a concentration of 10<sup>5</sup> cells/ml in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10 % fetal bovine serum (FBS) (Invitrogen) and incubated at 37 °C and 5% CO<sub>2</sub> for 48 h.

Following incubation, the Tuija cells were washed twice with phosphate buffered solution (PBS). Overnight cultures of each bacterial strain were centrifuged at 4000 rpm for 5 min and washed twice using PBS. The bacterial pellets were re-suspended in 10 ml DMEM. 500 µl bacterial suspensions containing  $\sim 2 \times 10^7$  cfu were added to the Tuija cells ( $2 \times 10^4$  cells). The exact cell counts of each treatment at 0 h is graphically represented in Figure. 5A. The treated cells were incubated for 6 h at 37 °C and 5% CO<sub>2</sub> to allow probiotic cell attachment. Following incubation, the Tuija cells were washed with PBS three times to remove all non-adherent bacteria. 500 µl of 0.1% Triton X-100 (v/v) were added to each well. The Tuija-probiotic cell suspensions were then incubated at 25 °C on a rotary shaker (LW210, LW Scientific, Georgia, USA) set at 150 rpm to induce Tuija cell lysis and detachment. The suspension containing the Tuija cells and adhered bacterial cells were then collected and the bacterial viability (bacterial attachment) was determined ( $T_{6h}$ ) by standard colony counting methods using MRS agar plates. The viability of the initial bacterial cell suspension was determined ( $T_{0h}$ ). The MRS agar plates were incubated for 48 h at 37 °C and 5% CO<sub>2</sub>. The percent bacterial attachment was then calculated for each bacteria using the following formula ( $n = 3$ ):

$$\% \text{ bacterial attachment} = (T_{6h} / T_{0h}) \times 100$$

#### **4.3.10 Quantification of NO production by *Lactobacilli* and *S. mutans***

We investigated the NO bacterial production for all the aforementioned *Lactobacillus* strains and *S. mutans* NCIMB 702062. 1% (v/v) ( $10^7$  cfu/ml) of overnight cultures was used to inoculate SSF and the bacterial suspensions were incubated at 37 °C and 75 rpm. Following 24 h of incubation, the cultures were centrifuged at 4000 rpm for 5 min at 4 °C. The supernatant was then filtered using a 0.22 µm filter to remove any

residual bacterial cells. The bacterial supernatant were processed for NO production using the Griess assay (n = 3). A standard curve was generated using sodium nitrite following the same method. The concentrations of sodium nitrite used were 1.46, 2.92, 5.85, 11.71, 23.43, 46.87, 93.75, 187.5, 375 and 750  $\mu\text{M}$  ( $R^2 = 0.990$ ).

#### **4.3.11 Determination of total antioxidant activity**

We investigated antioxidant production by the probiotic *Lactobacillus* strains using a QuantiChrom™ Antioxidant Assay Kit. SSF was inoculated with 1% (v/v) overnight culture of the aforementioned *Lactobacillus* strains and *S. mutans* NCIMB 702062. The cultures were incubated for 24 h at 75 rpm and 37 °C. The bacterial supernatant was collected by centrifugation at 4,000 rpm for 5 min at 4 °C for antioxidant measurement. The total antioxidant production by each strain was quantified following the protocol provided with the assay kit (n=3). A standard curve was generated using Trolox and the same method at concentrations of 100, 300, 600 and 1000  $\mu\text{M}$  ( $R^2 = 0.980$ ).

#### **4.3.12 Statistical analysis**

Experimental results are expressed as mean  $\pm$  standard error of the mean (SEM). Statistical analysis was carried out using Statistical Product and Service Solutions (SPSS) Version 17.0 (IBM Corporation, New York, NY, USA). Comparison of the means was performed using the general linear model and Tukey's post-hoc analysis. Statistical significance was set at  $p < 0.05$  and  $p$  -values less than 0.01 were considered highly significant.

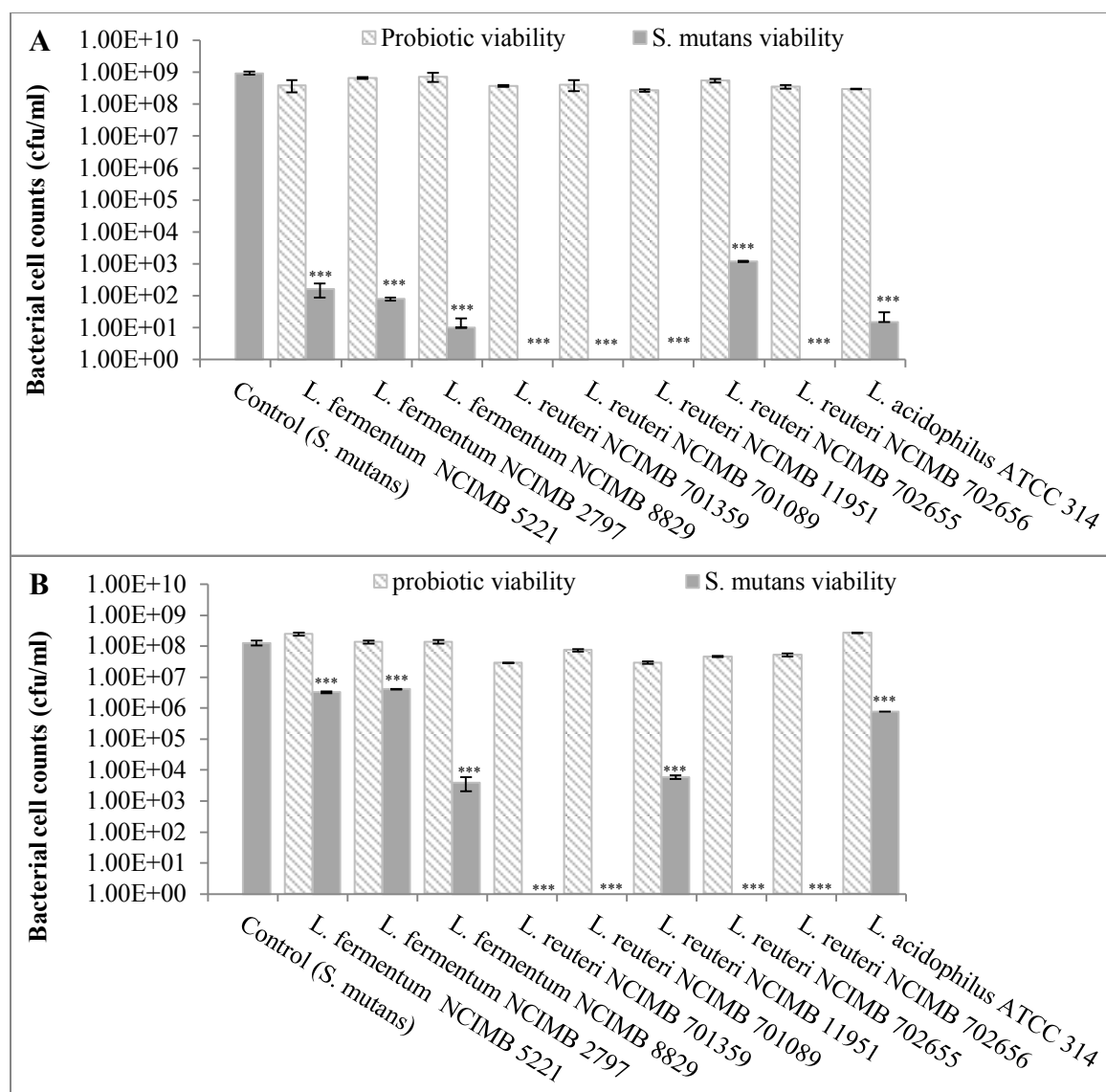


## 4.4 Results

### 4.4.1 Inhibition of *S. mutans* by probiotic live cells and probiotic bacteriocins

The initial viability of *S. mutans* alone (control) was  $5.63 \times 10^7 \pm 0.198 \times 10^7$  cfu/ml and  $9.35 \times 10^8 \pm 0.148 \times 10^8$  cfu/ml following 24 h. All the tested probiotic strains demonstrated significant inhibition of *S. mutans* NCIMB 702062 in MRS broth after 24 h of incubation ( $p < 0.001$ ). *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 702656 and *L. reuteri* NCIMB 11951 demonstrated the greatest inhibition of *S. mutans* amongst all of the tested probiotic bacteria with no detectable viable *S. mutans* cells ( $< 10$  cfu/ml) (**Figure.4.1A**). *L. fermentum* strains demonstrated significant inhibition but with greater counts of *S. mutans* remaining than the *L. reuteri* strains, suggesting a weaker inhibition by these strains.

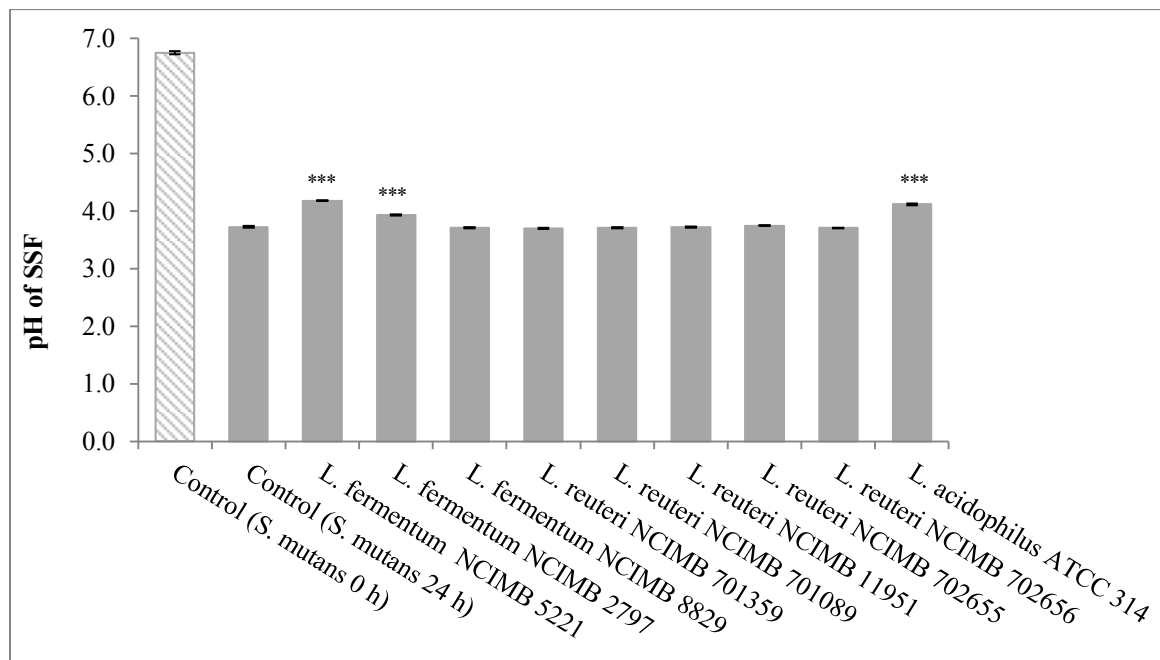
To replicate more closely oral conditions, the inhibition assay was repeated in SSF. Similar to the results in MRS, all the tested probiotic strains demonstrated significant inhibition of *S. mutans* NCIMB 702062 in SSF ( $p < 0.001$ ). The best inhibitors of *S. mutans* in SSF were *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 702656 and *L. reuteri* NCIMB 702655 with no detectable viable *S. mutans* cells remaining ( $< 10$  cfu/ml) (**Figure.4.1B**). The viability of *S. mutans* was  $2.26 \times 10^7 \pm 0.11 \times 10^7$  cfu/ml at the start of the assay. Following 24 h incubation, the viability of the pure *S. mutans* culture was  $1.29 \times 10^8 \pm 0.12 \times 10^8$  cfu/ml. The effect of probiotic bacteriocins extracted from 24 h and 48 h cultures were evaluated, for their ability to inhibit *S. mutans*. Successful bacteriocin inhibition of *S. mutans* was not observed by any of the probiotic strains (data not shown).



**Fig.4.1:** Inhibition of *S. mutans* NCIMB 702062 by probiotic bacteria following 24 h of incubation at 37°C and 75 rpm. (A) Bacterial cell viability at 0-24 h in MRS broth and (B) bacterial cell viability at 24 h in SSF. A pure culture of *S. mutans* without any probiotic bacteria was used as negative control. Data is presented as means of triplicate samples  $\pm$  SEM (\*\*\*)  $p < 0.001$ .

#### 4.4.2 pH modulation by probiotic bacteria

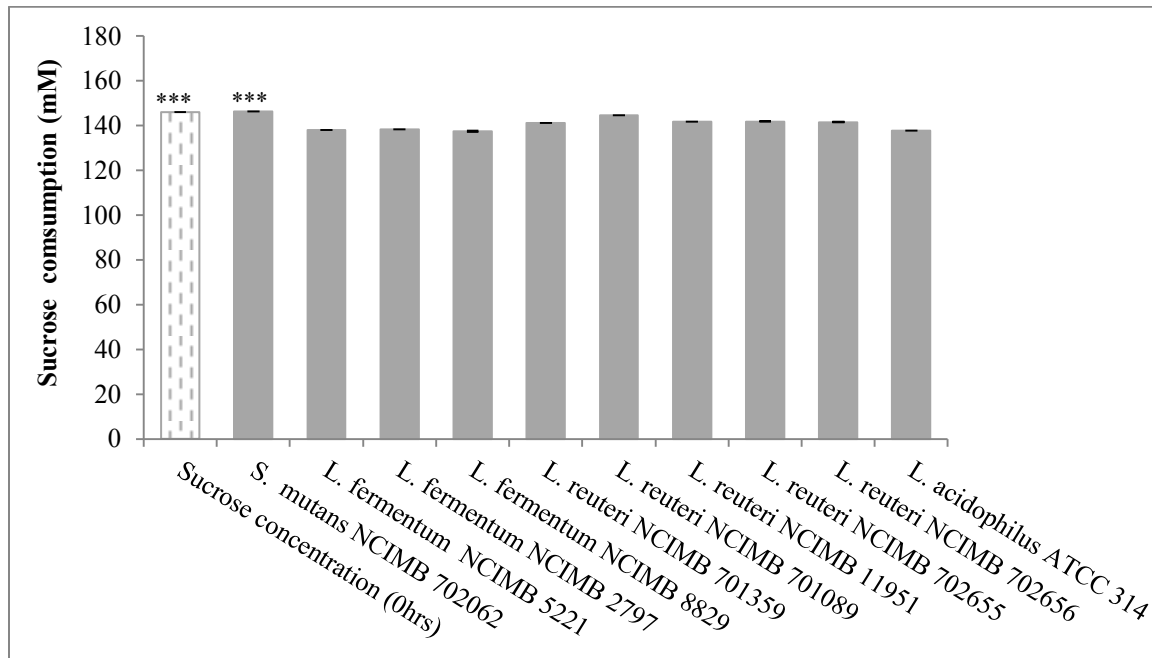
The pH of SSF for *S. mutans* without any probiotic was  $3.72 \pm 0.017$ . The pH of SSF for the *S. mutans* cultures with probiotic bacteria ranged from  $3.70 \pm 0.012$  to  $4.18 \pm 0.005$ , following 24 h of incubation, (**Figure.4.2**). The pH of SSF was significantly increased when *L. fermentum* NCIMB 5221 ( $4.18 \pm 0.005$ ), *L. fermentum* NCIMB 2797 ( $3.93 \pm 0.012$ ) and *L. acidophilus* ATCC 314 ( $4.12 \pm 0.018$ ) were grown with *S. mutans* ( $p < 0.001$ ).



**Fig.4.2:** Effect of probiotic *Lactobacillus* strains co-cultured with *Streptococcus mutans* on the pH of simulated salivary fluid after 24 h at 37°C and 75 rpm. A pure culture of *S. mutans* was used as control. Data is presented as means of triplicate samples  $\pm$  standard error of the mean (\*\*\* $p < 0.001$ ).

#### 4.4.3 Sucrose consumption by probiotic bacteria

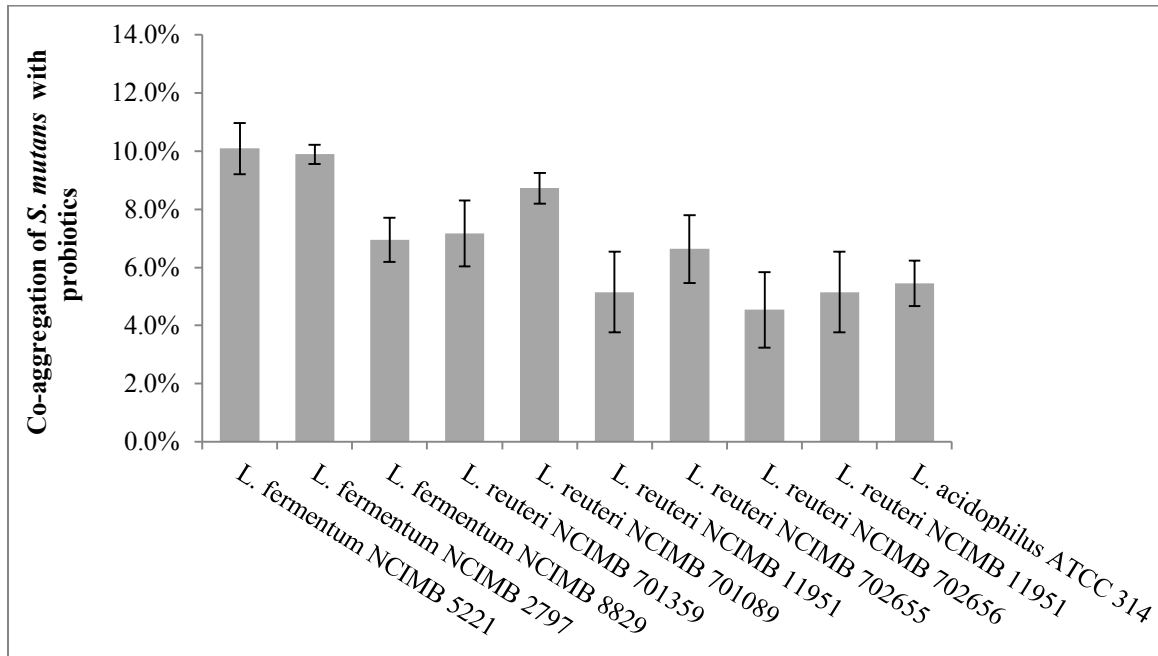
Consumption of sucrose by probiotic bacteria could prove beneficial for *S. mutans* inhibition, via competitive inhibition for nutrients. The sucrose consumption of *S. mutans* NCIMB 702062 was  $146.22 \pm 0.01$  mM, following 24 h of incubation in SSF (Figure.4.3). *S. mutans* consumed significantly more sucrose than all of the probiotic strains ( $p < 0.001$ ). *L. reuteri* NCIMB 701089 consumed the most sucrose ( $144.53 \pm 0.01$  mM) while *L. fermentum* NCIMB 8829 consumed the least ( $137.51 \pm 0.31$  mM), amongst all the tested probiotics.



**Fig.4.3:** Sucrose consumption by *S. mutans* and probiotic strains incubated for 24 h in simulated salivary fluid at 37 °C and 75 rpm, quantified by a colorimetric biochemical reaction. Data is presented as means of triplicate samples  $\pm$  SEM (\*\*\*)  $p < 0.001$ ).

#### 4.4.4 Probiotic co-aggregation of *S. mutans*

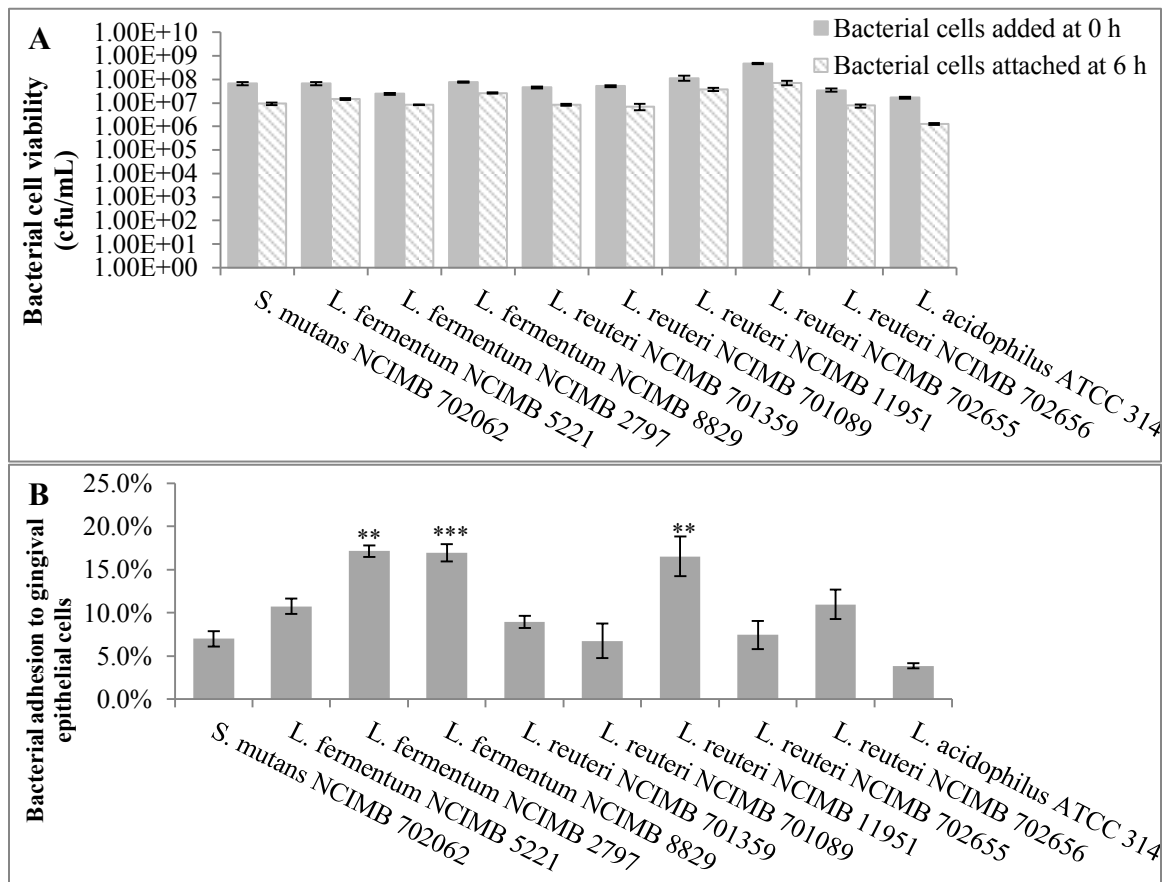
Co-aggregation of each probiotic strain with *S. mutans* was tested. *L. fermentum* NCIMB 5221 demonstrated the highest co-aggregation of *S. mutans* ( $11.24 \pm 1.09$  %) (Figure. 4.4). On the other hand, *L. reuteri* NCIMB 702656 demonstrated the least co-aggregation ( $4.80 \pm 1.43$  %). There was no significant difference in co-aggregation activity between any of the tested probiotics strains ( $p > 0.05$ ).



**Fig.4.4:** Percent co-aggregation of the tested probiotic strains with *S. mutans* in 0.85% (w/v) saline solution for 24 h at 37°C without shaking. Data is presented as means of triplicate samples  $\pm$  SEM (\*\*\*)  $p < 0.001$ ).

#### 4.4.5 Bacterial adhesion to human gingival epithelial cells

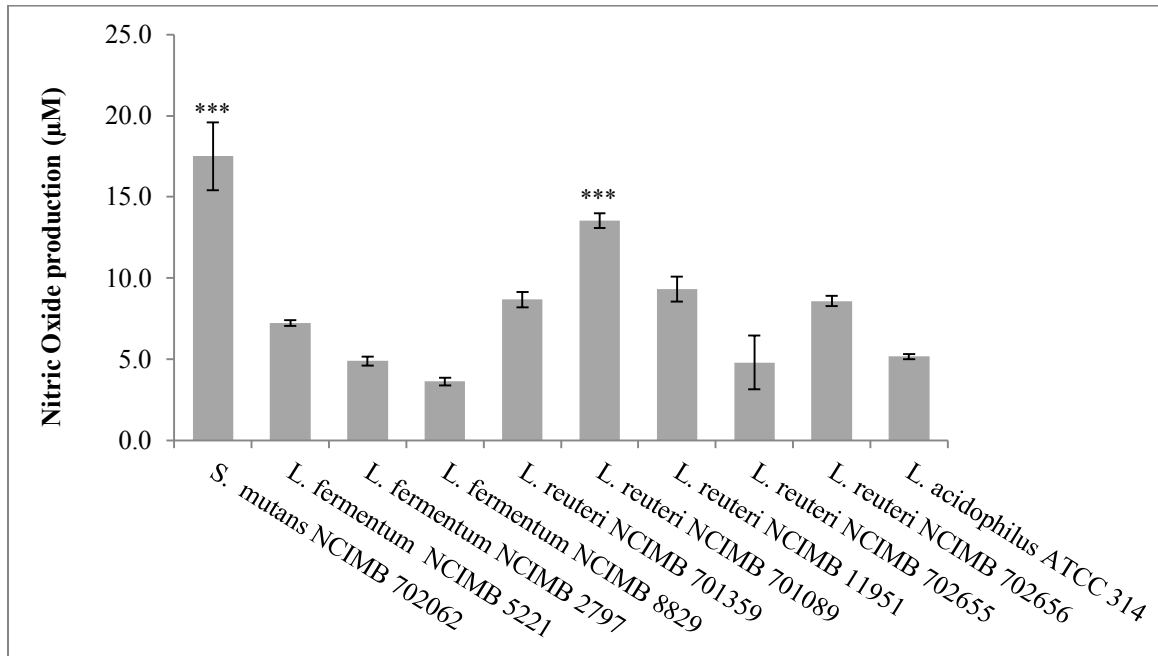
*S. mutans* significantly attached to the gingival epithelial cells ( $7.00 \pm 0.009$  %). The probiotic attachment ability ranged from  $4.00 \pm 0.003$  % for *L. acidophilus* ATCC 314 to  $17.00 \pm 0.006$  % and  $17.00 \pm 0.009$  % for *L. fermentum* NCIMB 2797 and *L. fermentum* NCIMB 8829, respectively. *L. fermentum* NCIMB 2797 ( $p = 0.002$ ), *L. fermentum* NCIMB 8829 ( $p = 0.001$ ) and *L. reuteri* NCIMB 11951 ( $p = 0.003$ ) demonstrated the highest bacterial adhesion compared to all the other tested probiotic strains to the pathogenic *S. mutans* (Figure.4.5B).



**Fig.4.5:** Adhesion of *Streptococcus mutans* and *lactobacillus* strains to epithelial keratinocytes (Tuija cells). (A) Viable bacterial cells counts at 0 h and at 6 h, (B) Percentage bacterial adhesion to Tuija cells at 6 h. Data is presented as means of triplicate samples  $\pm$  SEM (\*\* $p < 0.01$  and \*\*\* $p < 0.001$ ).

#### 4.4.6 Bacterial NO production

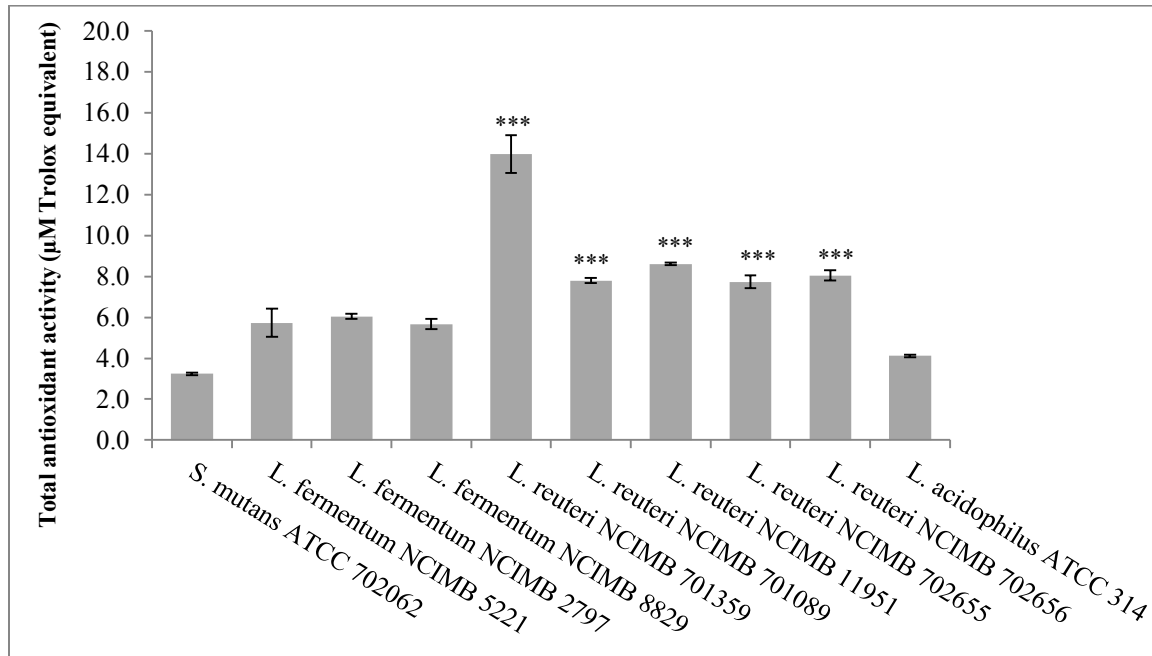
*S. mutans* NCIMB 702062 produced  $17.50 \pm 2.09$   $\mu\text{M}$  of NO following 24 h of incubation in SSF. Surprisingly, all the probiotic strains produced significantly less NO than *S. mutans*. *L. reuteri* NCIMB 701089 was the highest NO producers in terms of the probiotic strains ( $13.54 \pm 0.44$   $\mu\text{M}$  of NO) ( $p < 0.001$ ) and *L. fermentum* NCIMB 8829 produced the lowest levels of NO ( $3.63 \pm 0.23$   $\mu\text{M}$ ) (**Figure. 4.6**).



**Fig.4.6:** Nitric oxide production by the *Streptococcus mutans* and probiotic *Lactobacillus* strains following 24 h of incubation in simulated salivary fluid at 37°C and 75 rpm. Data is presented as means of triplicate samples  $\pm$  SEM (\*\*\*)  $p < 0.001$ ).

#### 4.4.7 Bacterial antioxidant activity

*S. mutans* demonstrated the least antioxidant activity of  $3.24 \pm 0.62$   $\mu\text{M}$  Trolox equivalents. The probiotic strain, *L. reuteri* NCIMB 701359 demonstrated the greatest antioxidant activity of  $13.99 \pm 0.94$   $\mu\text{M}$  Trolox equivalents (**Figure. 4.7**). All the *L. reuteri* strains demonstrated statistically significantly ( $p < 0.001$ ) higher antioxidant activity compared to the other tested bacterial strains.



**Fig.4.7:** The total antioxidant activity of *S. mutans* and the probiotic strains in simulated salivary fluid following 24 h of incubation at 37°C and 75 rpm. Data is presented as means of triplicate samples  $\pm$  SEM (\*\*\*)  $p < 0.001$ ).



## 4.5 Discussion

Dental caries and periodontal disease are the most prevalent oral diseases and pose important economic burdens [1]. Recent research has demonstrated the potential of probiotic biotherapeutics for the prevention and treatment of oral diseases but a successful probiotic formulation has yet to be developed. Our group has previously identified eleven putative probiotic bacteria that have the potential to inhibit the causative organisms of caries and oral candidiasis using a qualitative plate assay [144,223]. We selected nine putative probiotic strains for this study, as they demonstrated superior *S. mutans* inhibition. These were further investigated for the inhibition of *S. mutans* under simulated oral conditions. These strains were also characterized for other oral health benefits.

Putative probiotic bacteria were selected from the previous work in chapter 3. As MRS broth allows optimal growth of the probiotic strains, we first evaluated the probiotic inhibition of *S. mutans* in this media. *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 11951 and *L. reuteri* NCIMB 702656 demonstrated the highest *S. mutans* inhibition, with almost complete inhibition of *S. mutans* growth ( $< 10$  cfu/ml following 24 h) (**Figure. 4.1A**). Successful probiotic inhibition of *S. mutans* was confirmed under simulated oral conditions. *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 702655 and *L. reuteri* NCIMB 702656 lowered *S. mutans* viability to non-detectable levels ( $< 10$  cfu/ml after 24 h) (**Figure. 4.1B**). This is the first study to identify probiotic strains capable of inhibiting *S. mutans* under *in vitro* simulated oral conditions.

It has been well-documented that a lowering of pH increases acidogenic/aciduric organisms, such as *S. mutans*, associated with dental caries initiation/progression [63,231]. Probiotic bacteria, if successfully inhibiting *S. mutans*, may allow for a relatively less acidic pH when co-cultured with *S. mutans*. *Lactobacillus* strains also reduce pH, but the results demonstrated that when co-cultured with *S. mutans*, they decreased pH to a lesser degree than *S. mutans*, despite the probiotic production of lactic acid (**Figure.4.2**). We observed that the growth of *Lactobacillus* strains reached stationary phase in SSF at 24 h, at which point pH was evaluated. Future studies may investigate pH change over time. With the results that, *L. fermentum* NCIMB 5221, *L. fermentum* NCIMB 2797 and *L. acidophilus* ATCC 314 significantly ( $p < 0.001$ ) buffered the *S. mutans*-mediated acidic pH. We suggest that, although pH modulation via the tested probiotic bacteria would not significantly affect *S. mutans* viability, higher pH generated by the probiotic–pathogen co-cultures may slow the process of bone resorption, otherwise enhanced by the presence of acids, described in a recent review [232].

Interestingly, previous research has demonstrated that cell-free probiotic supernatant is effective at reducing oral biofilms likely via bacteriocin activity [233]. Bacteriocins are proteins with antibacterial properties extracted from bacteria, including reuterin, mutacin and nisin [139,173,234,235]. Bacteriocin production is associated with the exponential growth phase of bacteria [236]. We used 24-48 h cultures for bacteriocin extraction, to allow accumulation of bacteriocin produced during that total period. A pathogen inhibition would be observed regardless of the growth phase from which the bacteriocin were extracted from the culture, unless they were present in inadequate amounts or were disintegrated during the process of extraction. In our study, bacteriocin

extracted from the probiotic strains was unable to inhibit *S. mutans* (data not shown). More importantly, the extracted anti-microbial compound could be concentrated using methods such as freeze drying, in order to observe pathogen inhibitory effects. Despite these results, future studies should investigate probiotic bacteriocin activity using other oral pathogens including the red complex, important in the pathogenesis of periodontal disease.

It is well-documented that sucrose is the preferred carbohydrate source of *S. mutans* and its metabolism leads to acid production and tooth decay [237]. Hence, we investigated whether the selected putative probiotic bacteria could outcompete *S. mutans* for sucrose, promoting its inhibition via nutrient competition. The results demonstrate that *S. mutans* consumes statistically higher sucrose than the probiotic bacteria tested (**Figure. 4.3**). Even though nutrient competition does not appear to be the primary mode of inhibition, the probiotic bacteria may, nonetheless, reduce the availability of sucrose for *S. mutans*, contributing to its inhibition. This is the first study investigating probiotic sucrose consumption as a method of *S. mutans* inhibition. A method that would distinguish sucrose consumption by each bacterium in a mixed culture would provide a better understanding of nutrient competition as a mechanism of action.

An interesting phenomenon, co-aggregation, enable probiotic binding to *S. mutans* and the formation of masses/aggregates of bacteria, preventing *S. mutans* from binding to surfaces where it could proliferate to form oral biofilm, termed dental plaque [229]. A similar study was performed with other probiotic and *S. mutans* strains [238]. In this work *L. fermentum* NCIMB 5221 demonstrated the highest percent co-aggregation amongst all the tested probiotic strains (**Figure. 4.4**). However, all of the tested probiotic

bacteria expressed some potential for co-aggregation with *S. mutans*. Importantly, co-aggregation is not a mechanism relevant for the inhibition of *S. mutans* seen in the *in vitro* assay since the colonization of the pathogen was not adhesion-dependent [110]. Co-aggregation may prove of greater importance *in vivo*.

Both caries and periodontal disease pathogens reside and thrive in the biofilms that form on the oral surfaces such as gingiva, teeth and tongue [239]. It is important to note that most oral surfaces are composed of non-keratinized oral epithelium [240,241]. However, gingiva, hard palate and the dorsal surface of the tongue are composed of keratinized oral epithelium [240,241]. As gingiva is the most proximal tissue around the tooth, we hypothesize that probiotic modulation of the microbial community of the biofilm on gingiva would impact pathogen biofilm formation and potentially indicate pathogen displacement on other oral surfaces. This is the first study that evaluates and demonstrates the adhesion of probiotic bacteria to oral epithelial cells. *L. fermentum* NCIMB 2797, *L. fermentum* NCIMB 8829 and *L. reuteri* NCIMB 11951 had significantly superior affinity for Tuija cells than *S. mutans* (and the other tested probiotic strains) (**Figure. 4.5B**). Based on these results, we hypothesize that a higher affinity of the probiotic cells to adhere to the oral surfaces when compared to oral pathogens, would enable the modulation of the microbial community in the biofilm, eliminating pathogen growth. In a recent investigation, Gungor *et al.* reiterated the fact that probiotic bacteria reduce *S. mutans* counts on tooth surfaces, potentially through co-aggregation or competition for adhesion sites [242]. Hence, investigations to assess effects on both soft and hard tissue, alongwith cumulative effects of both pathogen and probiotic bacteria is imperative.

NO is known both for its antibacterial [175] and anti-inflammatory properties [243]. Probiotic bacteria have been shown to produce enough NO to inhibit bacterial pathogens [244] and, when produced in low levels, can reduce inflammation [243]. We hypothesize that a probiotic that would produce NO would be efficient in both inhibiting oral pathogens and reducing gingival/periodontal inflammation. We thus investigated whether the probiotic bacteria in our study could produce NO. The tested probiotic bacteria produced NO, but *S. mutans* produced the greatest levels of NO, suggesting a non-NO dependent inhibition of *S. mutans* (**Figure.4.6**). Further investigations are required to evaluate the anti-inflammatory properties of probiotic-produced NO on oral tissues, specifically in the context of periodontal disease.

In terms of probiotic anti-inflammatory properties several research groups have demonstrated that antioxidants have the ability to reduce inflammation via different mechanisms such as combatting reactive oxygen species (ROS) like superoxide produced as a result of inflammatory responses in periodontal disease. [245,246]. These molecules have ability to breakdown the extracellular matrix of connective tissue leading to the destruction of periodontal tissue, discussed in a review by Waddington *et al.*[247]. Certain probiotic bacteria such as *L. fermentum* NCIMB 5221 have the ability to produce antioxidants such as ferulic acid [174]. Interestingly, we observed that the *L. reuteri* strains demonstrated the greatest antioxidant activity (**Figure. 4.7**). This is the first study to propose probiotic antioxidants as oral health biotherapeutics.

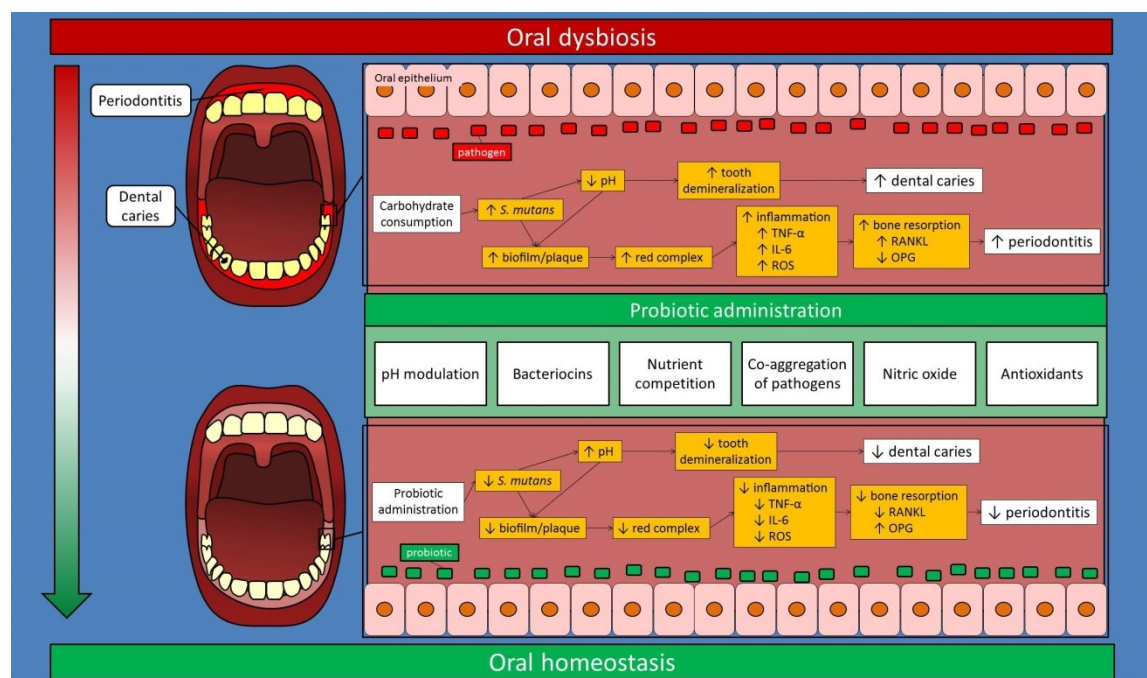
This work investigated nine Lactobacilli strains for the inhibition of *S. mutans* and oral health promoting properties. We demonstrated that *L. reuteri* strains were the best inhibitors of *S. mutans*. The probiotic strains grown with *S. mutans* had higher/equal pH,

potentially beneficial for limiting bone resorption. The investigated probiotic bacteria did not demonstrate bacteriocin activity against *S. mutans*. All the tested probiotic strains demonstrated high sucrose utilization beneficial for limiting the growth of *S. mutans*. The tested probiotic strains were also able to demonstrate their ability to co-aggregate with *S. mutans*, with *L. fermentum* NCIMB 5221 being the best, indicating their capability to facilitate the clearance of *S. mutans* from the mouth. All the tested probiotic strains had the capability to adhere to the gingival epithelial cells that would prove beneficial for limiting the growth of oral biofilm. NO production by the tested probiotic strains was lower than *S. mutans* and hence is not responsible for *S. mutans* inhibition. Lastly, all the tested probiotic strains exhibited higher total antioxidant activity than *S. mutans*, with the *L. reuteri* strains being the best strains producing antioxidants. **Table 4.2** is a schematic representation of investigated probiotic characteristics. In the presented work, the *Lactobacillus* strains were compared to *S. mutans*, the pathogen of interest. Experiments could have been designed with a non-probiotic strain as a control, to ensure probiotic specific effects. In this work *S. mutans* was used as a control since it is the dental caries-causing pathogen of interest that we aim to inhibit. In addition, several pathogens such as *Aggregatibacter actinomycetemcomitans* and the red complex are associated with periodontitis. It would be interesting to evaluate the inhibitory capabilities of probiotic bacteria with regards periodontopathogens. This will facilitate the probiotic mediated inhibition of both dental caries and periodontal disease causing organisms.

**Table 4.2:** Probiotic bacteria tested positive for oral health promoting characteristics represented in descending order. Grouped probiotic bacterial strains demonstrated similar effects for the tested characteristic.

pH Buffering	Sucrose Consumption	% Co-aggregation	Adhesion to epithelial keratinocytes	NO production	Antioxidant activity
<i>L. fermentum</i> NCIMB 5221	<i>L. reuteri</i> NCIMB 701089	<i>L. fermentum</i> NCIMB 5221	<i>L. fermentum</i> NCIMB 2797	<i>L. reuteri</i> NCIMB 701089	<i>L. reuteri</i> NCIMB 701359
<i>L. acidophilus</i> ATCC 314	<i>L. reuteri</i> NCIMB 702655	<i>L. fermentum</i> NCIMB 2797	<i>L. fermentum</i> NCIMB 8829	<i>L. reuteri</i> NCIMB 11951	<i>L. reuteri</i> NCIMB 11951
<i>L. fermentum</i> NCIMB 2797	<i>L. reuteri</i> NCIMB 11951	<i>L. reuteri</i> NCIMB 701089	<i>L. reuteri</i> NCIMB 11951	<i>L. reuteri</i> NCIMB 701359	<i>L. reuteri</i> NCIMB 702656
<i>L. reuteri</i> NCIMB 702655	<i>L. reuteri</i> NCIMB 702656	<i>L. reuteri</i> NCIMB 701359	<i>L. fermentum</i> NCIMB 5221	<i>L. reuteri</i> NCIMB 702656	<i>L. reuteri</i> NCIMB 701089
<i>L. reuteri</i> NCIMB 11951	<i>L. reuteri</i> NCIMB 701359	<i>L. fermentum</i> NCIMB 8829	<i>L. reuteri</i> NCIMB 702656	<i>L. fermentum</i> NCIMB 5221	<i>L. reuteri</i> NCIMB 702655
<i>L. reuteri</i> NCIMB 701089	<i>L. fermentum</i> NCIMB 2797	<i>L. reuteri</i> NCIMB 702655	<i>L. reuteri</i> NCIMB 701359	<i>L. acidophilus</i> ATCC 314	<i>L. fermentum</i> NCIMB 2797
<i>L. fermentum</i> NCIMB 8829	<i>L. fermentum</i> NCIMB 5221	<i>L. acidophilus</i> ATCC 314	<i>L. reuteri</i> NCIMB 702655	<i>L. fermentum</i> NCIMB 2797	<i>L. fermentum</i> NCIMB 5221
<i>L. reuteri</i> NCIMB 702656	<i>L. acidophilus</i> ATCC 314	<i>L. reuteri</i> NCIMB 11951	<i>L. reuteri</i> NCIMB 701089	<i>L. reuteri</i> NCIMB 702655	<i>L. fermentum</i> NCIMB 8829
<i>L. reuteri</i> NCIMB 701359	<i>L. fermentum</i> NCIMB 8829	<i>L. reuteri</i> NCIMB 702656	<i>L. acidophilus</i> ATCC 314	<i>L. fermentum</i> NCIMB 8829	<i>L. acidophilus</i> ATCC 314

In **Figure 4.8** we propose potential mechanisms by which an oral probiotic can potentially influence oral health. Ideally, a probiotic strain would possess, if not all, most of these biotherapeutic characteristics. Whether a single probiotic characteristic would aid in the prevention and treatment of oral diseases remains questionable as a number of properties are important and hence a probiotic cocktail formulation may be more beneficial. Further *in vivo* investigations are needed to investigate both the efficacy of probiotic bacteria for oral diseases and their mechanism(s) of action. This research is the first to identify probiotic bacteria for oral health applications based on thorough and comprehensive investigations into probiotic characteristics. These investigations should prove beneficial for the development and optimization of a successful probiotic biotherapeutic for the prevention and treatment of oral diseases.



**Fig.4.8:** The modes of action of probiotic bacterial cells that can potentially modulate oral microbiota and establish oral homeostasis for developing oral health biotherapeutics.

## 4.6 Acknowledgments

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#### **4.7 Conflict of Interest**

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

## CHAPTER 5: PROBIOTIC HYDROGEN PEROXIDE PRODUCTION INHIBITS *STREPTOCOCCUS MUTANS*: POTENTIAL DENTAL CARIES THERAPEUTIC

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**Preface:** In previous chapters we established that *L. reuteri* bacteria are superior *S. mutans* inhibitors. Additionally, H<sub>2</sub>O<sub>2</sub> is a well-known anti-bacterial compound. The aim of this study was to screen the selected *Lactobacillus* strains for hydrogen peroxide production. Both qualitative and quantitative colorimetric analyses were performed using trimethyl benzidine and horseradish peroxidase that determines H<sub>2</sub>O<sub>2</sub> production by the probiotic bacteria. Catalase that can destroy the H<sub>2</sub>O<sub>2</sub> produced was added with probiotic to prove that probiotic H<sub>2</sub>O<sub>2</sub> production inhibits *S. mutans*.

## 5.1 Abstract

Dental caries is the most common oral disease and its prevalence is on a rise. *Lactobacillus* probiotic bacteria can inhibit the growth of *Streptococcus mutans*, implicated in dental caries and considered to be the primary causative organism. The goal of the presented study was to screen probiotic bacteria for H<sub>2</sub>O<sub>2</sub> production and investigate their role to inhibit *S. mutans*. The results of qualitative H<sub>2</sub>O<sub>2</sub> screening demonstrated that all the tested *L. reuteri* strains and *L. acidophilus* ATCC 314 were H<sub>2</sub>O<sub>2</sub> producers. *L. reuteri* NCIMB 701359 was the highest H<sub>2</sub>O<sub>2</sub> producer among the tested strains (6379.45±31.28 µM H<sub>2</sub>O<sub>2</sub>). Interestingly, *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 702655 and *L. reuteri* NCIMB 702656 were the best *S. mutans* inhibitors with no viable *S. mutans* cells detectable following 24 h (< 10 cells/mL). The H<sub>2</sub>O<sub>2</sub> concentration, inhibitory for *S. mutans* was determined to be more than 1.3 µM. There was a notable co-relation between the best *S. mutans* inhibitors and the H<sub>2</sub>O<sub>2</sub> producers. The addition of catalase, an H<sub>2</sub>O<sub>2</sub> reducer, demonstrated reduced *S. mutans* inhibition by the four *L. reuteri* strains. Further studies are required to understand this mechanism completely. This study suggests that probiotic H<sub>2</sub>O<sub>2</sub> production is responsible for *S. mutans* inhibition with the potential of developing tested *L. reuteri* strains as dental caries biotherapeutics.

## 5.2 Introduction

Dental caries is the most prevalent oral disease and is characterized by the destruction of the hard surfaces of the teeth such as enamel and dentin. As reported by the World Health Organization (WHO), dental caries has a worldwide prevalence of almost 100% for adults and 60-90% for school children [216]. *Streptococcus mutans* is the

primary causative organism of dental caries [200,219]. The complications of dental caries include severe tooth pain, osteomyelitis and cellulitis [5]. In addition, *S. mutans* has been associated with infective endocarditis, a potentially fatal condition [214]. The etiology of dental caries is complex and is associated with multiple factors. Briefly, initiation of dental caries is due to the development of salivary pellicle on the tooth surfaces. Salivary pellicle originates from glycoproteins present in the saliva and enables the attachment of dental plaque on oral surfaces. Dental plaque, also known as oral biofilm, contains a diversity of microorganisms and elevated levels of exopolysaccharides. The microbial communities residing in the biofilm and the mouth are usually in homeostasis with the oral environment, but environmental changes initiated by poor oral hygiene, frequent intake of dietary carbohydrate and xerostomia leads to excess acid production [63,248]. Saliva acts as a buffer by providing ions such as calcium and fluoride but with increased carbohydrate breakdown and acid production, the buffering capacity of saliva is overwhelmed. In addition, cariogenic pathogens, such as *S. mutans*, are both acidogenic and aciduric, thrive in such an environment. This vicious cycle promotes the overgrowth of opportunistic cariogenic organisms, increased acid secretion, exopolysaccharide secretion and biofilm formation [63,200,248]. The lowered oral pH thus contributes to the destruction of the mineralized structures of the teeth due to leaching of ions. Ultimately, the disturbed oral ecosystem and the shift towards demineralization in combination with the destruction of organic components of the tooth, leads to cavitation [200,248].

The current treatment modalities focus mainly on prevention such as optimal oral hygiene, drinking water fluoridation, the use of fluoridated toothpastes/gels and the use of

dental sealants to limit pathogen attachment to tooth surfaces [5]. With the onset of dental caries, the treatment focuses on the removal of infected tissues and restoration of lost tooth structure [5]. With disease progression, treatment modalities include root canal treatment, surgical endodontic procedures and extraction of infected tooth to restrict disease progression to adjacent areas [5]. However, the current prevention and treatment procedures have shortfalls, as suggested by the current statistics. One of the most important reasons behind these shortfalls is the poor socioeconomic status of many patients, restricting patient's access to timely diagnosis and treatment [249,250]. Hence, an alternative biotherapeutic which is cost effective and easily accessible to patients would be beneficial.

Interestingly certain bacteria can produce  $H_2O_2$ , a well-established antimicrobial compound [251]. Indeed, Branton *et al.* have demonstrated that probiotic bacteria such as *Lactobacillus acidophilus* have the ability to produce  $H_2O_2$  [139]. Certain bacteria lack enzymes such as catalase that decompose  $H_2O_2$  to nontoxic compounds such as  $H_2O$  and  $O_2$ , required by microorganisms to counteract oxidative stress [252-254]. *S. mutans* is a catalase negative organism and hence,  $H_2O_2$  producing probiotic bacteria could inhibit *S. mutans* [255]. *Lactobacillus* is also a catalase negative organism but has inherent peroxidases responsible for  $H_2O_2$  breakdown, enabling them to be  $H_2O_2$  tolerant [255,256].  $H_2O_2$  is used for wound disinfection and tooth whitening and has shown to inhibit Streptococci species [251,257,258]. Moreover, low levels of  $H_2O_2$  can increase mammalian cell-cell interactions, promote sensory axon regeneration and prevent cytotoxicity and DNA damage in cells such as keratinocytes [176,177]. However, high concentrations of  $H_2O_2$  can damage soft tissue, like gingiva, and decrease enamel tensile

strength [259,260]. Hence, delivering high levels of pure H<sub>2</sub>O<sub>2</sub> to inhibit oral pathogens would prove more harmful than beneficial. A probiotic bio-therapeutic that provides continuous low levels of H<sub>2</sub>O<sub>2</sub> would provide a better alternative to treat oral microbial infections [200,261].

The goal of the presented study was to screen probiotic bacteria for H<sub>2</sub>O<sub>2</sub> production and to investigate their role to inhibit *S. mutans*. Probiotic bacteria were investigated, qualitatively and quantitatively, for their ability to produce H<sub>2</sub>O<sub>2</sub> both in bacterial culture media and in simulated salivary conditions. Further investigations were performed to determine the inhibition of *S. mutans* mediated by probiotic bacteria. The susceptibility to H<sub>2</sub>O<sub>2</sub> of the selected *S. mutans* strain was investigated to determine the amount of the antimicrobial required for inhibition. A correlation between H<sub>2</sub>O<sub>2</sub> production and *S. mutans* inhibition was drawn. Finally, to investigate H<sub>2</sub>O<sub>2</sub> as a mechanism of action responsible for the probiotic inhibition of *S. mutans*, we blocked H<sub>2</sub>O<sub>2</sub> activity using catalase, and investigated whether the probiotic inhibition of *S. mutans* was still observed.

### **5.3 Materials and methods**

#### **5.3.1 Bacterial strains and culture conditions**

*Lactobacillus fermentum* NCIMB 5221, *Lactobacillus fermentum* NCIMB 2797, *Lactobacillus fermentum* NCIMB 8829, *Lactobacillus reuteri* NCIMB 701089, *Lactobacillus reuteri* NCIMB 701359, *Lactobacillus reuteri* NCIMB 702655, *Lactobacillus reuteri* NCIMB 702656 and *S. mutans* NCIMB 702062 were purchased from the National Collection of Industrial Food and Marine Bacteria (NCIMB) (Aberdeen, Scotland, UK). *L. acidophilus* ATCC 314 was purchased from Cedarlane

Laboratories (Burlington, ON, Canada). All of the bacterial strains were maintained at -80°C as 20% (v/v) glycerol stocks. A MRS agar plate was streaked and incubated at 37°C for 24 h with 5% CO<sub>2</sub> to ensure the purity of the culture. One colony from the MRS agar plate was inoculated in MRS broth for 24 h at 37°C. A 1% (v/v) inoculum was subcultured into MRS broth and incubated at 37°C for 24 h immediately prior to any assays.

### **5.3.2 Bacterial growth media and chemicals**

De Man, Rogosa, Sharpe (MRS) broth and Rogosa agar were purchased from Fisher Scientific (Ottawa, ON, Canada) and Sigma-Aldrich (Oakville, ON, Canada), respectively. All other chemicals used were of high-performance liquid chromatography or analytical grade purchased from commercial sources. An EasyPure reverse osmosis system and a NanoPure Diamond Life Science (UV/UF) ultrapure water systems from Barnstead (Dubuque, IA, USA) were used to purify water.

### **5.3.3 Determining probiotic H<sub>2</sub>O<sub>2</sub> production**

A qualitative assay was performed to investigate H<sub>2</sub>O<sub>2</sub> production by the probiotic bacteria. The strains tested were *L. fermentum* NCIMB 5221, *L. fermentum* NCIMB 2797, *L. fermentum* NCIMB 8829, *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 702655, *L. reuteri* NCIMB 702656, *L. reuteri* NCIMB 11951, *L. acidophilus* ATCC 314 and *S. mutans* NCIMB 702062, as a control. An assay, modifications from previous work by Song *et al.* was performed [262]. MRS agar solution (15% (w/v)) was autoclaved, after which it was cooled to 55°C. TMB and HRP (50-150 U/mg) were added to the molten agar at 0.25 mg/mL and 0.01 mg/mL, respectively. Agar plates were poured and cooled. 100 µL of each tested bacterial strain were streaked and the agar plates were incubated at 37°C with 5 % CO<sub>2</sub> for 48 h (n=3).

#### 5.3.4 Quantifying probiotic H<sub>2</sub>O<sub>2</sub> production in glucose buffered solution

A quantitative assay was performed to determine H<sub>2</sub>O<sub>2</sub> levels produced by probiotic bacteria, using a modified protocol by Martin *et al.* [256]. The aforementioned *Lactobacillus* strains were tested. Overnight bacterial cultures (10 mL) were centrifuged at 4000 rpm for 5 min at 4°C. The supernatant was discarded and the bacterial pellets were washed twice with 50 mM phosphate buffered saline (PBS). The washed pellets were re-suspended in 9 mL PBS (50 mM) containing 5 mM glucose. 500 µL of the resulting solution was used to inoculate 9 mL of PBS (50 mM), containing 5 mM glucose and incubated for 24 h at 37°C with shaking. Following incubation, the samples were centrifuged at 4000 rpm for 5 min at 4°C to collect the bacterial supernatant which were filtered using 0.22 µm filters.

Concentrations of H<sub>2</sub>O<sub>2</sub> in the bacterial supernatant were quantified using a colorimetric assay. For the colorimetric assay, 961.3 µL of TMB (2.5 mg/mL) and 8.3 µL of HRP (2.4 mg/mL) were added to 1 mL of bacterial supernatant. The solutions were incubated for 5 min at room temperature and absorbance was measured at 620 nm using a spectrophotometer (PerkinElmer 1420 Multilabel Counter) (n=3). The H<sub>2</sub>O<sub>2</sub> concentrations were determined by generating a standard curve using 30% (v/v) H<sub>2</sub>O<sub>2</sub> (9.812 M). The concentrations of H<sub>2</sub>O<sub>2</sub> used were 78.12, 156.25, 312.50, 625.0, 1250.0, 2500.0 µM ( $R^2 = 0.999$ ).

#### 5.3.5 Determining probiotic H<sub>2</sub>O<sub>2</sub> production in simulated oral conditions

To determine H<sub>2</sub>O<sub>2</sub> release in simulated salivary fluid (SSF), a qualitative assay was performed using SSF agar. SSF was prepared as in our previous study (**Table.4.1**). The aforementioned *Lactobacillus* strains in section 2.3 and *S. mutans* NCIMB 702062



were tested for H<sub>2</sub>O<sub>2</sub> production. SSF agar solution (15% (w/v)) was autoclaved, after which it was cooled to 55°C. TMB and HRP (50-150 units/mg) were added to the molten agar at 0.25 mg/mL and 0.01 mg/mL, respectively. Agar plates were poured and cooled. 100 µL of each tested strain were streaked and the agar plates incubated at 37°C with 5 % CO<sub>2</sub> for 16 h (n=3).

### **5.3.6 Determining the probiotic inhibition of *Streptococcus mutans***

The capability of the *Lactobacillus* strains to inhibit *S. mutans* NCIMB 702062 was investigated, under simulated oral conditions. *S. mutans* and the probiotic bacteria were added to SSF at 1% (v/v) and 5% (v/v) respectively, of the overnight cultures. *S. mutans* NCIMB 702062, without any probiotic bacteria, was used as a control. The test and control samples (n=3) were placed on an orbital shaker (Napco, Fisher Scientific, Suwanee, GA, USA) set at 75 rpm and 37°C for 24 h. Bacterial counts were determined by standard cfu methods

### **5.3.7 Determining bacterial viability using selective agar**

Selective agar was used for differentiating the probiotic cells from the *S. mutans* cells in mixed cultures. Rogosa agar was used for probiotic selectivity. Manufacturer's instructions were followed to prepare Rogosa agar. Mitis-sucrose-bacitracin (MSB) agar was used for the selection of *S. mutans*. MSB agar was prepared according to previous work by Gold *et al.* [225]. The cell counts of the pathogens and probiotic bacteria were measured using standard colony forming units (cfu). 0.85% (w/v) NaCl was used to attain 10-fold serial dilutions. Each sample was streaked on both Rogosa agar and MSB agar plates at 0 and 24 h and incubated at 37°C and 5% CO<sub>2</sub> for 48 h (n=3). All the tested *L.*

*reuteri* strains were incubated using AnaeroGen gas bags from Sigma-Aldrich (Oakville, ON, Canada) to allow optimal bacterial growth.

### **5.3.8 Determining the H<sub>2</sub>O<sub>2</sub> concentration inhibitory for *S. mutans***

The H<sub>2</sub>O<sub>2</sub> inhibitory concentration required to observe a significant reduction in *S. mutans* growth was investigated. H<sub>2</sub>O<sub>2</sub> was used in concentrations ranging from 0–166.67 µM and was incubated for 24 h at 37°C with 1% (v/v) of an overnight culture of *S. mutans* NCIMB 702062 in MRS broth. Following incubation, *S. mutans* turbidity, as an indicator of bacterial cell biomass, was obtained at 620 nm using a spectrophotometer (n=3).

### **5.3.9 Investigating the role of probiotic H<sub>2</sub>O<sub>2</sub> for the inhibition of *S. mutans***

To determine whether H<sub>2</sub>O<sub>2</sub> is responsible for the probiotic inhibition of *S. mutans*, catalase was used to block H<sub>2</sub>O<sub>2</sub> activity. Catalase breaks down H<sub>2</sub>O<sub>2</sub> to water and oxygen, molecules that are non-toxic to microbial cells. *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 702655 and *L. reuteri* NCIMB 702656 were selected to evaluate the effect of catalase, on *S. mutans* NCIMB 702062 inhibition. MRS agar (15 % (w/v)) was prepared, autoclaved and cooled. Catalase solutions (9 286.4, 18 622.5 and 37 245.0 U/mL) were prepared in MRS broth. All the solutions were sterilized using a 0.22 µm syringe filter. The overnight cultures of probiotic strains and *S. mutans* were centrifuged at 4°C and 4000 rpm for 5 min. The bacterial pellets were re-suspended in MRS solution for further use. 0.5% (v/v) of *S. mutans* overnight culture was added to the molten agar and 30 mL of agar plates were poured and allowed to cool. 5 wells were formed in each agar plate, which were filled with 100 µL of 1) sterile MRS broth 2) 50% (v/v) of probiotic/*S. mutans* overnight culture 3) 50% (v/v) of probiotic/*S.*

*mutans* overnight culture in 37 245.0 U/mL of catalase solution 4) 50% (v/v) of probiotic/*S. mutans* overnight culture in 18 622.5 U/mL of catalase solution and 5) 50% (v/v) of probiotic/*S. mutans* overnight culture in 9286.42 U/mL of catalase solution. The agar plates were then incubated at 37°C with 5 % CO<sub>2</sub> for 16 h to allow bacterial growth (n=3). The clearance zones, indicating *S. mutans* inhibition, were measured from the edge of the wells to the edge of the clearance zones. The clearance zones were quantified using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-2011).

### **5.3.10 Statistical analysis**

Experimental results are expressed as mean±standard error of the mean (SEM). Triplicate samples were measured to ensure accuracy and reproducibility. Statistical analysis was carried out using Statistical Product and Service Solutions (SPSS) Version 17.0 (IBM Corporation, New York, NY, USA). Linear regression was performed for standard curves. Comparisons of the means were performed using the general linear model and Tukey's post-hoc analysis. Statistical significance was set at p<0.05 and p<0.001 were considered highly significant.

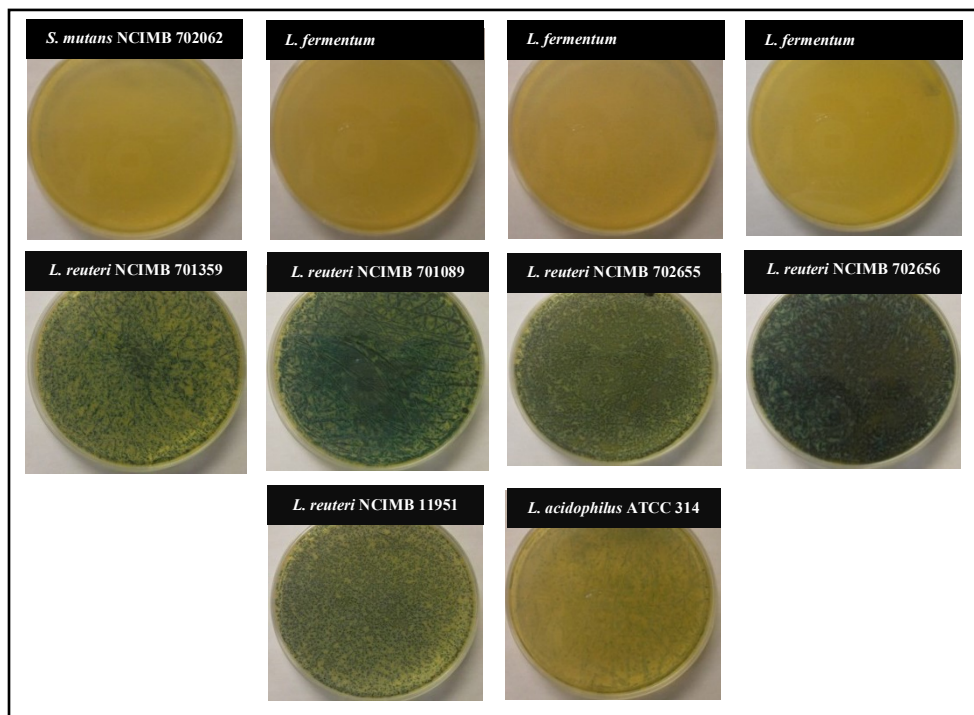
## **5.4 Results**

### **5.4.1 H<sub>2</sub>O<sub>2</sub> production by probiotic bacteria in MRS broth**

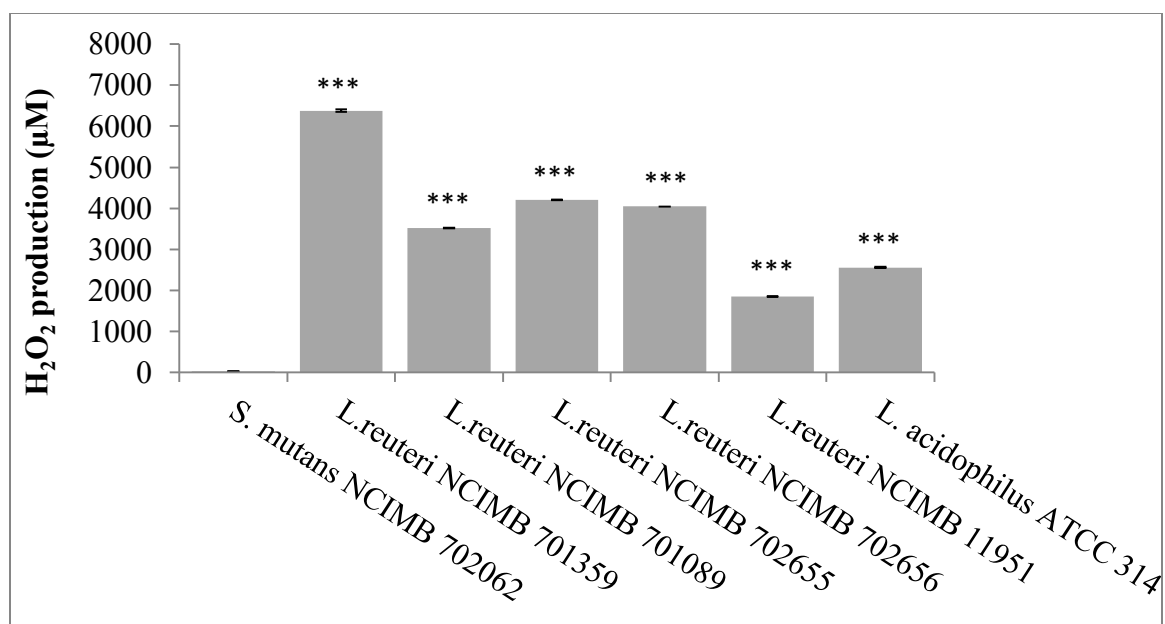
Certain probiotic strains have the capability to produce H<sub>2</sub>O<sub>2</sub>, an antimicrobial capable of inhibiting *S. mutans* growth. *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 702655, *L. reuteri* NCIMB 702656, *L. reuteri* NCIMB 11951 and *L. acidophilus* ATCC 314 produced detectable amounts of H<sub>2</sub>O<sub>2</sub>, as indicated by the formation of blue colonies when incubated with HRP and TMB (**Figure 5.1**). *L.*

*fermentum* NCIMB 5221, *L. fermentum* NCIMB 2797, *L. fermentum* NCIMB 8829 and *S. mutans* NCIMB 702062 did not produce any H<sub>2</sub>O<sub>2</sub>, as seen by the absence of blue colonies.

The probiotic H<sub>2</sub>O<sub>2</sub> production was further quantified. *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 702655, *L. reuteri* NCIMB 702656, *L. reuteri* NCIMB 11951 and *L. acidophilus* ATCC 314 produced significant quantities of H<sub>2</sub>O<sub>2</sub>, as was suggested by the qualitative assay (**Figure 5.2**). *L. reuteri* NCIMB 701359 produced the highest levels of H<sub>2</sub>O<sub>2</sub> with a production of 6379.45±31.28 µM H<sub>2</sub>O<sub>2</sub> ( $p<0.001$ ) following 24 h. None of the tested *L. fermentum* strains nor *S. mutans* NCIMB 702062 produced detectable levels of H<sub>2</sub>O<sub>2</sub>.



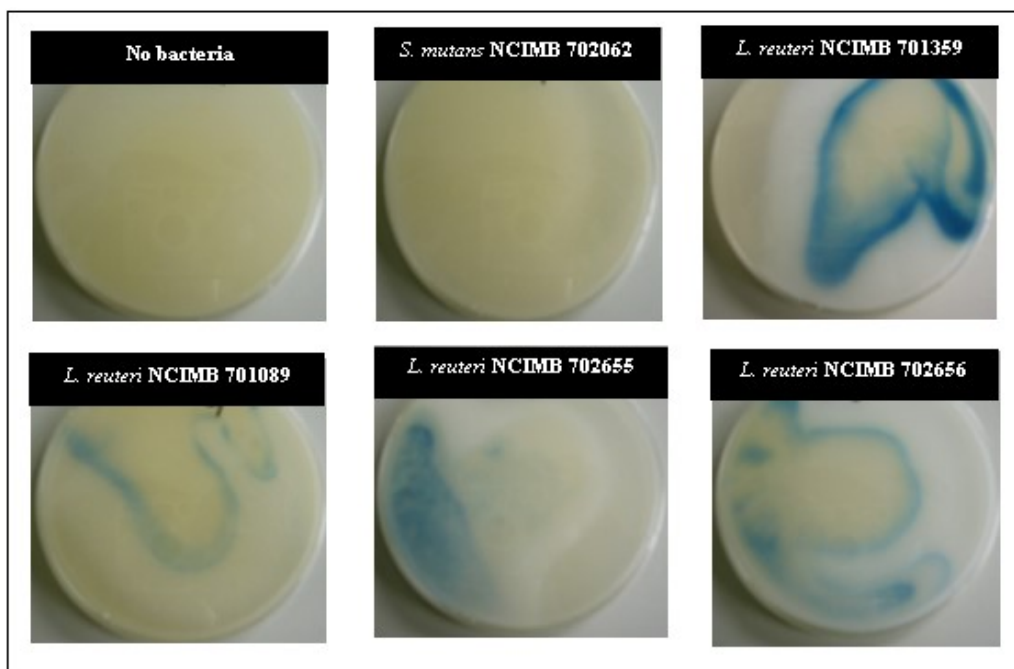
**Figure 5.1:** H<sub>2</sub>O<sub>2</sub> production by the probiotic bacterial strains. Probiotic bacteria were incubated on a MRS agar plate impregnated with trimethyl benzidine and horseradish peroxidase for 24 h at 5% CO<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> production was identified by the blue colonies. *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 702655, *L. reuteri* NCIMB 702656, *L. reuteri* NCIMB 11951 and *L. acidophilus* ATCC 314 demonstrated H<sub>2</sub>O<sub>2</sub> production.



**Figure 5.2:** Quantitative evaluation of probiotic H<sub>2</sub>O<sub>2</sub> production, using *S. mutans* as a control. Probiotic strains were incubated in phosphate buffered saline containing 5 mM glucose at 37°C for 24 h. A trimethyl benzidine spectrophotometric assay was used to quantify H<sub>2</sub>O<sub>2</sub>. Data is presented as mean ± SEM (\*\*\*  $p < 0.001$ ),  $n = 3$ .

#### 5.4.2 H<sub>2</sub>O<sub>2</sub> production by the probiotic bacteria under simulated oral conditions

To confirm that probiotic H<sub>2</sub>O<sub>2</sub> production also occurs under simulated oral conditions, we evaluated probiotic H<sub>2</sub>O<sub>2</sub> production on SSF-agar. *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 702655 and *L. reuteri* NCIMB 702656 produced detectable amounts of H<sub>2</sub>O<sub>2</sub> in SSF, as indicated by the formation of blue colonies when incubated with HRP and TMB (Figure 5.3).



**Figure 5.3:** Qualitative evaluation of probiotic H<sub>2</sub>O<sub>2</sub> production in simulated salivary fluid. Probiotic strains plated on SSF agar plate impregnated with trimethyl benzidine and horseradish peroxide for 16 h. H<sub>2</sub>O<sub>2</sub> production was identified by the blue colonies produced by *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 702655 and *L. reuteri* NCIMB 702656.

#### 5.4.3 Probiotic inhibition of *Streptococcus mutans* in SSF

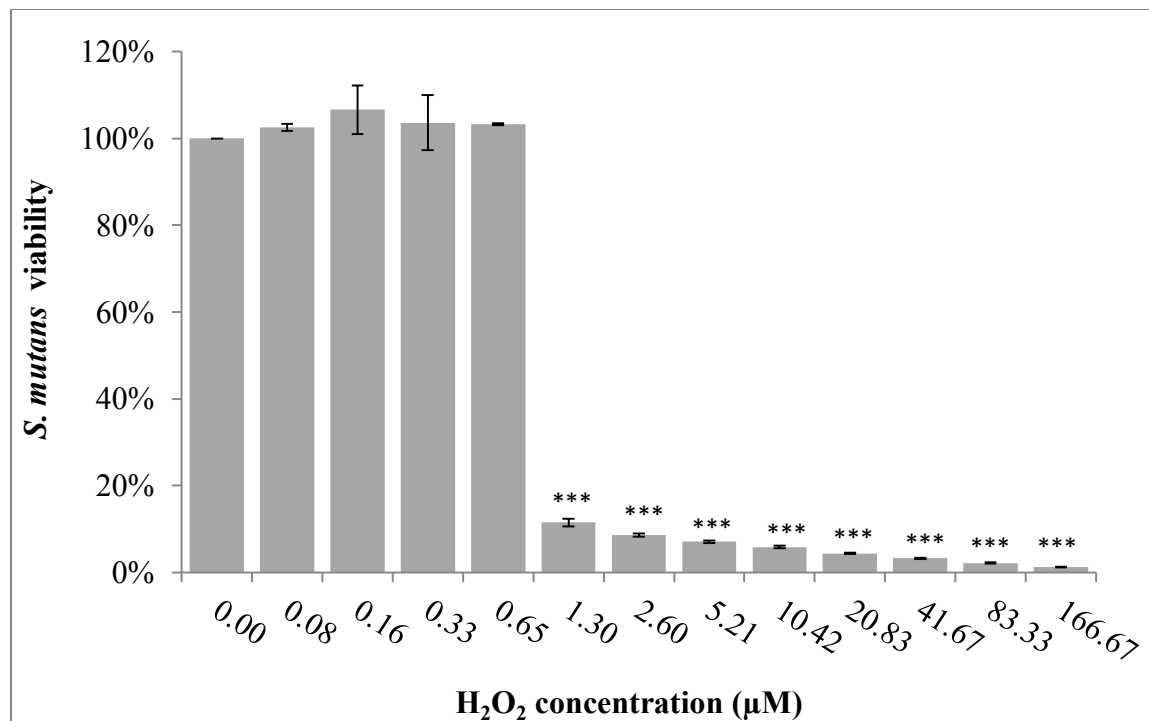
We investigated the probiotic bacteria for their capability to inhibit dental caries-causing *S. mutans*, as shown in **Table 5.1**. The viability of *S. mutans* was  $2.26 \times 10^7 \pm 1.1 \times 10^6$  cfu/mL at the start of the assay and  $1.29 \times 10^8 \pm 2.12 \times 10^7$  cfu/mL following 24 h of incubation. The best inhibitors of *S. mutans* viability were *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 702656 and *L. reuteri* NCIMB 702655, with no detectable viable *S. mutans* cells remaining following 24 h (<10 cfu/mL). The probiotic strain that demonstrated the weakest inhibition of *S. mutans* viability was *L. fermentum* NCIMB 2797 with  $4.07 \times 10^6 \pm 7.69 \times 10^4$  cfu/mL *S. mutans* cells remaining. The associated viability of all the probiotic strains was higher than *S. mutans* viability.

**Table 5.1:** Probiotic-mediated *S. mutans* NCIMB 702062 inhibition in simulated salivary fluid, co-incubated for 24 h at 37°C. A pure culture of *S. mutans* was used as negative control (no probiotic). Data is represented as means of triplicate samples  $\pm$  SEM, n = 3

Treatment	<i>S. mutans</i> viability (cfu/mL)	Probiotic viability (cfu/mL)	Significance
No probiotic	$1.29 \times 10^8 \pm 2.12 \times 10^7$	-	-
<i>L. fermentum</i> NCIMB 5221	$3.27 \times 10^6 \pm 2.63 \times 10^5$	$2.43 \times 10^8 \pm 2.73 \times 10^7$	$p < 0.001$
<i>L. fermentum</i> NCIMB 2797	$4.07 \times 10^6 \pm 7.69 \times 10^4$	$1.36 \times 10^8 \pm 1.56 \times 10^7$	$p < 0.001$
<i>L. fermentum</i> NCIMB 8829	$3.97 \times 10^3 \pm 1.87 \times 10^3$	$1.38 \times 10^8 \pm 1.79 \times 10^7$	$p < 0.001$
<i>L. reuteri</i> NCIMB 701359	<10	$2.85 \times 10^7 \pm 9.7 \times 10^5$	$p < 0.001$
<i>L. reuteri</i> NCIMB 701089	<10	$7.40 \times 10^7 \pm 6.66 \times 10^6$	$p < 0.001$
<i>L. reuteri</i> NCIMB 702655	<10	$4.60 \times 10^7 \pm 2.08 \times 10^6$	$p < 0.001$
<i>L. reuteri</i> NCIMB 702656	<10	$5.27 \times 10^7 \pm 6.57 \times 10^6$	$p < 0.001$
<i>L. reuteri</i> NCIMB 11951	$5.97 \times 10^3 \pm 7.80 \times 10^2$	$2.93 \times 10^7 \pm 2.91 \times 10^6$	$p < 0.001$
<i>L. acidophilus</i> NCIMB 314	$7.63 \times 10^5 \pm 1.77 \times 10^3$	$2.65 \times 10^8 \pm 6.07 \times 10^6$	$p < 0.001$

#### 5.4.4. H<sub>2</sub>O<sub>2</sub> required for *S. mutans* inhibition

As H<sub>2</sub>O<sub>2</sub> is an antimicrobial agent, we investigated how much H<sub>2</sub>O<sub>2</sub> is required to inhibit the growth of *S. mutans* NCIMB 702062. The results show that a concentration of H<sub>2</sub>O<sub>2</sub> greater than 1.3  $\mu$ M can significantly ( $p < 0.001$ ) inhibit the growth of *S. mutans* NCIMB 702062, indicated by bacterial turbidity (**Figure 5.4**).

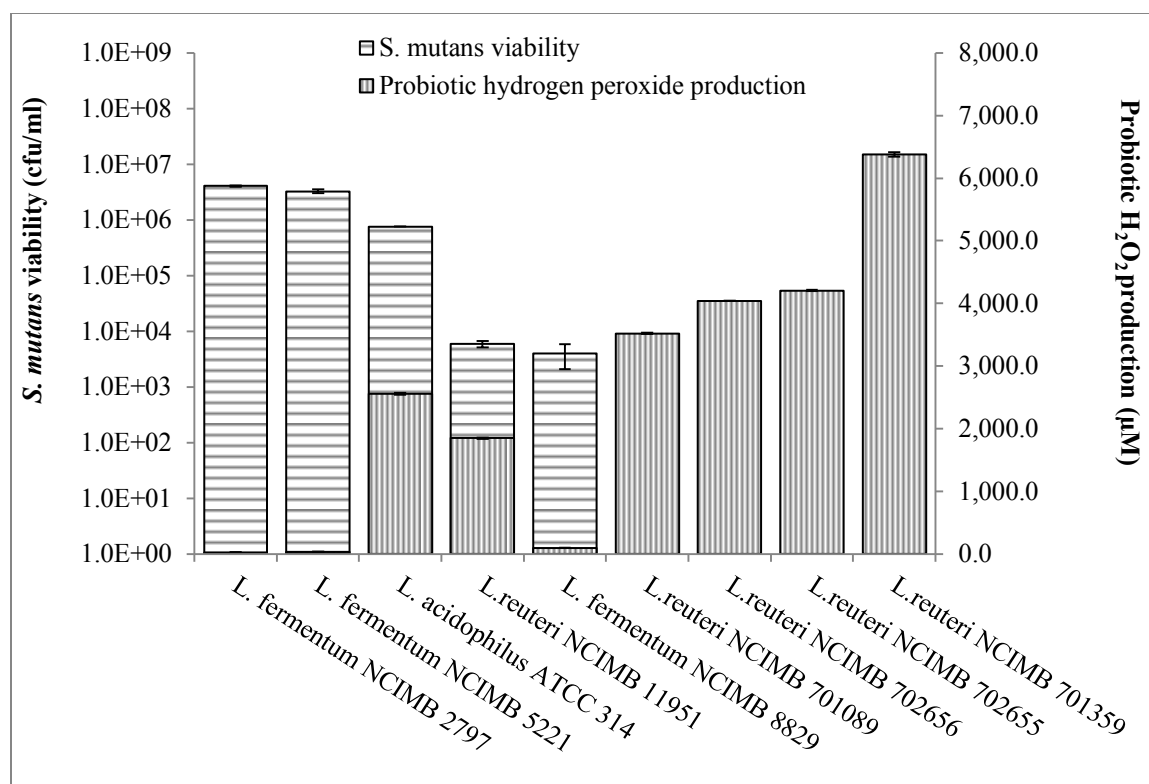


**Figure 5.4:** Determining the amount of H<sub>2</sub>O<sub>2</sub> required for the inhibition of *S. mutans* NCIMB 702062 grown in MRS broth at 37°C for 24 h. *S. mutans* NCIMB 702062 was significantly reduced at H<sub>2</sub>O<sub>2</sub> concentrations  $\geq 1.30$   $\mu$ M. Data is presented as mean  $\pm$  SEM (\*\*\*)  $p < 0.001$ ,  $n = 3$

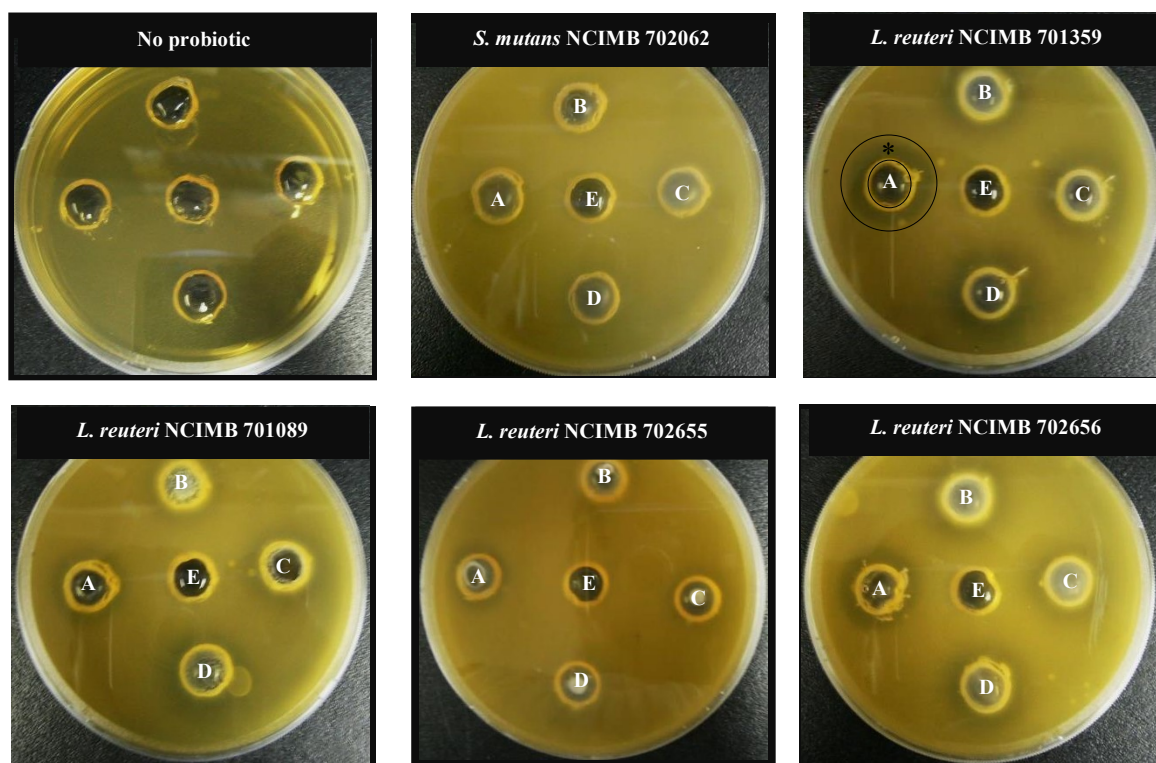


#### **5.4.5 Catalase deactivation of probiotic H<sub>2</sub>O<sub>2</sub> production demonstrating their role in *S. mutans* inhibition**

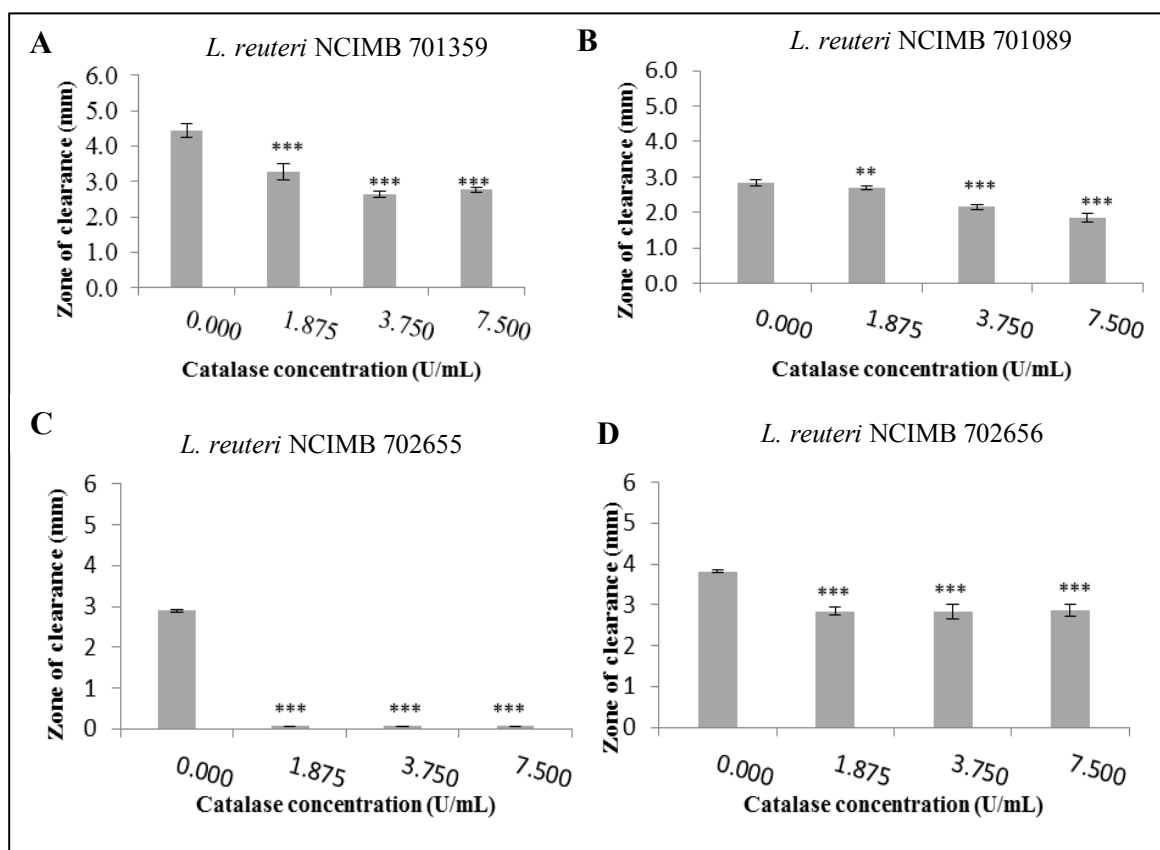
To identify the role of probiotic H<sub>2</sub>O<sub>2</sub> production for the inhibition of *S. mutans*, we first correlated probiotic H<sub>2</sub>O<sub>2</sub> production with *S. mutans* viability. We observed increased *S. mutans* inhibition by the H<sub>2</sub>O<sub>2</sub> producing probiotic strains (**Figure 5.5**). We then investigated whether removing/blocking H<sub>2</sub>O<sub>2</sub> activity would limit probiotic inhibition of *S. mutans*. Catalase can break down H<sub>2</sub>O<sub>2</sub> into non-antimicrobial products, and so we evaluated its effect on probiotic-mediated *S. mutans* inhibition. The viability of the incorporated *S. mutans* was  $1.04 \times 10^7 \pm 1.3 \times 10^6$  cfu/mL of agar. Our control samples, containing the probiotic strains demonstrated the successful inhibition of *S. mutans* NCIMB 702062, as observed by clearing zones around the wells (**Figure 5.6**). The activity of catalase on H<sub>2</sub>O<sub>2</sub> was shown to prevent the probiotic mediated inhibition of *S. mutans*, suggesting that H<sub>2</sub>O<sub>2</sub> is indeed responsible for the probiotic inhibitory activity on *S. mutans*. This result was demonstrated by a significant decrease ( $p < 0.001$ ) in clearing zones observed with catalase addition in wells containing probiotic bacteria (**Figure 5.7**). These results were observed for the tested probiotic strains, *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 702655 and *L. reuteri* NCIMB 702656, previously demonstrating significant H<sub>2</sub>O<sub>2</sub> production.



**Figure 5.5:** Graphical representation of the correlation between *S. mutans* viability and probiotic H<sub>2</sub>O<sub>2</sub> production. Data is represented as means of triplicate samples  $\pm$  SEM.



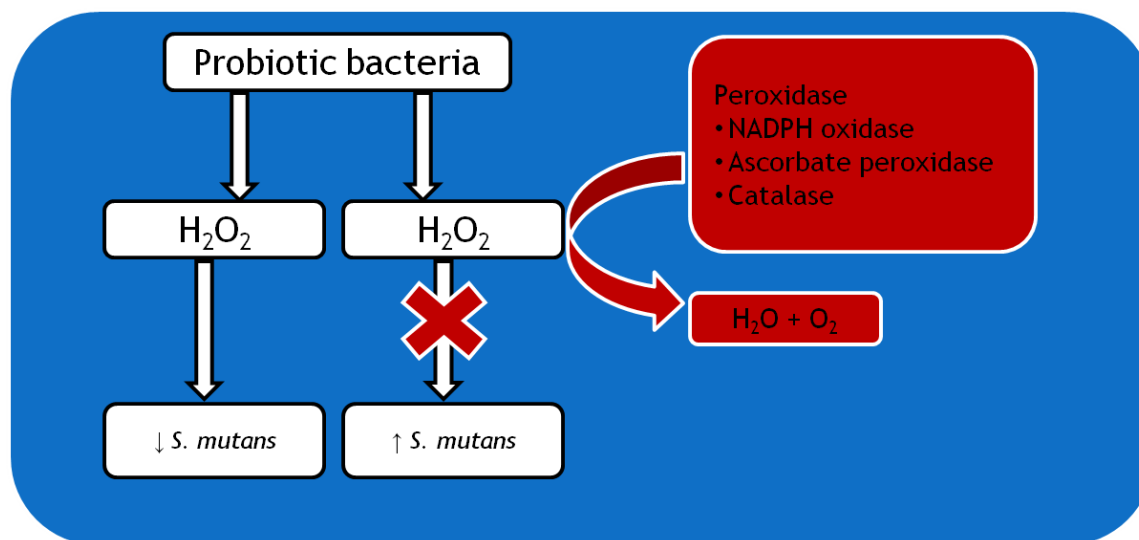
**Figure 5.6:** The effect of catalase on probiotic inhibition of *S. mutans* NCIMB 702062. Three different concentrations (37245, 18622.5 and 9286.42 U/mL) of catalase were tested. Inhibition assay for *S. mutans* NCIMB 702062 demonstrating the inhibition around the wells containing (A) Overnight culture of test strain (50% (v/v)) (B) Overnight culture of test strain (50% (v/v)) supplemented with 37245 U/mL of catalase (C) Overnight culture of test strain (50% (v/v)) supplemented with 18622.5 U/mL of catalase (D) Overnight culture of test strain (50% (v/v)) supplemented with 9286.42 U/mL of catalase (E) Sterile MRS broth and \* indicating the clearance zone (inhibition). The wells containing catalase with the probiotic strains demonstrated lower *S. mutans* inhibition represented by lower clearance zones compared to the wells containing probiotic bacteria with no catalase



**Figure 5.7:** The effect of catalase on probiotic inhibition of *S. mutans* NCIMB 702062, using a plate assay. Probiotic strains without any catalase were used as control and three different concentrations (37245, 18622.5 and 9286.42 U/mL) of catalase were used as tests. There was a reduction in probiotic mediated *S. mutans* inhibition, indicated by zone of clearance, in the presence of catalase. Tested probiotics were (A) *L. reuteri* NCIMB 701359 (B) *L. reuteri* NCIMB 701089 (C) *L. reuteri* NCIMB 702655 (D) *L. reuteri* NCIMB 702656. Data is presented as mean  $\pm$  SEM (\*\*\*  $p < 0.001$ ),  $n = 3$ .

## 5.5 Discussion

Probiotic bio-therapeutic demonstrates potential for dental caries, the most prominent oral disorder with multifactorial etiology [200]. Recent studies involving probiotic bacteria have demonstrated their potential in several health disorders including oral diseases [144]. Previous research has demonstrated that *S. mutans* could be successfully inhibited by probiotic bacteria [223]. Interestingly, probiotic bacteria can also produce  $H_2O_2$ , a well-documented antimicrobial agent used for wound disinfection and tooth whitening [139,251,257,258,263]. Probiotic Lactobacilli such as *L. acidophilus* produce  $H_2O_2$  via complex metabolic pathways [139]. Lactobacilli strains use flavoproteins via cytochrome-independent systems, which utilize  $O_2$  to produce  $H_2O_2$  [264,265]. Bacterial  $H_2O_2$  thus formed, can inhibit other pathogens lacking peroxidases. The ability to produce  $H_2O_2$  may allow for the strains to outcompete other bacterial species. Interestingly, Lactobacilli strains possess peroxidase activity via enzymes such as lactoperoxidases and NADH oxidases, breaking down  $H_2O_2$  to non-antimicrobial compounds, preventing its own destruction (**Figure 5.8**). Moreover,  $H_2O_2$  producing probiotic bacteria have shown potential in inhibiting enteric, vaginosis-associated and uropathogenic pathogens [266]. Keeping this in mind, the selection of probiotic bacteria producing  $H_2O_2$  to inhibit *S. mutans* could prove beneficial as a novel dental caries therapy.



**Figure 5.8:** Probiotic H<sub>2</sub>O<sub>2</sub> production for *S. mutans* inhibition

This study investigated nine *Lactobacillus* strains, selected from previous studies, for the inhibition of *S. mutans* NCIMB 702062: *L. fermentum* NCIMB 5221, *L. fermentum* NCIMB 2797, *L. fermentum* NCIMB 8829, *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 11951, *L. reuteri* NCIMB 702655, *L. reuteri* NCIMB 702656, and *L. acidophilus* ATCC 314 [223]. These *Lactobacillus* strains were screened for H<sub>2</sub>O<sub>2</sub> production. For the initial screening process, a qualitative assay for H<sub>2</sub>O<sub>2</sub> production was performed. A plate assay based on the enzymatic reaction between H<sub>2</sub>O<sub>2</sub>, TMB and HRP was used, where the formation of blue colonies indicated a probiotic strain capable of producing H<sub>2</sub>O<sub>2</sub>. The results demonstrate that the *L. reuteri* strains and *L. acidophilus* were H<sub>2</sub>O<sub>2</sub> producing probiotic bacteria, with blue colonies, while none of the *L. fermentum* strains exhibited H<sub>2</sub>O<sub>2</sub> production (**Figure. 5.1**). However, it is important to note that other mechanism(s) are involved by which probiotic bacteria can inhibit *S. mutans*. Probiotic production of nisin, reuterin and nitric oxide might also be responsible for their antibacterial properties [144].

For a quantitative assessment of H<sub>2</sub>O<sub>2</sub> production, the *L. reuteri* strains, which demonstrated production qualitatively, were further investigated. In agreement with the qualitative assay, all the *L. reuteri* strains produced H<sub>2</sub>O<sub>2</sub> (**Figure 5.2**). With further qualitative investigations, these strains demonstrated H<sub>2</sub>O<sub>2</sub> production under simulated oral conditions (**Figure 5.3**). However, due to the absence of glucose in the SSF, we were unable to quantify H<sub>2</sub>O<sub>2</sub> in SSF.

The next step, was to evaluate if the tested probiotic bacterial strains, were able to inhibit *S. mutans* when co-incubated with the pathogen in simulated oral conditions. The best inhibitors of *S. mutans* viability were *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 702655 and *L. reuteri* NCIMB 702656 to non-detectable *S. mutans* levels of  $\leq 10$  cfu/mL (**Table 5.1**). Further investigations were performed to determine the H<sub>2</sub>O<sub>2</sub> concentrations required for *S. mutans* inhibition. The results demonstrate that any H<sub>2</sub>O<sub>2</sub> concentration equal to or higher than 1.30  $\mu$ M, inhibited the viability/growth of *S. mutans* (**Figure 5.4**). A probiotic inhibitory effect on *S. mutans* due to H<sub>2</sub>O<sub>2</sub> production would be observed if the probiotic strains produce 1.30  $\mu$ M or higher H<sub>2</sub>O<sub>2</sub>. Interestingly, the probiotic strains produced H<sub>2</sub>O<sub>2</sub> at levels higher than 1.30  $\mu$ M after 24 h (**Figure 5.2**).

There was a clear association between the probiotic strains that inhibited *S. mutans* the most and those that produced H<sub>2</sub>O<sub>2</sub> the most, correlated graphically in **Figure 5.5**. This observation lead to further investigations, to prove our hypothesis that probiotic bacterial H<sub>2</sub>O<sub>2</sub> production was indeed responsible for the inhibition of *S. mutans*. The enzyme catalase has the ability to breakdown H<sub>2</sub>O<sub>2</sub> to non-toxic compounds [267]. We exploited this principle, to block the effect of H<sub>2</sub>O<sub>2</sub> produced by probiotic bacteria. If

H<sub>2</sub>O<sub>2</sub> is indeed responsible for the probiotic inhibition of *S. mutans*, blocking of H<sub>2</sub>O<sub>2</sub> by catalase would diminish the probiotic mediated *S. mutans* inhibition. The four best H<sub>2</sub>O<sub>2</sub> producing probiotic bacteria, the *L. reuteri* strains, were selected and an *in vitro* assay was developed. MRS agar incorporated with *S. mutans*, was treated with probiotic bacterial strains in wells at three different concentrations of catalase. The inhibition of *S. mutans* was visible with zones of clearing around the wells. Interestingly, all the probiotic strains demonstrated significantly reduced zones of clearance around the catalase treated wells, suggesting a significant reduction in probiotic-mediated *S. mutans* inhibition in the presence of catalase (**Figure 5.6** and **Figure 5.7**). Hence, we concluded that probiotic H<sub>2</sub>O<sub>2</sub> production is responsible for *S. mutans* inhibition. Catalase activity can be blocked in the presence of metallic ions, therefore, the probiotic H<sub>2</sub>O<sub>2</sub> activity with catalase blocking could not be tested in broth due to the presence of metallic ions [268].

This is the first study that demonstrates that H<sub>2</sub>O<sub>2</sub> producing probiotic strains are responsible for *S. mutans* inhibition and proves one of the many mechanisms of action suggested previously by various research groups. Future work may focus on the investigation of other strains capable of producing H<sub>2</sub>O<sub>2</sub> and other mechanism(s) responsible for *S. mutans* inhibition by strains such as the *L. fermentum*. In addition, various delivery methods for probiotic delivery in the oral cavity should be investigated, including dissolvable films, developed in recent work and microcapsules [269,270]. These delivery vehicles may have the capability of enhancing the effect of probiotic bacterial function, for example by shielding the probiotic from the different enzymes present in the mouth that would breakdown H<sub>2</sub>O<sub>2</sub>. More *in vitro* and *in vivo* studies are required to fully understand this pathway and investigate their effect using appropriate



delivery vehicle. This study demonstrates the use of *L. reuteri* probiotic strains for the development of a successful dental caries therapeutic.

## **5.6 Acknowledgments**

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## **5.7 Conflict of Interest**

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

**CHAPTER 6: INHIBITION OF DENTAL CARIES CAUSING *STREPTOCOCCUS MUTANS* BIOFILM BY PROBIOTIC *LACTOBACILLUS REUTERI* NCIMB 701359**

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**Preface:** As established in the previous chapters, *Lactobacillus reuteri* NCIMB 701359 was a potent H<sub>2</sub>O<sub>2</sub> producing probiotic and inhibited planktonic *S. mutans* cultures. The goal of this study was to evaluate *Lactobacillus reuteri* NCIMB 701359 for *S. mutans* biofilm inhibition. *S. mutans* biofilms were formed in 24 well plates and were treated with probiotic bacteria using transwell systems. The study was performed for 5 days. Selective agar was used for bacterial enumeration. Biofilm mass, *S. mutans* aggregates in the biofilms and *S. mutans* viability in the biofilms were evaluated to determine probiotic mediated biofilm inhibition.

## 6.1 Abstract

Oral biofilm, termed dental plaque, is associated with the development of oral diseases, including dental caries. *Streptococcus mutans*, which thrives in oral biofilms, is the primary causative agent of dental caries. For a successful dental caries therapeutic, it is essential to limit *S. mutans* biofilm. Current caries therapies focus on prevention such as tooth brushing, flossing, mouthwashes, water fluoridation and sealants. In terms of treatment, physical disruption of biofilm is the only effective method. Probiotic bacteria can inhibit *S. mutans* in planktonic cultures. One potent probiotic *S. mutans* inhibitor, *Lactobacillus reuteri* NCIMB 701359, produces a number of antibacterial molecules, including H<sub>2</sub>O<sub>2</sub>. The goal of this work is to investigate the capability of *L. reuteri* NCIMB 701359 to limit *S. mutans* biofilm formation, using a novel *in vitro* model. *S. mutans* biofilms were established with a viability of  $1.36 \times 10^5 \pm 0.40 \times 10^5$  cfu/well and 100.00 $\pm$ 16.51% increase in biofilm mass, following 5 days. Probiotic *L. reuteri* NCIMB 701359 significantly reduced biofilm mass (61 $\pm$ 17 %) (p=0.03) and *S. mutans* viability ( $1.35 \times 10^5 \pm 0.39 \times 10^5$  cfu/well)(p=0.02) at day 5. The results indicate that *L. reuteri* NCIMB 701359 has great potential as a dental caries biotherapeutic, by reducing *S. mutans* oral biofilm.

## 6.2 Introduction

Oral biofilm also termed dental plaque, is a matrix of diverse bacterial populations and bacterial exopolysaccharides, associated with the development of various oral diseases[110]. Dental caries and periodontal disease, whose aetiologies have been correlated with dental plaque propagation are amongst the most common oral disorders [216]. Dental plaque hinders simple host mechanisms, targeting oral pathogens contained

in the biofilm [36,93]. Moreover, biofilms prevent the penetration of saliva, preventing the removal of pathogens from the oral cavity. As biofilm formation is a microbial driven process, it is evident that the oral microbiota plays an important role in the initiation and progression of oral diseases. Typically, most oral commensals are in homeostasis with the host and do not cause disease [36,93]. However, changes in the oral cavity that cause a decreases of oral pH due to increased breakdown of dietary carbohydrates (example: sucrose), and reduced salivary flow (xerostomia), alter the oral ecosystem and lead to the growth of opportunistic organisms including those that cause dental caries and periodontal disease [36,93].

There is a vast presence of *Streptococcus* species in the oral cavity. Although most of these are in harmony with the oral environment, increased *Streptococcus mutans* counts have been associated with dental caries [271,272]. Most importantly, it is the primary causative organism of dental caries and is a highly acidogenic and aciduric organism. *S. mutans* overgrowth leads to the utilization of simple dietary sugars, like sucrose, and the production of excess acid which leads to the demineralisation of tooth, initiating dental caries [36,93]. It is important to note that the spread of infection to the surrounding teeth structures can further lead to other oral diseases such as periodontal diseases that includes both gingivitis and periodontitis [273]. In addition, *S. mutans* is also associated with endocarditis and periodontal diseases is associated with atherosclerosis [273]. The current dental caries therapeutic measures have little efficacy, highlighted by the near 100% worldwide prevalence of dental caries in adults, reported by the World Health Organization.

Probiotic therapeutics have shown promise for a wide range of diseases including metabolic syndrome, cardiovascular diseases, non-alcoholic fatty liver disease colon cancer, allergies and oral diseases [140-147]. Probiotic bacteria are naturally found in food products and provide a natural, safe and cost-effective approach. They have demonstrated great potential in the prevention and treatment of oral disorders such as dental caries and periodontal diseases [144,223]. Although previous studies have demonstrated that probiotic bacteria can inhibit planktonic *S. mutans* cultures [223], for the successful development of therapeutics the focus should lie on the inhibition of *S. mutans* biofilm formation and progression, the goal of this study.

In this study we investigate the potential of probiotic *Lactobacillus reuteri* NCIMB 701359 to reduce *S. mutans* biofilm formation. A novel system, adapted from Stander *et al.* was used to study probiotic-*S. mutans* biofilm interactions [198]. This system consists of transwell inserts that allow for physical separation of the pathogen and probiotic, without restricting the transfer of probiotic bacterial by-products. This transwell system was used to assess *S. mutans* biofilm formation with and without probiotic bacterial treatment. Biofilm mass, *S. mutans* viability and density of *S. mutans* aggregates were monitored, with and without probiotic treatment over a period of 5 days.

## **6.3 Materials and methods**

### **6.3.1 Bacterial growth media and chemicals**

De Man, Rogosa, Sharpe (MRS) broth was purchased from Fisher Scientific (Ottawa, ON, Canada) for the growth of *Lactobacillus*. Mitis-sucrose-bacitracin (MSB) agar was prepared based on previous work by Gold *et al.* [225] for *S. mutans* NCIMB 702062 enumeration. Simulated salivary fluid (SSF) was prepared according to our

previous work [269]. Water was purified with an EasyPure reverse osmosis system and a NanoPure Diamond Life Science (UV/UF) ultrapure water system from Barnstead (Dubuque, IA, USA). All other chemicals used were of HPLC or standard analytical grade purchased from Sigma-Aldrich (Oakville, ON, Canada).

## 2.2 Bacterial strain and culture conditions

*L. reuteri* NCIMB 701359 and *S. mutans* NCIMB 702062 NCIMB 702062, extracted from carious dentin, were purchased from the National Collection of Industrial, Food and Marine Bacteria (NCIMB, Aberdeen, Scotland, UK). The bacterial strains were stored at -80°C in MRS broth containing 20% (v/v) glycerol. An agar plate was streaked to ensure purity and incubated for 24 h at 37°C with 5% CO<sub>2</sub>. 10 mL of MRS broth was inoculated with a single colony and incubated for 24 h at 37°C. The overnight cultures were used for all experiments.

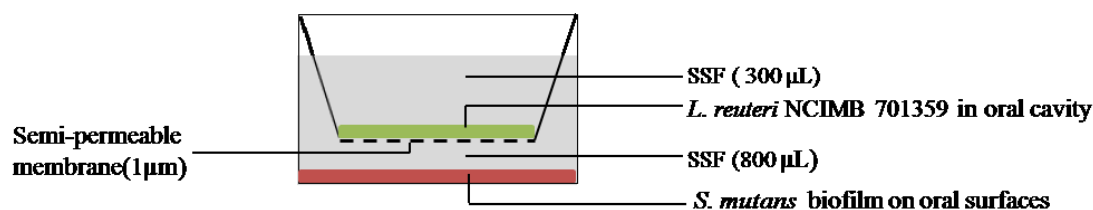
### 6.3.2 Establishing a *S. mutans* NCIMB 702062 biofilm

To study the probiotic inhibition of *S. mutans* NCIMB 702062 biofilm, we established *S. mutans* NCIMB 702062 biofilms in 24 well plates. *S. mutans* NCIMB 702062 overnight cultures were washed twice with 0.85 % (w/v) NaCl by centrifuging the cultures for 5 min at 4000 g and 4°C. The resulting bacterial pellets were collected and re-suspended in equal volumes of 0.85 % (w/v) NaCl. A 1 % (v/v) ( $\sim 10^6$  cfu/mL) *S. mutans* NCIMB 702062 suspension was prepared in SSF for all experiments. 800 µL of *S. mutans* NCIMB 702062 suspension was used to start the *S. mutans* NCIMB 702062 biofilms at day zero. Used SSF was replaced by sterile SSF every day for 5 days to maintain the viability of the *S. mutans* NCIMB 702062 biofilms, by providing nutrients. Biofilm formation, biofilm mass and *S. mutans* NCIMB 702062 viability were evaluated

daily for a period of 5 days. The experiment was performed with  $n = 4$ . The method for determining biofilm mass and *S. mutans* NCIMB 702062 viability is detailed in the following sections.

### 6.3.3 Set-up of the probiotic-biofilm model

A transwell system was used to isolate the *S. mutans* NCIMB 702062 biofilm from the *Lactobacillus* treatment (**Fig 6.1**). The transwell inserts (1  $\mu\text{m}$  pore size and 6.4 mm diameter) were purchased from Fisher Scientific (Ottawa, ON, Canada). Overnight cultures of *S. mutans* NCIMB 702062 and *L. reuteri* were washed twice with 0.85 % (w/v) NaCl by centrifuging the cultures for 5 min at 4000 g and 4°C. The resulting pellets were collected and re-suspended in equal volumes of 0.85 % (w/v) NaCl. A 1 % (v/v) suspension corresponding to  $4.63 \times 10^6 \pm 0.89 \times 10^6$  cfu/mL of *S. mutans* NCIMB 702062 and 2 % (v/v) suspension corresponding to  $9.13 \times 10^6 \pm 0.36 \times 10^6$  cfu/mL of *L. reuteri* NCIMB 701359 were prepared in SSF for all the following experiments. 800  $\mu\text{L}$  ( $7.3 \times 10^6 \pm 0.29 \times 10^6$  cfu/well) of *S. mutans* NCIMB 702062 suspension was used to start the biofilms at day 0 in 24 well plates. 300  $\mu\text{L}$  ( $2.74 \times 10^6 \pm 0.11 \times 10^6$  cfu/well) of *L. reuteri* NCIMB 701359 were added to the apical side of the transwell inserts daily for 5 days. SSF was prepared according to our previous study (composition presented in **Table 4.1**). Sterile SSF was replaced every 24 h to maintain *S. mutans* NCIMB 702062 biofilm viability. *S. mutans* NCIMB 702062 without any probiotic treatment was used as a control.



**Figure 6.1:** Biofilm set up for *S. mutans* NCIMB 702062 biofilm formation with probiotic bacteria *L. reuteri* NCIMB 701359 treatment using transwell inserts and 24 well plates. *S. mutans* NCIMB 702062 biofilm was formed on the basolateral side while the probiotic treatment was added on the apical side.

### 6.3.4 Determining the effect of *L. reuteri* NCIMB 701359 on *S. mutans* NCIMB

#### 702062 biofilm mass and viability

We investigated the potential of *L. reuteri* NCIMB 701359 to reduce *S. mutans* NCIMB 702062 viable cell counts and biofilm mass. At each time point (0, 1, 2, 3, 4, 5 days), the biofilms were washed twice with 0.85 % (w/v) NaCl. Following washing, 800 µL of 0.85 % (w/v) NaCl were added to each well and the films were scraped off using cell scrapers. *S. mutans* NCIMB 702062 viability was determined using standard colony forming units (cfu). 0.85% (w/v) NaCl was used to attain 10-fold serial dilutions. Each sample was streaked on MSB agar daily and incubated at 37°C and 5% CO<sub>2</sub> for 48 h. To determine the biofilm mass, the resulting suspensions were measured for turbidity at 620 nm using a spectrophotometer. Samples were investigated with n = 4 to ensure accuracy and reproducibility.

### 6.3.5 Determining the effect of *L. reuteri* NCIMB 701359 on *S. mutans* NCIMB

#### 702062 biofilm density

*S. mutans* NCIMB 702062 grow in oral biofilms and increase in proportion, thereby, increasing their density. We investigated whether *L. reuteri* NCIMB 701359, in our biofilm model, could reduce the density of *S. mutans* NCIMB 702062 aggregates in



the biofilm. The *S. mutans* NCIMB 702062 biofilms were washed twice with 0.85 % (w/v) NaCl, at each time point for 5 days. Following washing, they were viewed under a light microscope (Leica Microsystems Inc., Ontario, Canada ) and micrographs were collected for determining biofilm density, using a magnification of 40X. The densities of the *S. mutans* NCIMB 702062 aggregates in the biofilms were quantified by Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Maryland, USA). Briefly, all the radiographs were transformed to 8-bit images. The 8-bit images were further transformed to binary images. These binary images were analysed to evaluate the area covered by *S. mutans* NCIMB 702062 aggregates in the biofilms. Samples were investigated with  $n = 4$  to ensure accuracy and reproducibility.

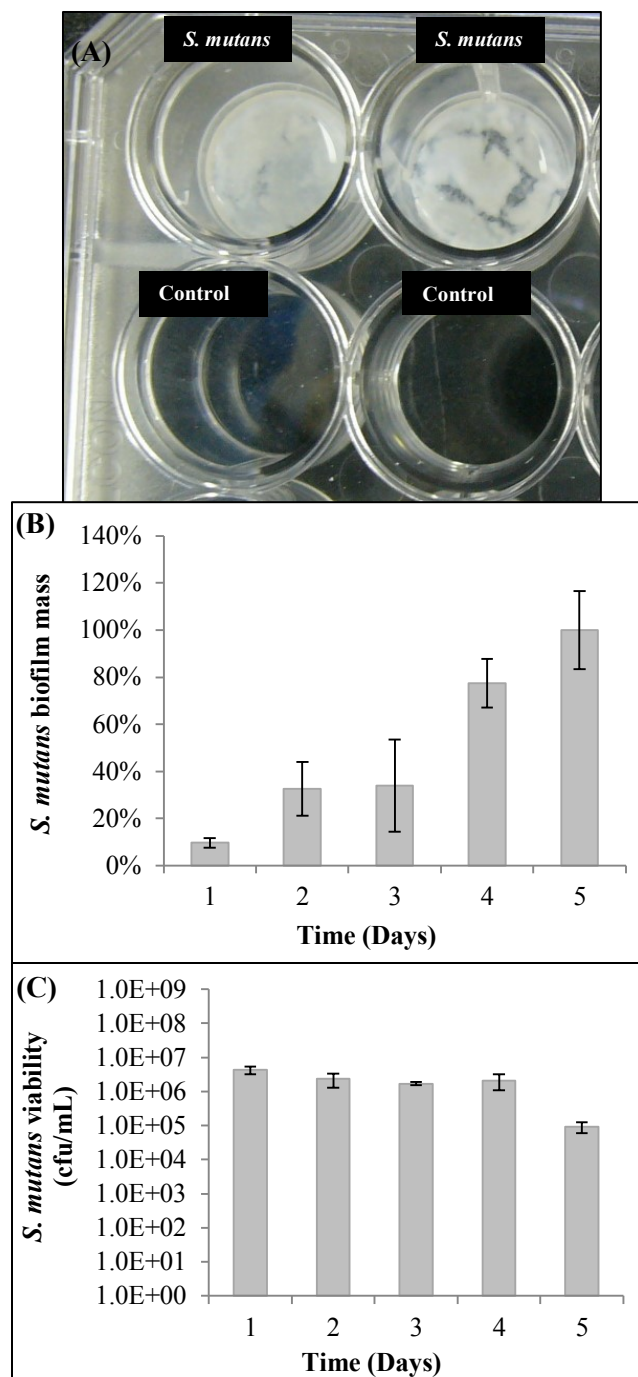
#### **6.3.6 Statistical analysis**

Experimental results are expressed as mean  $\pm$  standard error of the mean (SEM). Samples with  $n = 4$ , were measured to ensure accuracy and reproducibility. Statistical analysis was carried out using Statistical Product and Service Solutions (SPSS) Version 17.0 (IBM Corporation, NY, USA). Comparison of the means was performed using the independent student's t-test. Statistical significance was set at  $p < 0.05$  and  $p$  -values less than 0.01 were considered highly significant.

## 6.4 Results

### 6.4.1 *S. mutans* NCIMB 702062 biofilm formation *in vitro* using the transwell model

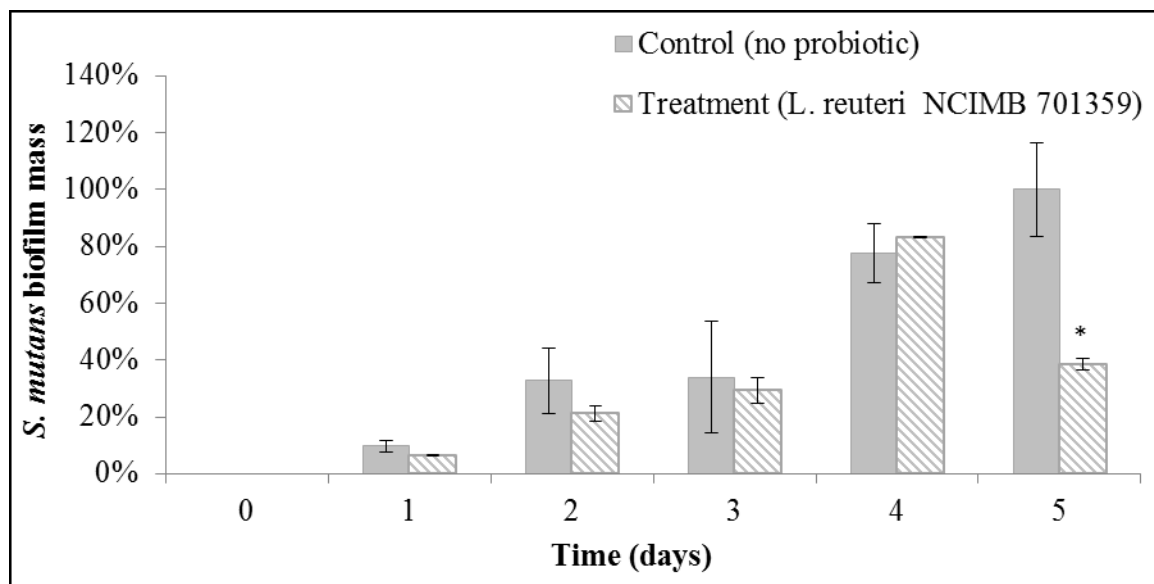
An *in vitro* model to study biofilm formation would aid in evaluating probiotic *S. mutans* NCIMB 702062 biofilm propagation. We established an *in vitro* biofilm model using 24 well plates and transwell inserts. We monitored *S. mutans* NCIMB 702062 biofilm mass for 5 days. *S. mutans* NCIMB 702062 growth in the formed biofilms was evaluated. The *S. mutans* NCIMB 702062 biofilms were stable for 5 days *in vitro* (**Fig 6.2**). The biofilm formation was also evident visually when wells containing *S. mutans* NCIMB 702062 were compared with ones without *S. mutans* NCIMB 702062 (**Fig. 6.2**). The viability of *S. mutans* NCIMB 702062 in the biofilm at day 5 was  $1.36 \times 10^5 \pm 0.40 \times 10^5$  cfu/well.



**Figure 6.2:** *S. mutans* NCIMB 702062 biofilm formation, monitored over 5 days. (A) *S. mutans* biofilm following 24 hours. (B) Biofilm mass and (C) *S. mutans* viability monitored for 5 days. Results are presented as mean  $\pm$  SEM, n=4.

#### 6.4.2 *L. reuteri* NCIMB 701359 can reduce *S. mutans* NCIMB 702062 biofilm mass

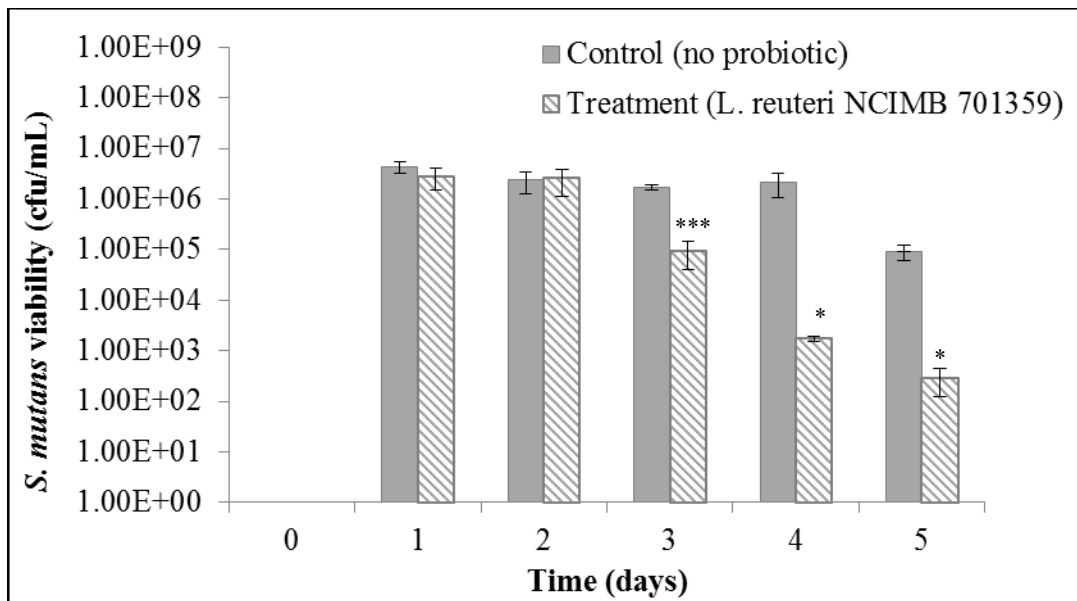
Previous studies by our group suggest that *L. reuteri* NCIMB 701359 is a potent inhibitor of *S. mutans* NCIMB 702062. In this work, we investigated its potential in inhibiting *S. mutans* NCIMB 702062 biofilm formation. Incubation of *S. mutans* NCIMB 702062 with *L. reuteri* NCIMB 701359 demonstrated a significant decrease ( $p=0.01$ ) in *S. mutans* NCIMB 702062 biofilm mass as compared to the non-treated biofilms. The increase in biofilm mass of *L. reuteri* treated *S. mutans* NCIMB 702062 was  $39 \pm 2\%$  as compared to non-treated biofilm which was at  $100 \pm 17\%$ , at day 5 (**Fig. 6.3**).



**Figure 6.3:** Reduction of *S. mutans* NCIMB 702062 mass in the biofilms, when treated with *L. reuteri* NCIMB 701359 compared to controls (no probiotic) monitored for 5 days. Significant reductions were observed at day 5. Results are presented as mean  $\pm$  SEM,  $n = 4$ . Comparisons of the means were performed using the independent student t-test analysis where  $p < 0.05$  was considered to be significant (\*).

#### 6.4.3 *L. reuteri* NCIMB 701359 can reduce *S. mutans* NCIMB 702062 counts

A decrease in *S. mutans* NCIMB 702062 viability in the biofilms treated with *L. reuteri* would prove beneficial in limiting biofilm propagation. We compared the *S. mutans* NCIMB 702062 viability with and without probiotic treatment. There was a statistically significant decrease ( $p = 0.02$ ) in *S. mutans* NCIMB 702062 counts in the biofilms treated with *L. reuteri* NCIMB 701359 at day 5. The most significant difference ( $p = 0.0001$ ) was noted following three days of probiotic treatment. We observed that the *L. reuteri* treated *S. mutans* NCIMB 702062 biofilms had  $2.80 \times 10^2 \pm 1.57 \times 10^2$  cfu/mL of *S. mutans* NCIMB 702062 counts, as compared to non-treated biofilms that contained  $9.14 \times 10^4 \pm 3.20 \times 10^4$  cfu/mL at day 5 (Fig. 6.4).

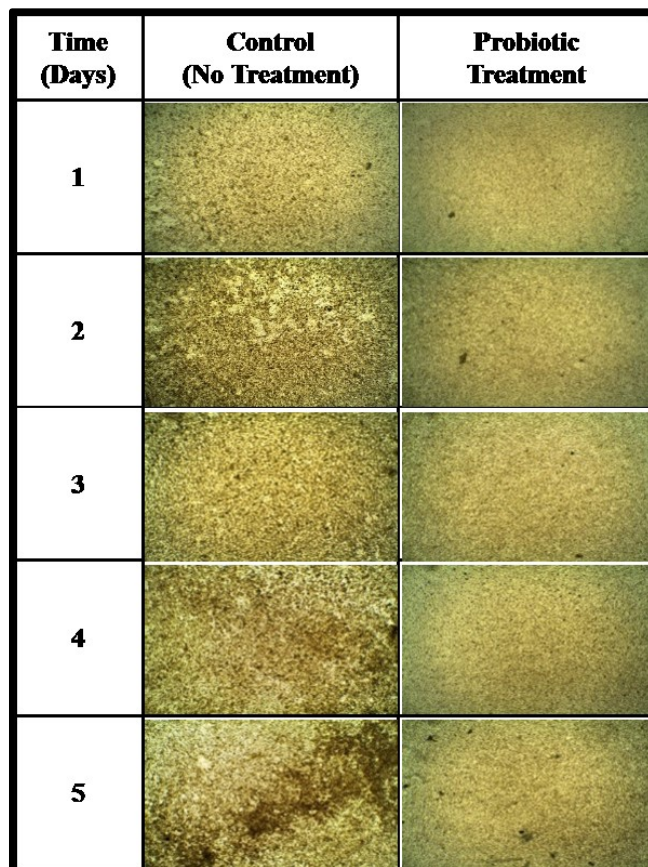


**Figure 6.4:** Reduction of *S. mutans* NCIMB 702062 viability in the biofilms, when treated with *L. reuteri* NCIMB 701359 compared to controls monitored for 5 days. Significant reductions were observed from day 3 to day 5. Results are presented as mean  $\pm$  SEM,  $n = 4$ . Comparisons of the means were performed using the independent student t-test analysis where  $p < 0.001$  was considered to be highly significant (\*\*\*) and  $p < 0.05$  to be significant (\*).

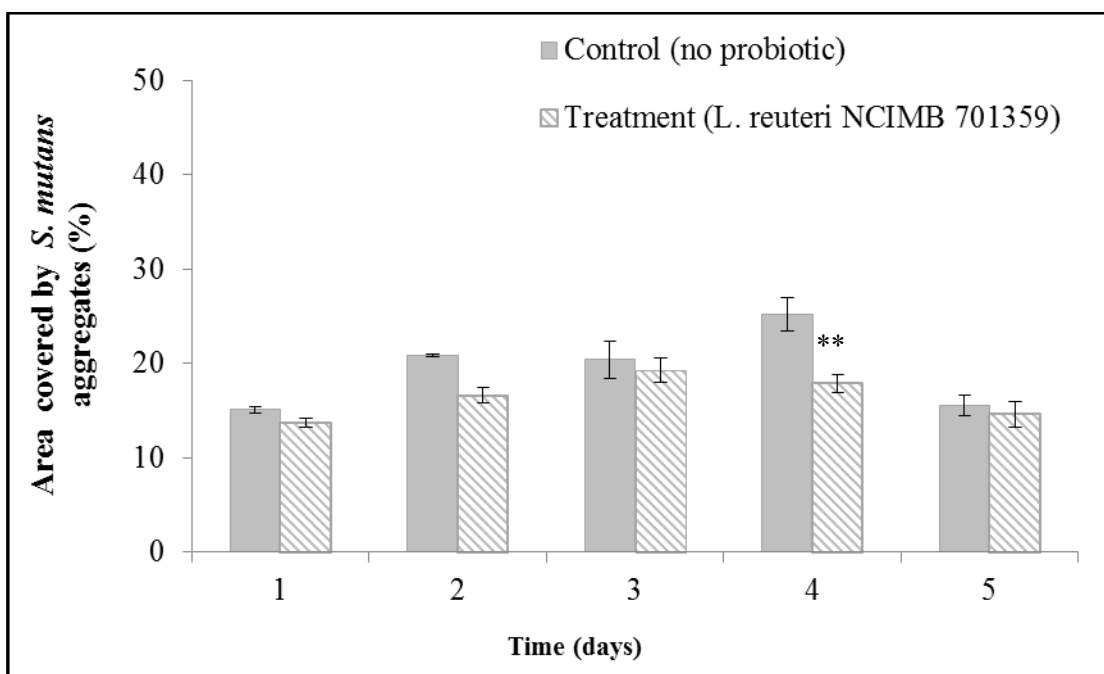
#### 6.4.4 *L. reuteri* NCIMB 701359 can reduce the density of *S. mutans* NCIMB

##### 702062 aggregates

*L. reuteri* NCIMB 701359 can also limit *S. mutans* NCIMB 702062 aggregates in biofilms. *S. mutans* NCIMB 702062 biofilm micrographs when analysed by image J, demonstrated a significant difference in *S. mutans* NCIMB 702062 aggregates between non-treated and probiotic treated biofilms. As seen in **Fig. 6.5**, less area was covered by *S. mutans* NCIMB 702062 aggregates treated with *L. reuteri* NCIMB 701359 ( $7.89 \pm 0.97$  %) when compared to non-treated ( $25.20 \pm 1.74$  %), a significant difference observed at day 4 ( $p=0.01$ ) (**Fig. 6.6**).



**Figure 6.5:** Reduction in the density of *S. mutans* NCIMB 702062 aggregates with probiotic *L. reuteri* NCIMB 701359 treatment. Light microscopy pictures (40X) of *S. mutans* NCIMB 702062 biofilm monitored for 5 days with and without *L. reuteri* NCIMB 701359 treatment.



**Figure 6.6:** Reduction in density of *S. mutans* NCIMB 702062 aggregates with *L. reuteri* NCIMB 701359 treatment. The image analysis of the *S. mutans* NCIMB 702062 biofilm micrographs demonstrates significant reduction of *S. mutans* NCIMB 702062 aggregates with *L. reuteri* NCIMB 701359 treatment. Results are presented as mean  $\pm$  SEM,  $n = 4$ . Comparisons of the means were performed using the independent student t-test analysis where  $p < 0.01$  was considered to be significant (\*\*).

## 6.5 Discussion

The oral cavity houses a wide variety of microorganisms including some housed in oral biofilms, also known as dental plaque [36]. Oral biofilm is a collection of microorganisms with increased proportions of exopolysaccharides which are formed by a much researched phenomenon of quorum sensing [36]. Bacterial cells, upon reaching a certain density, express quorum sensing and manifest altered gene expression via signal transduction, to adapt to their new environment [274]. Moreover, changes in the oral environment due to factors such as poor oral hygiene and reduced salivary flow, allow the proliferation of oral microorganisms which have the ability to cause opportunistic

infections such as dental caries [275]. *S. mutans*, an acidogenic and aciduric organisms is one of these opportunistic pathogens [275]. Although antimicrobial agents such as chlorhexidine mouthwashes are used to decrease the counts of oral pathogens, the most effective strategy to remove oral biofilm is physical disruption via techniques such as tooth brushing and flossing [5,70]. However, patient compliance is limited for both prevention and treatment modalities and hence success of the current therapeutic methods is inadequate.

A therapeutic that would prevent and treat dental caries, should not only inhibit the viability of oral pathogens such as *S. mutans* but, more importantly, limit *S. mutans* oral biofilm formation. Recently, probiotic bio-therapeutics have gained significant interest for the prevention and treatment of oral diseases [144]. Our group has previously demonstrated that probiotic bacteria can reduce *S. mutans* counts in planktonic cultures, with *L. reuteri* NCIMB 701359 as one of the best inhibitors. Several mechanisms have been associated with probiotic inhibition of *S. mutans* such as nutrient competition for sucrose, probiotic co-aggregation with *S. mutans*, capability of probiotic bacteria to attach to oral epithelial cells, probiotic nitric oxide production and probiotic H<sub>2</sub>O<sub>2</sub> production. Interestingly, in our previous study, we found that *L. reuteri* NCIMB 701359 was one of the best H<sub>2</sub>O<sub>2</sub> producers, a well-established antimicrobial agent. H<sub>2</sub>O<sub>2</sub> production via *L. reuteri* NCIMB 701359 would be beneficial in limiting both *S. mutans* counts and *S. mutans* biofilm. In this study, we investigated the capability of *L. reuteri* NCIMB 701359 to reduce *S. mutans* biofilm propagation as a candidate for dental caries therapeutic.

Several *in vitro* models have been used to study oral biofilms, including glass slides, coverslips and hydroxyapatite beads [233,276,277]. We opted to evaluate *S.*



*mutans* biofilm using a transwell system using a protocol adapted from a study by Stander *et al.* to separate the probiotic bacteria from the pathogen [198]. The rationale behind this approach was to evaluate the reduction in *S. mutans* biofilm mass and *S. mutans* aggregates in the absence of probiotic bacterial biofilm contribution. The work presented here is also the first to evaluate the impact of probiotic bacteria on *S. mutans* biofilm formation under simulated oral conditions, using SSF. To further mimic oral conditions, probiotic bacteria were added to the model daily, as these have limited residence time in the oral cavity, and would require daily consumption of probiotics *in vivo* using microcapsules and oral thin films [140,269].

We successfully established *S. mutans* biofilm in 24 well plates and maintained their viability for 5 days (**Fig 6.2**). The biofilm mass increased gradually for 5 days, however, we observed a reduction in *S. mutans* viability following 4 days. As aforementioned, quorum sensing might play a role in the observed increase in biofilm mass, via production of extracellular matrix in which the bacteria are encased [274,278,279]. Moreover, quorum sensing has growth arresting and cell death effects, which could be the reason behind the observed decrease in *S. mutans* viability in non-treated biofilms, following 5 days [274,278,279]. In addition, the loss in *S. mutans* viability could also be due to the fact that the surface area needed for further growth of *S. mutans* was limited. The set up used was a static model which does not allow the movement of bacteria in the growth media. A continuous flow system that would resemble the oral cavity more closely would likely be a better model for investigating oral biofilms. However, a continuous flow system is only suitable for long term studies and is not cost effective. Research groups such as Goeres *et al.* and Gorynia *et al.* are

amongst very few groups who are working towards establishing such a continuous flow system that would resemble an oral biofilm more closely [280,281]. Our study focuses on *L. reuteri* NCIMB 701359 inhibition of *S. mutans* biofilm formation and it was important to separate both the species, as aforementioned. Hence an optimized model with continuous flow system that would allow for the separation of two bacterial species could be beneficial for future investigations.

To assess the ability of *L. reuteri* NCIMB 701359 for limiting *S. mutans* biofilm propagation, we investigated the probiotic effect on biofilm mass, *S. mutans* viability in the formed biofilms and the area covered by *S. mutans* aggregates using a transwell system. As expected, with probiotic *L. reuteri* NCIMB 701359 treatment, there was a significant decrease in *S. mutans* biofilm formation. We observed a significant decrease ( $p = 0.01$ ) in biofilm mass at day 5 with probiotic treatment in SSF. Similar results were also observed with *S. mutans* counts in biofilms with treatment. Interestingly, reduced *S. mutans* counts were observed with probiotic treatment following just one day of co-incubation. Lastly, the formation of *S. mutans* aggregates were assessed following a similar approach used by Sharma *et al.* [282]. We observed significant reduction of *S. mutans* aggregates with probiotic treatment.

One of the most important hurdles in the investigation of oral biofilms with multiple species is the choice of the *in vitro* models. As stated earlier, the use of a continuous flow system with multiple species would be extremely valuable for these investigations. However, the use of transwell systems allows investigations into dual species models and hence we could successfully investigate *L. reuteri* inhibition of *S. mutans*. Future *in vitro* and *in vivo* studies should include investigation of biofilm with

multiple oral pathogens in biofilms, studies over a long period of time and optimization of probiotic dose.

This study is the first to investigate the inhibition of *S. mutans* biofilm by probiotic *L. reuteri* NCIMB 701359, using a transwell system. The developed *in vitro* system should be used to screen all previously screened probiotic bacteria for planktonic *S. mutans* inhibition to assess their capability to limit biofilm formation [181,223]. In addition, this study opens avenues for investigating pathogen interactions with other bacterial species, mammalian cells and drug effects, *in vitro*. Lastly, the use of SSF is pivotal to understand *in vivo* conditions more closely. This is the first study to investigate biofilm formation and inhibition under simulated oral conditions. Finally from our results, we conclude that *L. reuteri* NCIMB 701359 holds great potential as a dental caries therapeutic as it demonstrated the reduction of both planktonic *S. mutans* and *S. mutans* biofilms.

## **6.6 Acknowledgments**

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## **6.7 Conflict of interest**

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

**CHAPTER 7: *LACTOBACILLUS FERMENTUM* NCIMB 5221 AS A PERIODONTAL DISEASE BIO-THERAPEUTIC: *IN VITRO* AND *IN VIVO* INVESTIGATIONS USING F344 RATS**

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**Preface:** As studied in previous chapters and earlier studies, *Lactobacillus fermentum* NCIMB 5221 have demonstrated the potential to inhibit oral pathogens and host immunomodulation. Moreover, periodontal disease is characterised by chronic inflammation and loss of connective tissue such as alveolar bone. The aim of the present investigation was to evaluate *L. fermentum* NCIMB 5221 for antioxidant activity, ferulic acid and nitric oxide production that would prove beneficial for reducing inflammation. *L. fermentum* NCIMB 5221 was then evaluated for its potential in modulating inflammatory cytokine production by inflamed pre-osteoblasts. Potential of probiotic bacteria to enhance bone growth was also evaluated. We investigated the probiotic effects on F344 rats for serum C-reactive protein, serum alkaline phosphatase and serum calcium levels using Hitachi 911 automated clinical chemistry autoanalyzer. Femur and mandibular bone mineral density and alveolar bone support using radiography and image j analysis.

## 7.1 Abstract

Periodontal disease is a chronic condition with a high prevalence of almost 20% in worldwide adults, as reported by the World Health Organization. The host immune system responds to combat disease by releasing inflammatory cytokines that result in the destruction of host connective tissues. These contribute to a compromised tooth support system and dissemination of disease. Current therapeutic strategies focus on prevention. With the onset of periodontitis, characterised by bone and periodontal ligament loss, invasive surgical therapies are essential. Probiotic biotherapeutics have shown potential for periodontal therapy. The aim of this study was to investigate probiotic *Lactobacillus fermentum* NCIMB 5221, for its immune-modulatory and regenerative effects on preosteoblasts and its potential to modulate bone metabolism in F344 rats. The results indicate that *L. fermentum* NCIMB 5221 significantly reduced ( $p = 0.00008$ ) the pro-inflammatory cytokines IL-1 $\beta$  level ( $0.029 \pm 0.001$  pg/ $\mu$ g) as compared to non-treated ( $0.111 \pm 0.015$  pg/ $\mu$ g) at 24 h and IL-6 level ( $2.41 \pm 0.21$  pg/ $\mu$ g,  $p=0.004$ ) as compared to non-treated ( $3.256 \pm 0.13$  pg/ $\mu$ g) at 4 h in inflamed preosteoblasts. Interestingly, the IL-12p70 levels, found in decreased levels in disease, were elevated (non-significant) with probiotic treatment. Most importantly, probiotic treatment significantly ( $p < 0.005$ ) promoted preosteoblast growth ( $\sim 140$  % compared to control at 72 h). As for their effects in F344 rats, there was no significant difference in serum C-reactive protein, alkaline phosphatase levels, femur and mandibular bone mineral densities and alveolar bone support. However, the probiotic treatment significantly reduced levels of serum calcium ( $02.87 \pm 0.036$  mmol/L,  $p = 0.02$ ). Taken together, the results demonstrate the potential of probiotic *L. fermentum* NCIMB 5221 as a periodontal disease biotherapeutic.

## 7.2 Introduction

The World Health Organization estimates that 15-20% of the world's adult population is affected by severe periodontitis [2]. Moreover, although outdated, a previous study suggests that 50 -100% of the population has gingivitis [2,34]. Periodontal diseases include both gingivitis and periodontitis. Gingivitis is a milder form of periodontal disease that is characterised by inflamed and bleeding gingiva. Gingivitis, a reversible condition, when not treated optimally, can progress to an irreversible condition, periodontitis [3,4,283,284]. Periodontitis is characterised by a destruction of the supporting structures of the tooth such as alveolar bone and periodontal ligaments (PDL). The current therapeutic measures focus on prevention such as optimal oral hygiene via tooth brushing, flossing, mouth washes, scaling and root planing. These impede the bacterial stagnation and growth in susceptible tooth surfaces such as pits and fissures [3,4,283]. Antibiotics have also been used as therapeutics [285]. However, with disease progression, more invasive surgeries are required for debridement of plaque and calculus in deeper subgingival areas, to reduce periodontal pockets and to regenerate lost connective tissue [3]. However, the success of such therapy is limited by the socio-economic conditions of the patient which hamper timely diagnosis and treatment [286]. Moreover, current statistics and the association of periodontal disease with diabetes, cardiovascular diseases and pre-term babies, call for substitute therapies [121,287].

To develop an alternative therapeutic, it is crucial to comprehend the events that are responsible for and contribute to periodontal disease. In terms of disease etiology, inflammation plays an important role. The abundance of periodontal pathogens and their by-products activates the host immune system and the hyperactive immune state leads to

the release of various cytokines in the gingival crevicular fluid (GCF). These cytokines, in turn, contribute to pathogen elimination but, in the process, destroy host connective tissue [35,288]. Proper regulation of these events is required for minimizing host tissue destruction. Periodontal tissue destruction includes the destruction of the PDL and the alveolar bone, ultimately leading to tooth exfoliation and the spread of disease to surrounding structures giving rise to other complications such as cellulites or osteomyelitis [3]. Moreover, the compromised periodontal status facilitates the entry of other oral commensals such as *Streptococcus* species that have the ability to cause endocarditis with potential to be fatal [286,289].

Probiotic bacteria have potential as an alternative or adjunct therapy for periodontitis. Probiotic bacteria are naturally found in foods and are safe and cost effective. Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”, according to the Food and Agriculture Organization of the United Nations (FAO) and WHO [20]. Most importantly, probiotic bacteria have demonstrated the capability to modulate inflammation in various diseases such as non-alcoholic fatty liver disease and *Helibacter pylori* infections [290,291]. Moreover, antioxidant molecules such as ferulic acid (FA) produced by probiotic bacteria have the potential to scavenge reactive oxygen species, key players in inflammation [178,292]. Probiotic bacteria such as *Lactobacillus fermentum* NCIMB 5221 and *Lactobacillus fermentum* NCIMB 2797 has the capability of producing FA [178]. Previously, Tomaro-Duchesneau *et al.* established that *L. fermentum* NCIMB 5221 produced greater amounts of FA as compared to *L. fermentum* NCIMB 2797. Similarly, probiotic bacteria have demonstrated both antibacterial and anti-inflammatory properties



via nitric oxide (NO) production [293]. Hence, the aim of this study was to investigate the role of *Lactobacillus fermentum* NCIMB 5221, a FA producing bacteria, for the modulation of the host immune system using *in vitro* and *in vivo* studies in F344 rats. *L. fermentum* NCIMB 5221 was evaluated for the production of anti-oxidant molecules, their effect on inflamed preosteoblasts and their effect on the growth of preosteoblasts, pivotal for periodontal tissue regeneration. This was followed by investigations into the effects of *L. fermentum* NCIMB 5221 on systemic markers and bone markers of bone remodeling.

### **7.3 Materials and methods**

#### **7.3.1 Bacterial strains and culture conditions**

De Man, Rogosa, Sharpe (MRS) broth was purchased from Fisher Scientific (Ottawa, ON, Canada). SSF was prepared as in our previous study, **Table 4.1**. All other chemicals were of high-performance liquid chromatography (HPLC) or analytical grade and purchased from commercial sources. Water was purified with an EasyPure reverse osmosis system and a NanoPure Diamond Life Science (UV/UF) ultrapure water system from Barnstead (Dubuque, IA, USA). Simulated salivary fluid (SSF) was prepared according to our previous work [294].

*Lactobacillus fermentum* NCIMB 5221 and *Streptococcus mutans* NCIMB 702062 were purchased from the National Collection of Industrial Food and Marine Bacteria (NCIMB) (Aberdeen, Scotland, UK). Both the bacterial strains were kept at -80°C as 20% (v/v) glycerol stocks. A MRS agar plate was streaked and incubated at 37°C for 24 h with 5% CO<sub>2</sub> to ensure the purity of the culture. One colony from the MRS agar plate was

inoculated in MRS broth for 24 h at 37°C. A 1% (v/v) inoculum was subcultured into MRS broth and incubated at 37°C for 24 h immediately prior to any assay.

### **7.3.2 Mammalian cell culture conditions**

MC 3T3-E1 subclone 14 was purchased from the American Type Culture Collection (ATCC, Virginia, USA). Minimum Essential Medium  $\alpha$  (MEM  $\alpha$ ) purchased from Invitrogen (Ontario, Canada) was supplemented with 10% (v/v) fetal bovine serum (FBS) also purchased from Invitrogen and was used to grow the MC 3T3-E1 mouse osteoblast like cells. They were allowed to grow at 37°C with 5% CO<sub>2</sub> until they reached 85% confluence and then the cells were used for experiments.

### **7.3.3 Determining bacterial cell viability**

The cell counts of *L. fermentum* NCIMB 5221 were measured using standard colony forming units (cfu). Briefly, 0.85% (w/v) NaCl was used to attain 10-fold serial dilutions and all samples were plated in triplicate on MRS agar plates to ensure accuracy and reproducibility. The plates were incubated at 37°C and 5% CO<sub>2</sub> for 48 h prior to colony counting.

### **7.3.4 Determining the production of total antioxidants, ferulic acid and nitric oxide**

#### *7.3.4.1 Ferulic acid production*

FA, an antioxidant molecule, has the ability to scavenge reactive oxygen species and can reduce inflammation [174,295]. FA production by *L. fermentum* NCIMB 5221 was determined by HPLC. 1% (v/v) overnight culture of *L. fermentum* NCIMB 5221 was used to inoculate SSF and MRS broth, both containing 1.33 mM (0.2956 mg/mL) of ethyl ferulate (ethyl 4-hydroxy-3-methoxycinnamate, EFA). *L. fermentum* NCIMB 5221 was

incubated for 24 h at 75 rpm and 37°C. The HPLC method to assess FAE activity was adapted from Tomaro-Duchesneau *et al.* [174]. Briefly, 500 µL of samples were collected at 24 h and centrifuged at 10,000 rpm for 7 min at 4°C. The supernatants were collected and acidified with 100 µL 0.35 M H<sub>2</sub>SO<sub>4</sub> and vortexed. 300 µL of 1 mM benzoic acid, an internal standard, were added to each tube, followed by addition of 100 µL of 0.7 M NaOH to neutralize the pH. Finally, the samples were stored at -20°C for further HPLC analysis.

For HPLC analysis, each sample was thawed at room temperature and filtered in two steps. A 0.8 µm syringe filter was used to filter the samples, followed by filtering using a 0.45 µm syringe filter. A Varian HPLC system equipped with a Varian 9012 tertiary pump module, refrigerated auto-sampler model 410 and UV/Vis variable wavelength detector module 9050 operated by a Varian Star 5.3 software was used for sample analysis. Phenolic compounds were separated with a protocol adapted from Shakya *et al.*, using a reverse phase HPLC Gemini-NX (5 µm, 100 mm × 4.6 mm) column (Phenomenex, Torrance, CA,) and a 4.6 mm × 2.0 mm guard column [296]. The mobile phase was composed of solvent buffer A (10 mM formic acid, pH 3.5, with NH<sub>4</sub>OH) and buffer B (100% (v/v) methanol with 5 mM ammonium formate). The solvent gradient was as follows: 0-1 min 100% buffer A, 1-5 min 0-30 % buffer B, 5-8.5 min 30-70 % buffer B, 8.5-12 min 70-100 % buffer B. UV detection was conducted at 280 nm. A flow rate of 1.0 mL/min was used and 20 µL of sample were injected. Triplicate samples were measured twice. Duplicate samples of standards were used for generating standard curves to assess the FA quantity in the samples. Both FA and EFA of concentrations 0.09, 0.18, 0.37, 0.75 and 1.5 mM were plotted against peak area with R<sup>2</sup>

= 0.990 and  $R^2 = 0.983$ , respectively. Standards were processed and analyzed exactly the same way as the test samples.

#### 7.3.4.2 Nitric oxide production

Nitric oxide (NO), like FA, can act as an anti-inflammatory molecule. 1% (v/v) of overnight *L. fermentum* NCIMB 5221 culture was used to inoculate SSF and MRS broth. The bacterial suspensions were incubated at 37°C and 75 rpm for 24 h. Following 24 h of incubation, the cultures were centrifuged at 4000 rpm for 5 min at 4°C. NO production by *L. fermentum* NCIMB 5221 was quantified using the Griess assay. The supernatant was then filtered using a 0.22 µm filter to remove any bacterial cell residues. 60 µL of supernatant were treated with 60 µL of a solution containing sulfanilamide (1% w/v) and phosphoric acid (0.5% v/v). The samples were then incubated for 10 min at 25°C. Following incubation, 60 µL of naphthylethylenediamine dihydrochloride (NED) (0.1% w/v) were added to the previous mixture and incubated for 30 min at 25°C. Triplicate samples were measured at 570 nm using a spectrophotometer. A standard curve was generated using sodium nitrite treated the exact same way. The concentrations of sodium nitrite used were 1.46, 2.92, 5.85, 11.71, 23.43, 46.87, 93.75, 187.5, 375 and 750 µM ( $R^2 = 0.990$ ).

#### 7.3.4.3 Total Antioxidant production

Antioxidant production by the probiotic *L. fermentum* NCIMB 5221 in both MRS broth and simulated oral conditions was determined via a QuantiChrom™ Antioxidant Assay Kit, purchased from BioAssay systems (CA, USA). SSF and MRS broth were inoculated with 1% (v/v) overnight culture of *L. fermentum* NCIMB 5221. The cultures were incubated for 24 h at 75 rpm and 37 °C. The bacterial supernatant was collected by

centrifugation at 4000 rpm (Napco 2028R centrifuge) for 5 min at 4 °C for antioxidant measurement. The total antioxidant production by each strain was quantified following the protocol provided with the assay kit. Briefly, 20 µL of the bacterial supernatants were incubated with 100 µL of the working reagent. Following 10 min of incubation, the absorbance of the samples was measured at 570 nm using a spectrophotometer (PerkinElmer 1420 Multilabel Counter). A standard curve was generated using Trolox and the same method at concentrations of 100, 300, 600 and 1000 µM ( $R^2 = 0.980$ ). The experiment was performed in triplicate.

#### **7.3.5: Determining the effect of *L. fermentum* NCIMB 5221 on inflamed osteoblast-like cells**

The potential of *L. fermentum* NCIMB 5221 to reduce inflammation induced in osteoblast like cells (MC 3T3) was investigated. Lipopolysaccharide (LPS) extracted from *Escherichia coli* O55:B5 (Sigma Aldrich, Ontario, Canada) was added to MC 3T3 cells to induce inflammation. To prepare the probiotic treatment, an overnight culture of *L. fermentum* NCIMB 5221 was centrifuged at 4000 rpm for 10 min at 4°C. The resulting supernatant was filtered using a 0.22 µm syringe filter to eliminate any bacterial cell residues. The supernatant was diluted to 1% (v/v) containing approximately  $10^8$  cfu/mL of probiotic bacteria in MEM  $\alpha$  with 5 µg/mL of LPS. MEM  $\alpha$  with 5 µg/mL of LPS without any probiotic supernatant and MEM  $\alpha$  without any probiotic supernatant nor LPS were used as controls. Prior to the addition of treatments or control, MC 3T3 cells were seeded at a concentration of 20,000 cells per well in 96 well tissue culture treated plates. The cells were allowed to attach for 24 h at 37°C and 5% CO<sub>2</sub>. Following LPS addition, 200 µL of probiotic treatment and controls were added to three wells each and incubated

at 37°C and 5% CO<sub>2</sub>. The cell supernatant was collected from each well following 2, 4, 8 and 24 h of treatment exposure. The samples were centrifuged for 2 min at 4,000 rpm and 4 °C and stored at -80°C for determining cytokines levels. Cytokine quantification was performed using a multiplex ELISA, proinflammatory panel 1 (mouse kit) V-PLEX generously gifted by Mesoscale discovery (Maryland, USA). Measurements were made using a Multi-Spot® Assay System with a Sector® Imager 2400 (Meso Scale Discovery®, MD, USA)

A Bradford assay was purchased from Sigma Aldrich (Ontario, Canada) to perform total protein quantification. Briefly, 5 µL of sample were incubated with 250 µL of coomassie reagent for 10 min. Triplicate samples were measured at 595 nm using a spectrophotometer. A standard curve was generated using sodium nitrite treated the exact same way. Bovine serum albumin provided in the kit was used for generating a standard curve with the following concentrations: 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg/mL ( $R^2 = 0.976$ ). The ratio of cytokine to total protein levels was calculated following the formula.

$$\text{Cytokine levels (pg/}\mu\text{g total protein)} = \text{Cytokine levels (pg/mL)} / \text{Total protein (}\mu\text{g/mL)}$$

#### **7.3.6 Determining the effect of *L. fermentum* NCIMB 5221 on osteoblast like cell growth**

We investigated the potential of *L. fermentum* NCIMB 5221 to promote the growth of preosteoblast cells (MC 3T3). An overnight culture of *L. fermentum* NCIMB 5221 was centrifuged at 4000 rpm for 10 min at 4 °C. The resulting supernatant was filtered using a 0.22 µm syringe filter to eliminate any bacterial cell residues. The supernatant from approximately 10<sup>8</sup> cfu/mL of probiotic bacteria was diluted 1% (v/v)

using MEM  $\alpha$  to be used as treatments. MEM  $\alpha$  without any probiotic supernatant was used as control. These were then used on MC 3T3 cells. Prior to the addition of treatments or control, MC 3T3 cells were seeded at a concentration of 20,000 cells per well in 96 well tissue culture treated plates. The cells were then allowed to attach for 24 h at 37°C and 5% CO<sub>2</sub>. 200  $\mu$ L of probiotic treatments and control were added to five wells each and allowed to grow for 72 h at 37 °C with 5% CO<sub>2</sub>. Cell viability assays were performed at 0, 24 48 and 72 h using a CellTiter 96® AQueous One Solution Cell Proliferation Assay purchased from Promega (Wisconsin, USA). Briefly, 100  $\mu$ L of cell culture media were treated with 20  $\mu$ L of CellTiter 96® AQueous One Solution Reagent and incubated for 1 h, following which the absorbance was measured using a Varian multiplate reader set at 490 nm. Percent cell viability was calculated according to the formula:

$$\% \text{ Cell viability}_x = (\text{Absorbance}_x / \text{Absorbance}_{24h \text{ of control}}) \times 100$$

where, % Cell viability<sub>x</sub> is the % cell viability of the desired time point, Absorbance<sub>x</sub> is the absorbance of cell supernatant at the desired time point and Absorbance<sub>24h</sub> is the absorbance of cell supernatant at 24 h.

### **7.3.7 Animals**

Five week old male F344 rats were purchased from Charles River (Wilmington, MA, USA) and were randomly housed two rats per cage. The environmental conditions of the room used to house the animals were controlled for temperature (22-24°C) and humidity. All the experimental protocols were in accordance with the Animal Care Committee of McGill University and the Canadian Council on Animal Care guidelines.

### **7.3.8 *In vivo* experimental protocol and serum collection**

The animals were allowed free access to food (standard diet) and water upon arrival to get acclimatized to the environment. Following a week of acclimatization, the rats were weighed and randomly assigned into two groups ( $n = 4$ ). The treatment group received 2 mL of *L. fermentum* NCIMB 5221 suspension in 0.85% (w/v) saline solution containing approximately  $1 \times 10^9$  cells, once daily. The control group was gavaged with 2 mL of 0.85% (w/v) saline solution once daily. The doses were administered via intragastric gavage using plastic gavage needles for a period of 38 days. Animal mass was monitored weekly. In addition, blood was collected bi-weekly following a 16 h fasting period, from the lateral saphenous vein using 23-gauge/19-mm needles into Microtainer<sup>®</sup> serum separator tubes from Becton Dickinson (Franklin Lakes, NJ, USA). To obtain serum samples, the blood was allowed to clot for 30 min followed by 5 min centrifugation at 10,000 g. The collected serum samples were then stored at  $-80^{\circ}\text{C}$  for further analysis. At the end of the treatment period the animals were sacrificed by carbon dioxide asphyxiation. Blood was collected at end point via cardiac puncture after the sacrifice. Serum was separated and stored at  $-80^{\circ}\text{C}$  for further analysis.

### **7.3.9 Alveolar bone and femur collection and storage**

Following animal sacrifice the rat heads were decapitated and the femurs removed immediately and transferred into 10% (w/v) neutral buffered formalin solution for further processing. Following 48 h of formalin fixation the alveolar bones and femurs were defleshed, keeping the samples moist at all times using phosphate buffered solution. The bone samples were then transferred into a freshly prepared 70% (v/v) ethanol solution for densitometry and x-ray processing.



### **7.3.10 Determining rat serum C-reactive protein, alkaline phosphatase and calcium levels**

The fasted serum samples obtained at end point were analysed for C-reactive protein (CRP), alkaline phosphatase (ALP) and calcium levels, important markers of inflammation and bone metabolism. The serum samples were assayed by conventional enzymatic methods on a Hitachi 911 automated clinical chemistry autoanalyzer (Roche Diagnostics, USA) using reagent kits supplied by Roche Diagnostics (Laval, QC, Canada).

### **7.3.11. Determining bone mineral density via bone densitometry**

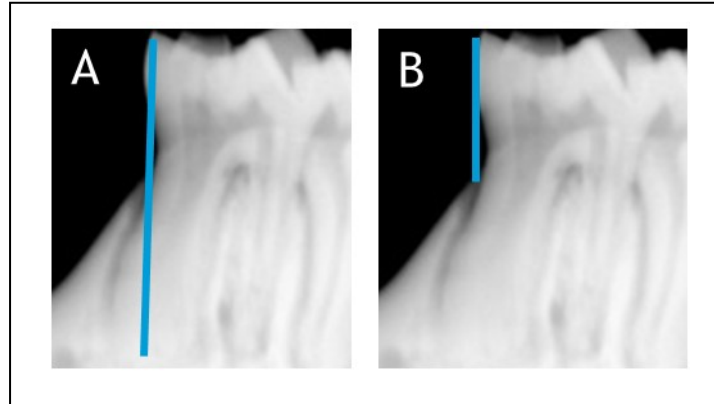
Probiotic treatment has been shown to increase bone mineral density (BMD), an important marker of bone remodeling. We investigated the effect of *L. fermentum* NCIMB 5221 on the BMD of healthy F344 rats. Both alveolar and femur bones were scanned for BMD measurements using a GE Lunar PIXImus II consisting of an X-ray tube with a 0.3 mm focal spot, and dual-energy supply of 80/35 kVp at 500  $\mu$ A. Image resolution for all the samples was kept at 0.18 x 0.18 mm (pixel size = 180  $\mu$ m)

### **7.3.12 Determining alveolar bone support via X-ray**

The onset of periodontitis provokes the host immune system and contributes to alveolar bone destruction. We investigated the effect of *L. fermentum* NCIMB 5221 on alveolar bone resorption. The alveolar bones were radiographed using a Kubtec XPERT 80 with 24.8 kV/735  $\mu$ A dual energy supply. The magnification for all the samples was kept constant at 5X. The images were analysed using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>,

1997-2011). The X-ray images (**Figure 7.1**) were analysed to assess the alveolar bone support as a measurement of bone resorption, according to the formula:

$$\text{Bone support} = \text{Total tooth length} - \text{Distance from tooth apex to alveolar bone crest}$$



**Figure 7.1:** Quantification of alveolar bone support. (A) Total tooth length and (B) Distance from tooth apex to alveolar bone crest.

### 7.3.13 Statistical analysis

Experimental results are expressed as mean  $\pm$  standard error of the mean (SEM). Statistical analysis was carried out using SPSS Version 17.0 (Statistical Product and Service Solutions, IBM Corporation, New York, NY, USA). Independent student t-test, general linear model and Tukey's post-hoc analysis were performed to compare means between controls and treatment groups. Linear regression was performed for generating standard curves. Statistical significance was set at  $p = 0.05$  and  $p$ -values less than 0.01 were considered highly significant.

## 7.4 Results

### 7.4.1 Anti-inflammatory properties of *L. fermentum* NCIMB 5221

Antioxidants scavenge reactive oxygen species and play an important role in combatting host inflammation. *L. fermentum* NCIMB 5221 has demonstrated the ability to produce anti-oxidant compounds [292]. Investigations were performed to quantify the amount of antioxidant molecules secreted by *L. fermentum* NCIMB 5221 in both MRS broth and under simulated oral conditions. *L. fermentum* NCIMB 5221 demonstrated  $461.22 \pm 08.02$   $\mu$ M Trolox equivalents of total antioxidant activity by  $\sim 10^9$  cfu/mL probiotics in MRS broth, compared to  $5.57 \pm 0.37$   $\mu$ M Trolox equivalents in SSF with associated probiotic  $5.17 \pm 0.32 \times 10^7$  cfu/mL. In terms of FA production, *L. fermentum* NCIMB 5221 produced  $961.37 \pm 42.15$   $\mu$ M of FA in MRS broth compared to  $676.63 \pm 197.51$   $\mu$ M of FA in SSF. Investigations into NO production by *L. fermentum* NCIMB 5221, demonstrated a production of  $41.81 \pm 9.08$   $\mu$ M of NO in MRS compared to a production of  $7.23 \pm 0.18$   $\mu$ M NO in SSF (**Table 7.1**). Interestingly, the total antioxidant activity and production of FA and NO in MRS broth via *L. fermentum* NCIMB 5221 was significantly different from production of these in SSF.

**Table 7.1:** Anti-inflammatory molecules produced by *L. fermentum* NCIMB 5221, following 24 h of incubation in simulated salivary fluid and MRS. Means were compared for values corresponding to  $10^9$  cfu to generate p values using independent student t-tests.  $P < 0.05$  was considered significant.

	SSF observed	SSF normalized	MRS Observed	MRS normalized	p-value
Cell viability	$5.17 \pm 0.32 \times 10^7$ cfu/mL	$10^9$ cfu	$1.01 \pm 0.13 \times 10^9$ cfu/mL	$10^9$ cfu	—
Ferulic acid ( $\mu$ M)	$676.63 \pm 197.51$	$13021.70 \pm 3650.37$	$961.37 \pm 42.15$	$977.01 \pm 97.18$	0.081
Nitric oxide ( $\mu$ M)	$7.2342 \pm 00.18$	$141.42 \pm 11.53$	$41.81 \pm 9.08$	$41.92 \pm 3.86$	0.003**
Antioxidant ( $\mu$ M Trolox equivalent)	$5.57 \pm 0.37$	$108.51 \pm 17.99$	$461.22 \pm 08.02$	$474.99 \pm 70.95$	0.034*

Results are presented as mean  $\pm$  SEM, n=3 where \*\*p < 0.01 and \*P < 0.05.

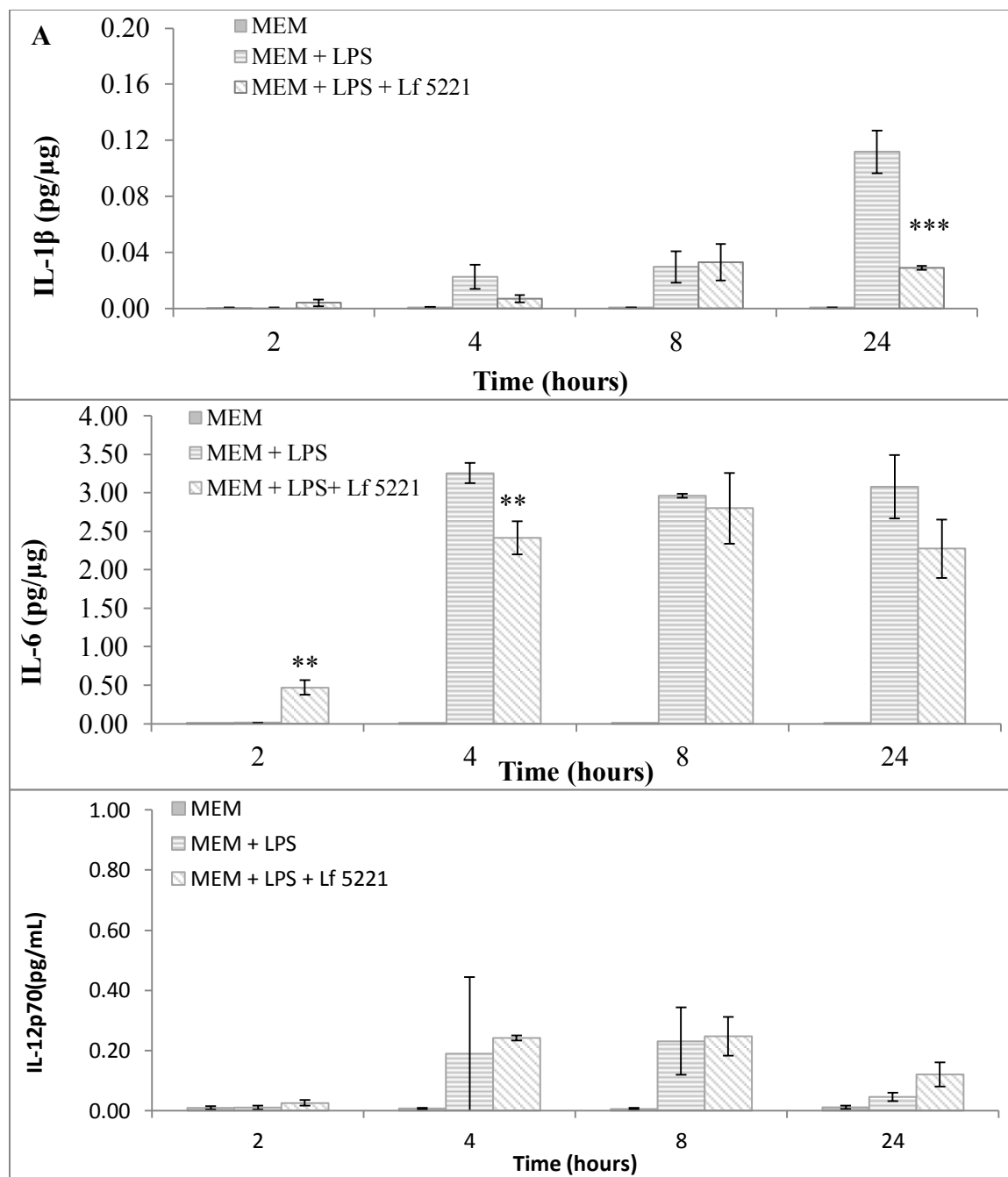
#### 7.4.2 *In vitro* effect of *L. fermentum* NCIMB 5221 on osteoblast inflammation

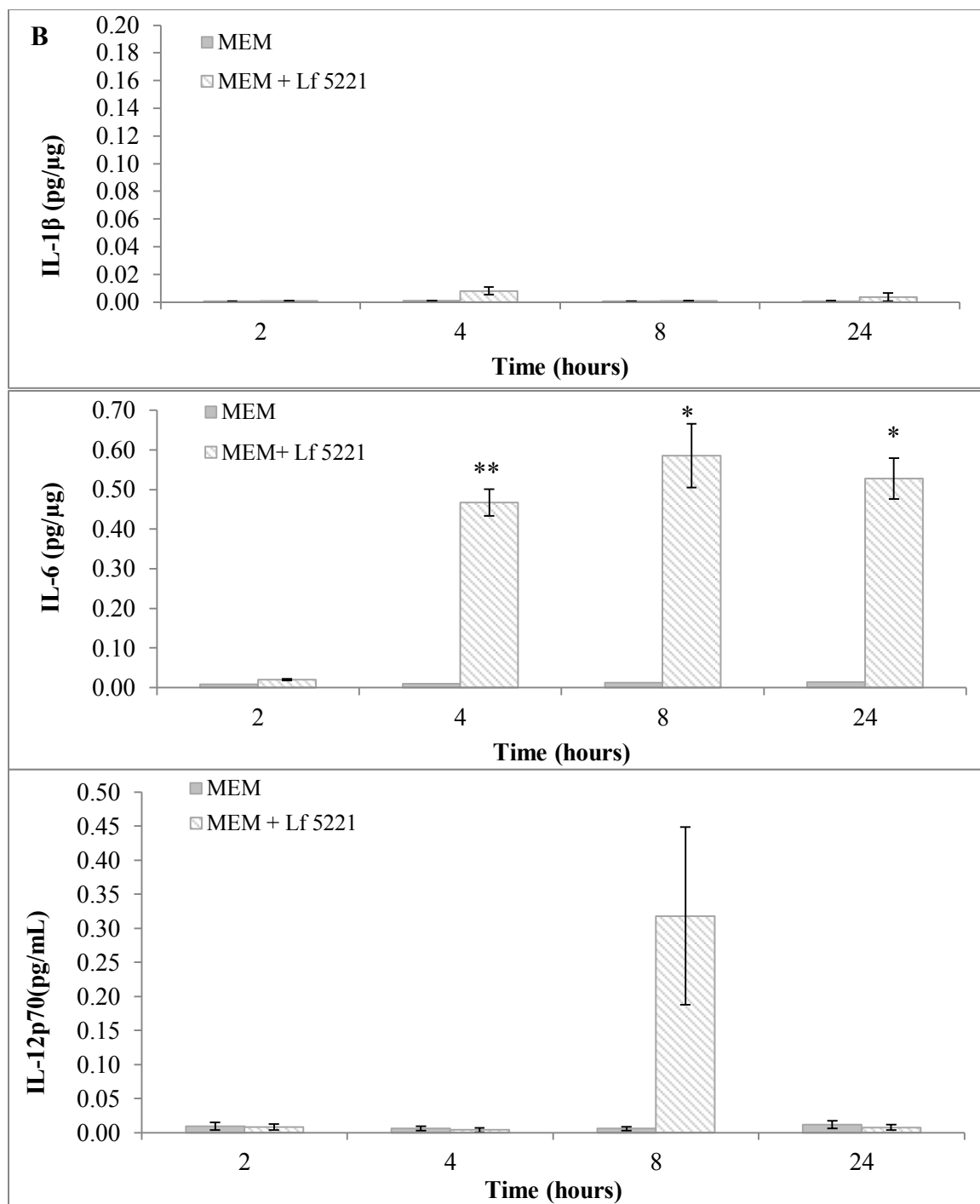
FA-producing compounds have demonstrated anti-inflammatory characteristics [295]. We investigated the effects of FA-producing *L. fermentum* NCIMB 5221 on inflamed osteoblast like cells. *L. fermentum* NCIMB 5221 treatment reduced the levels of the inflammatory cytokine IL-1 $\beta$  ( $0.0289 \pm 0.0014$  pg/ $\mu$ g), significantly ( $p < 0.05$ ), secreted by inflamed MC 3T3 cells, as compared to non-treated cells ( $0.1118 \pm 0.0153$  pg/ $\mu$ g) following 24 h. Interestingly, a significant difference was not observed in IL-1 $\beta$  levels in non-inflamed MC 3T3 cells with *L. fermentum* NCIMB 5221 treatment ( $0.0036 \pm 0.0029$  pg/ $\mu$ g) as compared to non-treated cells ( $0.0008 \pm 6.7526$  pg/ $\mu$ g) at 24 h or at any time point (**Figures 7.2B**).

A similar trend was also observed for IL-6 production by inflamed MC 3T3 cells. *L. fermentum* NCIMB 5221 treatment reduced the levels of the inflammatory cytokine IL-6 ( $2.41 \pm 0.21$  pg/ $\mu$ g) significantly ( $p < 0.05$ ), secreted by inflamed MC 3T3 cells, as compared to non-treated cells ( $3.26 \pm 0.13$  pg/ $\mu$ g) following 4 h (**Figures 7.2 A**). A significant difference was observed in IL-6 levels in non-inflamed MC 3T3 cells with *L. fermentum* NCIMB 5221 treatment as compared to non-treated cells at 4, 8 and 24 h (**Figures 7.2 B**).

Previous studies have demonstrated increased levels of IL-12p70 observed following periodontal therapy [297]. *L. fermentum* NCIMB 5221 treatment elevated the levels of the inflammatory cytokine IL-12p70 non-significantly ( $0.1209 \pm 0.0399$  pg/ $\mu$ g), secreted by inflamed MC 3T3 cells, as compared to non-treated cells ( $0.0458 \pm 0.0321$  pg/ $\mu$ g) following 24 h. A similar trend was also observed following 2 and 4 h. In addition, a significant difference was not observed in IL-12p70 levels in non-inflamed

MC 3T3 cells with *L. fermentum* NCIMB 5221 treatment as compared to non-treated cells at any time point (**Figures 7.2**). TNF- $\alpha$  and IL-10 levels were not detectable in *L. fermentum* NCIMB 5221 treated and non-treated MC 3T3 cells.

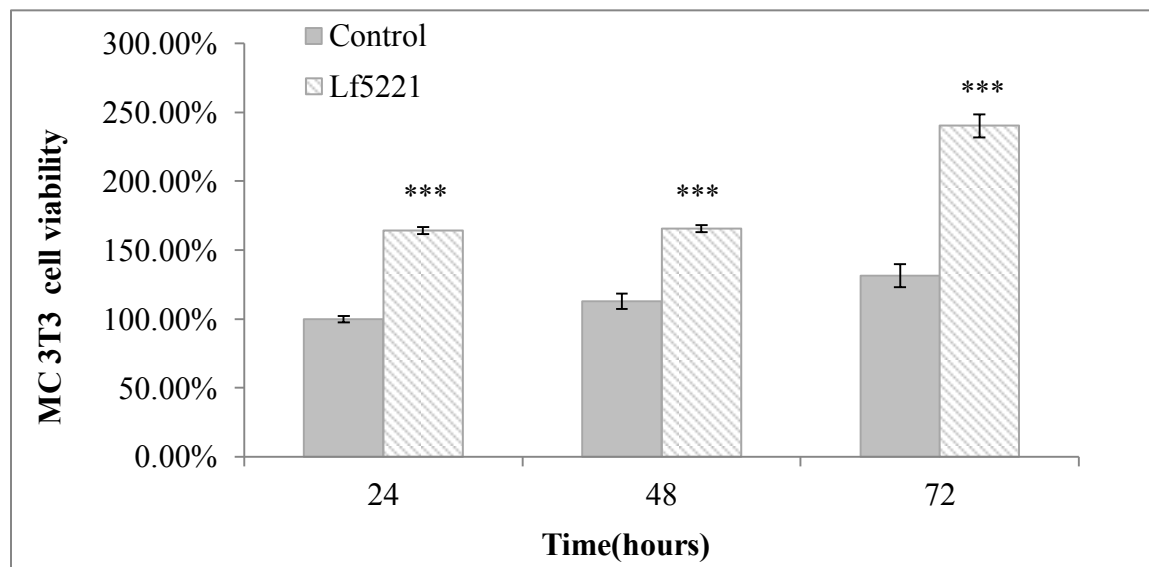




**Figure 7.2:** (A) Anti-inflammatory and (B) Pro-inflammatory affects of *L. fermentum* NCIMB 5221 on inflamed MC3T3 cells. Results are presented as mean  $\pm$  SEM, n = 3. Comparisons of the means were performed using independent student t-tests, where p < 0.001 was considered to be highly significant (\*\*\*).

### 7.4.3 Stimulation of osteoblast proliferation by *L. fermentum* NCIMB 5221 *in vitro*

FA compounds have demonstrated the potential to promote endothelial and neuron progenitor cell growth [298,299]. We investigated whether FA-producing *L. fermentum* NCIMB 5221 could promote the growth of osteoblast-like cells. Interestingly, our results demonstrate that *L. fermentum* NCIMB 5221 treatment promoted the growth of MC 3T3 cells significantly ( $p < 0.005$ ), ( $240.10 \pm 8.31$  % , compared to control at 24 h) as compared to non-treated cells ( $131.52 \pm 08.26$  % , compared to control at 24 h) following 72 h (**Figure 7.3**). Similar results were also observed following 24 and 48 h.



**Figure 7.3:** *L. fermentum* NCIMB 5221 promotes MC 3T3 cell growth. *L. fermentum* NCIMB 5221 treatment promoted osteoblast cell growth. 1% (v/v) cell free extract were used to treat MC 3T3 cells. MTS assay for cell viability was performed following 24, 48 and 72 h. Results are presented as mean  $\pm$  SEM,  $n = 5$ . Comparisons of the means were performed using general linear model and Tukey's post-hoc analysis, where  $p < 0.001$  was considered to be highly significant (\*\*\*). Cell free extract of *L. fermentum* NCIMB 5221 significantly enhances osteoblast growth as compared to control ( $p < 0.001$ )



#### 7.4.4 Effect of *L. fermentum* NCIMB 5221 on periodontal disease markers in

##### F344 rats

Previous studies demonstrated anti-inflammatory effects of *L. fermentum* when administered to high-fat-fed hamsters [300]. We investigated the effect of *L. fermentum* NCIMB 5221 on F344 rats. The treated and the non-treated groups had no difference in animal mass throughout the trial period. However, a non-significant decrease was observed in the treated group, for serum CRP levels ( $1.805 \pm 0.162$  mg/L) at end point as opposed to the non-treated group ( $1.945 \pm 0.211$  mg/L). A similar trend was observed for serum ALP levels with the treated group exhibiting lower levels ( $243.525 \pm 4.632$  mg/L) compared to the non-treated group ( $268.512 \pm 17.200$  mg/L). Interestingly, we observed a significant ( $p < 0.05$ ) difference in serum calcium levels in treated animals ( $2.867 \pm 0.036$  mM) as compared to the control rodents ( $3.04 \pm 0.047$  mM), at endpoint.

In addition, we observed that the treated group exhibited non-significant increases in femur BMD ( $0.153 \pm 0.002$  g/cm<sup>2</sup>) and alveolar bone BMD ( $0.106 \pm 0.0003$  g/cm<sup>2</sup>) as compared to the non-treated group for femur BMD ( $0.146 \pm 0.005$  g/cm<sup>2</sup>) and alveolar BMD ( $0.108 \pm 0.001$  g/cm<sup>2</sup>), at endpoint. Similar observations were also made for alveolar bone support in the treated group ( $02.823 \pm 0.157$ ) as opposed to the non-treated group ( $02.702 \pm 0.108$ ), at endpoint (**Table 7.2**).

**Table 7.2:** Effect of *L. fermentum* NCIMB 5221 on F344 rats at endpoint. Means were compared to generate p values using independent student t-tests. P < 0.05 was considered significant.

Parameter	Control (saline)	<i>L. fermentum</i> NCIMB 5221	p-value
Body mass (g)	208.35 ± 3.18	216.27 ± 5.32	0.258
Serum CRP concentration (mg/L)	1.945 ± 0.211	1.805 ± 0.161	0.619
Serum ALP concentration (U/mL)	268.512 ± 17.200	243.525 ± 4.632	0.244
Serum calcium concentration (mmol/L)	3.040 ± 0.047	2.867 ± 0.036	0.028*
Bone mineral density (Femur) (g/cm <sup>2</sup> )	0.146 ± 0.005	0.153 ± 0.0020	0.308
Bone Mineral density (Alveolar Bone) (g/cm <sup>2</sup> )	0.108 ± 0.001	0.106 ± 0.0003	0.460
Alveolar bone support	2.702 ± 0.108	2.823 ± 0.157	0.552

## 7.5 Discussion

Periodontal disease weakens the supporting framework of the tooth leading to tooth loss and disease progression to deeper structures causing complications such as tooth loss, cellulites and osteomyelitis. However, the most important aspect of periodontal disease is the chronic low grade inflammation leading to aforementioned complications and life-threatening conditions such as infective endocarditis. Probiotic bacteria have shown potential to modulate host immune responses. Moreover, their use in the prevention and treatment of various diseases such as inflammatory bowel diseases (IBD), diarrhoea, GIT cancers, constipation, hypersensitivity responses, peptic ulcers, cardiovascular diseases and urogenital tract disorders is promising [25,137,138,150,300-305]. *Bifibacterium* and *Lactobacillus* are the most commonly investigated probiotic strains. More importantly, certain bacterial strains such as *L. fermentum* NCIMB 5221 have been shown to enhance FA production via their FA esterase (FAE) activity [178,270]. FA is a potent antioxidant molecule that can limit reactive oxygen species such as superoxides, found in elevated levels in diseases such as diabetes (type 2) and atherosclerosis [306]. Apart from this property, they have also demonstrated anti-oxidant and anti-inflammatory properties helpful in the reduction of cerebral infarcts [307]. Moreover, several *L. fermentum* strains demonstrated the ability to produce nitric oxide (NO), another anti-inflammatory molecule [293].

The goal of this study was to investigate the potential of FAE active *L. fermentum* NCIMB 5221 to modulate the inflammatory cytokines secreted by inflamed preosteoblasts, on systemic inflammation and bone remodeling parameters in F344 rats. Hence, the first goal was to quantify FA production, NO production and the total

antioxidant activity production by *L. fermentum* NCIMB 5221 in simulated oral conditions. Tomaro-Duchesneau *et al.* reported FA production of approximately 783.86  $\mu\text{M}$  in MRS broth, following 24 hours incubation with EFA [178]. We observed similar results in this study where *L. fermentum* NCIMB 5221 produced non-significantly higher levels of FA both in SSF and MRS broth (**Table 7.1**). The discrepancies in the FA levels, in both the studies could just be due to the different HPLC methods used for FA quantification. However, the observed difference in FA production, particularly increased levels in SSF (in terms of FA production per cfu) could be attributed to the composition of SSF (**Table 7.1**). Moreover, greater counts of probiotic bacteria ( $1.01 \pm 0.13 \times 10^9$  cfu/mL) in the MRS broth as compared to ( $5.17 \pm 0.32 \times 10^7$  cfu/mL) probiotic bacteria in SSF possibly utilized the complete amount of EFA substrate available. Hence, the expected value of FA for  $10^9$  cfu in MRS was less compared to SSF with lower cfu. The lower ratio of cfu to available substrate was the limiting factor for FA production in MRS. In addition, SSF is a solution rich in several amino acids and casein which might contribute to the increased levels of FA detected, as FA can be derived from amino acids such as phenylalanine [308]. Hence, addition of more substrate in the MRS broth would have resulted in higher FA production. A similar trend was also observed with NO concentrations, with significantly higher levels of NO production by the probiotic in SSF (**Table 7.1**). It is important to note that NO is derived from nitrates and other amino acid sources such as arginine [309-312]. Therefore, the elevated levels of NO in amino acid rich SSF was expected compared to production in MRS broth where there is limited substrate for NO production. Importantly, both FA and NO have anti-inflammatory properties. FA possesses anti-inflammatory characteristics related to its antioxidant

properties to combat reactive oxygen species. FA's structure is crucial to its antioxidant properties, with a phenolic nucleus, a 3 methoxy and a 4-hydroxyl group, which enable electron donation, with the ability to remain stable through the formation of resonance structures [313]. Moreover, the carboxylic group imparts additional antioxidant properties by opposing lipid peroxidation via its ability to combine with cellular lipid bilayers [313]. Indeed, NO producing probiotic patches demonstrated anti-inflammatory properties when applied to infected skin wounds with compromised blood supply [293]. Thus the production of FA and NO could prove beneficial for reducing inflammation, one of the important factors of periodontal disease. The next step was to quantify the total antioxidant activity of *L. fermentum* NCIMB 5221 in both SSF and MRS. We observed that *L. fermentum* NCIMB 5221 demonstrated superior total antioxidant activity in MRS broth as compared to SSF (**Table 7.1**). Although puzzling, this result could simply be due to the presence of several chelators such as Haemin and vitamin B12 in SSF [314,315]. These chelators can interfere with the reduction of  $\text{Cu}^{2+}$  used as an antioxidant detection method with the QuantiChrom™ Antioxidant Assay Kit. A different antioxidant detection method may be preferred in the future. Taken together, it is clear that *L. fermentum* NCIMB 5221 produces FA, NO and possesses significant antioxidant properties beneficial for combatting inflammation in periodontal diseases.

The next step was to examine the effect of *L. fermentum* NCIMB 5221 cell free extract on inflammatory cytokines produced in inflamed preosteoblasts found in increased amounts in periodontal diseases. Inflammatory cytokines such as  $\text{TNF}\alpha$ , IL-1 $\beta$  and IL-6 are found in increased levels in inflammatory bone diseases [316]. Previous works by Saha *et al.* and Tomaro-Duchesneau *et al.* have demonstrated that both FA and

NO production correlate positively with the bacterial growth curve [294,317]. Previous studies by our group also established that *L. fermentum* NCIMB 5221 had faster growth kinetics in MRS broth as compared to SSF (data not shown). Hence, we used cell free extract from *L. fermentum* NCIMB 5221 grown in MRS broth for further experiments. Previous studies by Rosellia *et al.* and Rachmilewitz *et al.* demonstrated that probiotic treatment can reduce these aforementioned inflammatory cytokines in inflammatory bone diseases [318,319]. IL-10 is an anti-inflammatory cytokine that has demonstrated the potential to combat the release of the aforementioned inflammatory cytokines [320]. More importantly, probiotic formulations such as LGG and VSL#3 have demonstrated an increase in IL-10 associated with a reduction in pro-inflammatory cytokines [321,322]. Other studies demonstrated decreased IL-12p70 levels in gingivitis and periodontitis in humans [323]. As well, traditional periodontal therapy increased IL-12p70 levels in patients with periodontitis [324]. Based on these previous works, we investigated the effects of *L. fermentum* NCIMB 5221 cell free extract on inflamed preosteoblasts for their capability to modulate cytokines levels for the prevention and treatment of periodontal disease. It is well-established that LPS derived from periodontopathogens increases TNF $\alpha$  and IL-1 $\beta$  levels [325]. In addition, increased levels of IL-6 are also induced by LPS in preosteoblast cells [326]. In this work, as expected, induction of inflammation via LPS in MC 3T3 cells led to increased levels of IL-1 $\beta$  and IL-6 (**Figure 7.2A**). However, TNF $\alpha$  and IL-10 were below detection levels.

It is well-established that osteoblasts and osteoblast-like cells are of mesenchymal cell lineage [327]. As opposed to these cells, inflammatory cytokines are generously expressed by macrophage cells [328]. More importantly, TNF family proteins such as

Receptor activator of nuclear factor- $\kappa$  B ligand (RANKL), present in elevated levels in osteoclastic lesions and in bone resorption, are poorly expressed by osteoblast cells [329,330]. Hence, the amount of LPS used in our study was most likely not enough to observe detectable levels of TNF $\alpha$ . Unlike TNF $\alpha$ , IL-10, an anti-inflammatory cytokine, is known to regulate the expression of other pro-inflammatory cytokines [331]. However, IL-10 expression is usually slow and delayed [332]. Hence, detectable levels of IL-10 might have been observed at later time points.

*L. fermentum* NCIMB 5221 did not have any pro-inflammatory effects compared to control, in terms of IL-1 $\beta$  levels, however, demonstrated increased IL-6 levels (**Figure 7.2B**). On the other hand, we observed significant reductions in IL-1 $\beta$  and IL-6 levels with probiotic treatment in inflamed osteoblasts (**Figure 7.2A**). Interestingly probiotic treatment demonstrated non-significant increases in IL-12p70 with probiotic treatments, as also observed in patients with periodontitis following periodontal therapy (**Figure 7.2A**) demonstrating the anti-inflammatory effects of *L. fermentum* NCIMB 5221. Hence, *L. fermentum* NCIMB 5221 cell free extract was able to modulate inflammatory responses in preosteoblasts.

Apart from inflammation in periodontal disease, alveolar bone loss is an important aspect that ultimately leads to the weakening of tooth supporting structures and tooth loss. Probiotic bacteria have shown potential to play a role in epithelial tissue regeneration [333,334]. Thus, we investigated the effect of *L. fermentum* NCIMB 5221 cell free extract on preosteoblasts. We observed significant increases in preosteoblast growth with probiotic treatment for 72 h when compared to control (**Figure 7.3**). The exact mechanisms responsible for this observation are yet to be elucidated but this is the

first study that demonstrates that probiotic bacteria or their by-products can be used for bone regeneration or, at the very least, to limit bone resorption.

The next step was to identify the effects of *L. fermentum* NCIMB 5221 *in vivo* in F344 rats. As aforementioned, the most important feature of periodontal disease is chronic low-grade inflammation. Previous studies, by Saito *et al.*, have established a link between systemic inflammation and alveolar bone loss [335]. Moreover, D'Aiuto *et al.* demonstrated that periodontal disease can contribute to systemic inflammation [336]. ALP is associated with bone remodeling and decreased levels of ALP have been reported in GCF with reduced periodontal inflammation [337]. In addition, increased levels of ALP and serum calcium were reported by Rani *et al.* in patients with periodontitis [338]. We postulated that probiotic treatment could modulate systemic inflammatory markers and bone parameters in F344 rats with or without inflammation. In this study we observed non-significant decrease in serum CRP and ALP levels. However, there was a significant decrease in serum calcium. The exact reasoning requires further investigations.

Many researchers have looked at the relation between osteoporosis and periodontal disease, as discussed by Martinez-Maestre *et al.* [339]. Mohammad *et al.* demonstrated a positive correlation between decreased BMD and increased clinical attachment loss and tooth loss [340]. Recently, McCabe *et al.* demonstrated that probiotic treatment can increase BMD in normal male mice [341]. We observed a non-significant increase in femur BMD and alveolar bone support in rats treated with probiotic bacteria versus non treated group. However, it is important to note that McCabe *et al.* used mice in specific pathogen free conditions which were then placed into conventional non-sterile



conditions. As discussed by the group, the observed differences could be due to reduced bone formation because of a lack of the gut microbiome or due to their shift to standard facility inducing a pro-inflammatory state which was then improved by probiotic treatment [341].

Although we observed non-significant changes in systemic inflammatory markers and bone remodeling markers with probiotic treatment, we still observed a significant decrease in serum calcium in the treated group with non-significant increases in femur BMD and alveolar bone support (**Table 7.2**). Hence, further investigations should focus on understanding the changes in calcium metabolism and bone parameters using both *in vitro* and *in vivo* periodontitis models. This is the first study to demonstrate that *L. fermentum* NCIMB 5221 can modulate both pro and anti-inflammatory markers in periodontitis. We also demonstrated its potential for bone regeneration and modulation of systemic calcium absorption. This study opens up avenues to investigate the systemic and local mediators of inflammation and bone remodeling involved in periodontal disease. Even more importantly, this research demonstrates that *L. fermentum* NCIMB 5221 has great potential to prevent and treat periodontal disease and requires further investigations for the development of a successful biotherapeutic.

## **7.7 Acknowledgments**

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### **7.8 Conflict of Interest**

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

**CHAPTER 8: NOVEL PROBIOTIC DISSOLVABLE CARBOXYMETHYL  
CELLULOSE FILMS AS ORAL HEALTH BIOTHERAPEUTICS: *IN VITRO*  
PREPARATION AND CHARACTERIZATION**

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**Preface:** Based on previous findings that demonstrated the beneficial effects of probiotic bacteria, the goal of this study was to develop oral thin films (OTF) efficient at delivering probiotic bacteria to the mouth. Carboxymethyl cellulose (CMC) was used to develop the OTFs. Films were optimized and characterised for CMC concentration, weight, thickness, hygroscopicity and dissolving time. Following optimization CMC-OTF of wet film weight 10 g and CMC concentration 5 mg/mL were used for *L. fermentum* NCIMB 5221 incorporation, which were then stored in non-controlled conditions. The probiotic CMC-OTFs were evaluated for bacterial viability and anti-oxidant activity following long-term storage at room temperature.

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## 8.1 Abstract

Oral health is influenced by the mouth's resident microorganisms. Dental caries and periodontitis are oral disorders caused by imbalances in the oral microbiota. Probiotics have potential for the prevention and treatment of oral disorders. Current formulations, including supplements and foods, have limitations for oral delivery including short storage time, low residence time in the mouth, effects on food consistency, and low patient compliance. Oral thin films (OTFs) may be efficient in delivering probiotics to the mouth. This research aims to develop a novel carboxymethyl cellulose (CMC)-probiotic-OTF to deliver probiotics for the treatment/prevention of oral disorders. CMC-OTFs were developed with varying CMC concentration (1.25 – 10 mg/mL), weight (5 – 40 g), thickness (16 – 262  $\mu\text{m}$ ), hygroscopicity (30.8 – 78.9 mg/cm<sup>2</sup> film), and dissolving time (135 – 600 s). The 10 g 5 mg/mL CMC-OTF was selected and used to incorporate *Lactobacillus fermentum* NCIMB 5221 ( $6.75 \times 10^8$  cells/film), a probiotic with anti-inflammatory potential for periodontitis treatment and capable of inhibiting microorganisms responsible for dental caries and oral candidiasis. The CMC-OTF maintained probiotic viability and antioxidant activity following 150 days of storage with a production of  $549.52 \pm 26.08$   $\mu\text{M}$  Trolox equivalents. This research shows the successful development and characterization of a novel probiotic-CMC-OTF with potential as an oral health biotherapeutic.

## 8.2 Introduction

The oral microbial ecosystem plays a significant role in influencing human oral health and disease. Dental caries and periodontitis are two prominent oral disorders, with the World Health Organization estimating that almost 100% of the world's population is

affected by dental caries and 10-15% affected by periodontitis [1,342]. Current therapeutic methods for the prevention and treatment of both dental caries and periodontitis have important limitations. Recent research has turned to probiotics, “live microorganisms which when administered in adequate amounts confer a health benefit on the host” [343]. Probiotics have been shown to modulate the oral microbiota, specifically inhibiting the growth of pathogens such as *Streptococcus mutans*, a cariogenic bacterium, and *Candida albicans*, an organism responsible for the initiation and progression of oral candidiasis [24,26,60]. There are numerous techniques and methods currently used for the oral delivery of probiotics, including incorporation in foods, capsules and gels [27-30]. The current delivery methods, however, have important limitations with respect to oral delivery, such as short storage time without loss of probiotic viability, short probiotic residence time in the oral cavity, undesired effects on food consistency and patient non-compliance [344,345]. A delivery system capable of delivering probiotics to the oral cavity and allowing their slow release is required for the efficient modulation of the oral microbiota. A delivery system should allow for a slow release of viable and metabolically active probiotic bacteria to the oral cavity, and potentially the rest of the gastrointestinal (GI) system.

One delivery system, oral thin films (OTF) should prove promising for the oral delivery of probiotic bacteria. OTFs were first developed for children and geriatric patients who had difficulties swallowing tablets and capsules [346]. OTFs disperse or disintegrate in the mouth releasing the enclosed active ingredient and possess advantages such as fast disintegration, no water requirement for dissolution, accurate dosing and increased patient compliance [346-348]. OTFs are formed by a polymer matrix which can

contain active components. Current research has investigated the release of a number of drugs from these OTFs [348,349] but there are limited studies on the use of OTFs for the delivery of live bacterial cells.

For probiotic delivery using OTFs, a matrix capable of supporting bacterial viability and metabolic activity, while allowing the quick and efficient release of the cells, is required. Carboxymethyl cellulose (CMC), a natural polymer derived from cellulose, is easily dissolvable in water and has not presented any toxicity [350]. A CMC-OTF should hence prove suitable for the oral delivery of probiotic bacteria for the goal of inhibiting oral pathogens to treat and prevent oral diseases. This work demonstrates the development of a novel CMC-OTF for the delivery of *Lactobacillus fermentum* NCIMB 5221, a probiotic bacterium that presents antioxidant capability due to a feruloyl esterase (FAE) enzyme, releasing ferulic acid (FA) [178,270] potentially beneficial as a therapeutic for managing the inflammatory profile of periodontitis. In addition, this probiotic strain has been shown to inhibit the cariogenic *S. mutans* and also *C. albicans* [351]. The CMC-OTFs are characterized for their dissolution time, probiotic incorporation efficiency, CMC-OTF thickness and film hygroscopicity, all in the context of the oral delivery of the incorporated probiotic for oral health applications. We also investigate the amount of viable *L. fermentum* remaining following CMC-OTF formation and the capability of the CMC-OTFs to maintain probiotic viability and metabolic activity following long-term storage under non-refrigerated conditions.

## 8.3 Materials and Methods

### 8.3.1 Bacterial strain, culture conditions and chemicals

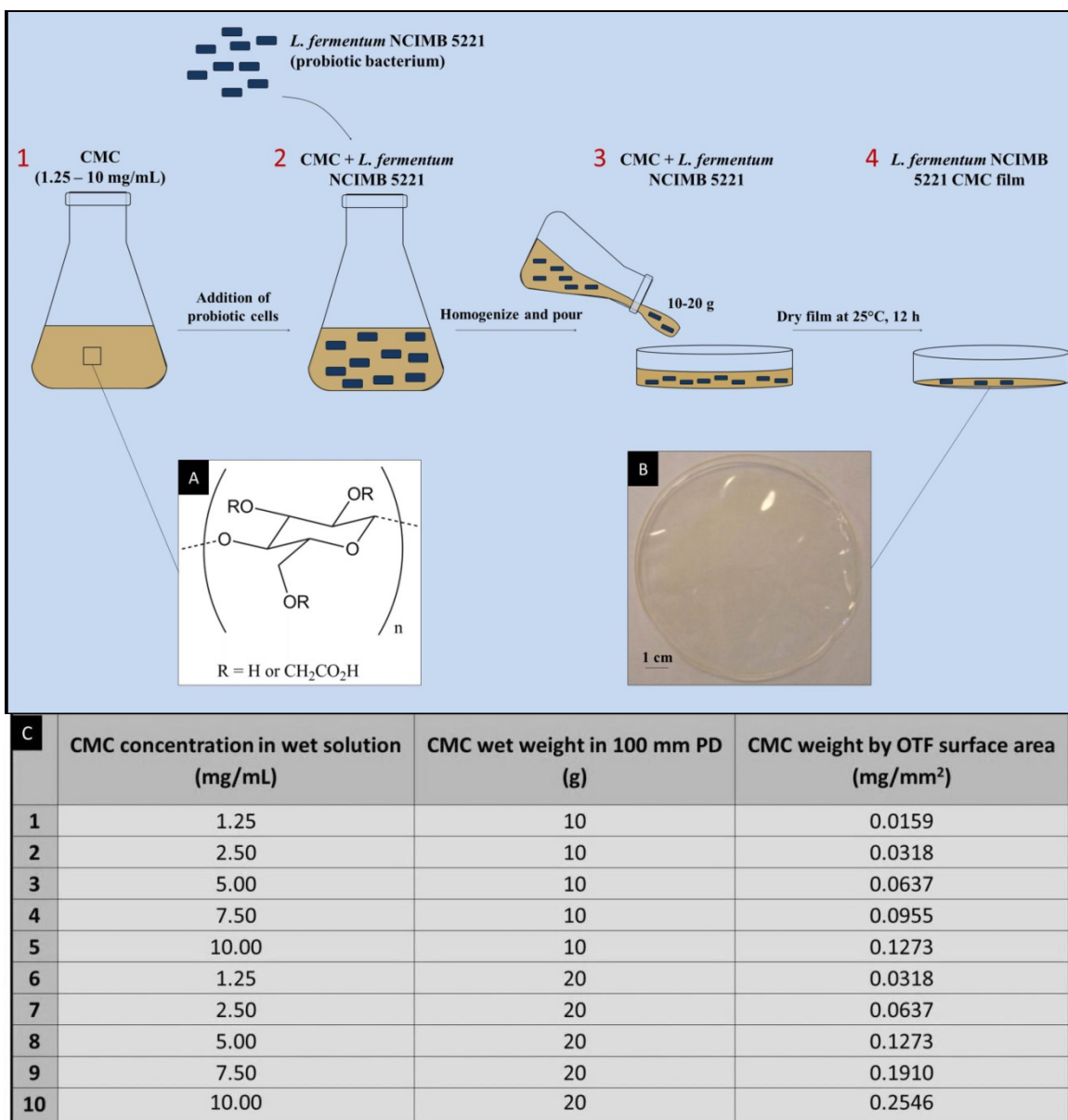
Potential probiotic bacteria, *L. fermentum* NCIMB 5221 was purchased from NCIMB (Aberdeen, Scotland, UK). The bacterial strain was stored at  $-80^{\circ}\text{C}$  in De Man, Rogosa, Sharpe (MRS) containing 20% (v/v) glycerol. MRS broth was purchased from Fisher Scientific (Ottawa, ON, Canada). A MRS-agar plate was streaked for isolation from the frozen stock and incubated at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  for 24 h to ensure purity. One colony from the MRS-agar plate was inoculated into 5 mL MRS broth and incubated at  $37^{\circ}\text{C}$  for 24 h. A 1% (v/v) inoculum was then used for subculturing and incubated at  $37^{\circ}\text{C}$  for 24 h immediately before use. CMC sodium salt was purchased from Sigma Aldrich (Oakville, ON, Canada). Water was purified with an EasyPure reverse osmosis system and a NanoPure Diamond Life Science (UV/UF) ultrapure water system from Barnstead (Dubuque, IA, USA). All other chemicals were of analytical or HPLC grade and purchased from commercial sources.

### 8.3.2 Development of CMC – probiotic OTFs

Five different concentrations of CMC films were prepared: 1.25, 2.50, 5.00, 7.50, 10.00 mg/mL and four different masses: 5, 10, 20 and 40 g wet weight, as described in **Figure 8.1**. CMC was dissolved in double-distilled water by continuous stirring at room temperature. The CMC solutions were autoclaved for 10 min at  $121^{\circ}\text{C}$  followed by cooling to room temperature. Following autoclaving, all procedures were performed under sterile conditions. For CMC films containing *L. fermentum* NCIMB 5221, an overnight bacterial culture was incorporated at 0.1 g of bacterial pellet/mL of CMC solution. Briefly, the overnight culture was pelleted by centrifuging at 4000 rpm for 20

min at 4°C (Napco 2028R centrifuge, Fisher Scientific, Ottawa, ON, Canada). The supernatant was discarded and the bacterial pellet was washed with 0.85% (w/v) NaCl. The pellet was then re-suspended in 0.85% (w/v) NaCl and was added to the CMC solution at a concentration of 0.1 g bacterial pellet/mL CMC solution. The CMC solution was then poured into individual 100 mm bacteriological Petri Dishes (PD) at a wet mass of 5, 10, 20 and 40 g of CMC solution. The CMC films were allowed to dry in the Biological Safety Cabinet with a continuous laminar air flow for 12 h at room temperature. The films were then stored at room temperature, with parafilm sealing the PD containing the OTF. To determine water loss and dry CMC film mass following drying, the formed CMC films were weighed on an analytical scale from Mettler Toledo (Mississauga, ON, Canada).





**Figure 8. 1:** Schematic representation of Carboxymethyl cellulose (CMC) - probiotic oral thin film preparation. (A) Chemical structure of CMC (B) 5.00 mg/mL CMC of 100 mm in diameter (C) Tabulated representation of the various concentrations and wet weight of CMC as well as CMC by OTF surface area

### 8.3.3 Determining the CMC film incorporation efficiency of viable *L. fermentum*

#### NCIMB 5221

The cell counts of bacteria incorporated in the wet film (WF, CMC film prior to drying) was calculated by standard colony counting methods, in terms of colony forming units (cfu)/g of CMC film. Briefly, this was done by 10-fold serial dilutions of the sample using 0.85% (w/v) NaCl and plating on MRS-agar plates which were incubated at 37°C and 5% CO<sub>2</sub> for 48 h. Viability of the dry CMC-*L. fermentum* NCIMB 5221 film was also determined at day 0, in terms of cfu/g of dry CMC film (DF). The film was dissolved in a known volume of 0.85% (w/v) NaCl followed by 10-fold serial dilutions and plating on MRS-agar plates which were incubated at 37°C and 5% CO<sub>2</sub> for 48 h for viability measurements. Triplicate samples were measured to ensure accuracy and reproducibility.

The loss of viability (LV) of *L. fermentum* NCIMB 5221 upon drying and formation of the CMC films was calculated. First, the cell counts of the wet film ( $V_{WF}$ ) and dry film ( $V_{DF}$ ) in cfu/g were determined using standard colony counting methods, described previously, and the values of  $V_{WF}$  and  $V_{DF}$  were adjusted for the loss of CMC mass upon drying.

$$LV = V_{WF} - V_{DF}$$

### 8.3.4 Method for measuring the thickness of CMC films

The final thicknesses of the dried CMC films were measured using digital calipers from Fisher Scientific (Ottawa, ON, Canada). The CMC films were measured at five different locations on three different films to obtain accurate and reproducible thickness values. Five different concentrations of CMC were measured: 1.25, 2.50, 5.00, 7.50 and 10.00 mg/mL of two different masses: 10 and 20 g (wet weight).

### 8.3.5 Methods for measuring CMC film hygroscopicity

The hygroscopicity of the CMC films was determined using simulated salivary fluid (SSF). The SSF formulation was prepared as in our previous study, with its composition listed in **Table 4.1**. Five different concentrations of CMC were used: 1.25, 2.50, 5.00, 7.5 and, 10.00 mg/mL of two different masses: 10 and 20 g (wet weight). CMC films of 1 cm<sup>2</sup> were sampled from the larger CMC films. A PD of known mass was filled with a known volume of SSF. The CMC films were submerged in SSF for 10 sec, until maximum liquid absorption. SSF absorption was measured with the following equation, where mass<sub>0 sec</sub> is the initial mass and mass<sub>10 sec</sub> is the mass in milligrams following submersion:

$$SSF\ absorbed = (PD\ mass + SSF\ mass_{10\ sec}) - (PD\ mass + SSF\ mass_{0\ sec})$$

The assay was performed at 25°C in triplicates, using three different CMC films for each concentration and mass, to ensure accuracy and reproducibility.

### 8.3.6 Method for determining the dissolving time of CMC films

To determine the dissolution time, dielectric spectroscopy, using conductivity measurements, was performed on 1.25, 2.5 and 5 mg/mL CMC films of 10 g and 20 g (wet weight). First, an 8 mm diameter CMC film was cut using a hole-puncher to ensure accuracy and reproducibility. The CMC film sample was then placed inside a cylindrical measurement cell, of 10 mm in diameter and 4.5 mm in height, enclosed by two parallel plate electrodes made of platinum deposited with platinum black. The measurement cell was filled with 300 µL of deionized water to allow for polymer dissolution to occur. The associated conductivity measurements were then measured using an Agilent 4294a impedance analyzer (Palo Alto, CA) at a fixed frequency of 100 kHz and a peak-to-peak

AC voltage of 300 mV. The polymer was allowed to dissolve for 600 secs while acquiring 50 conductivity measurement points, every 12 sec. The number of replicates of measured films was  $n = 5$  for all CMC-OTF samples. Complete dissolution was marked by the plateau and stabilization of the conductivity of the resulting solution.

### **8.3.7 Method for determining bacterial cell release from CMC films in SSF**

The time required for total bacterial release from the 5 mg/mL and 10 g (wet weight) CMC when added to SSF was determined. SSF was added in a ratio of 1/40 (CMC weight (g)/SSF volume (mL)) to the CMC film. The CMC-SSF solution was then incubated at 37°C on a rotary shaker set at 75 rpm. At each time point, the viability of the SSF solution (bacteria released from CMC) was determined using 10-fold serial dilutions and plating for cfu on MRS-agar plates which were incubated at 37°C and 5% CO<sub>2</sub> for 48 h. The time point at which bacterial viability in SSF was highest was determined as the time at which the CMC-OTF was completely dissolved. The experiment was performed using  $n = 3$  samples of CMC-OTF.

### **8.3.8 Method for determining the viability of probiotic bacteria embedded in CMC films**

CMC films of 5 mg/mL and 10 g (wet weight), containing *L. fermentum* NCIMB 5221, prepared as described previously, were used for storage experiments. Following CMC film formation, the CMC-*L. fermentum* NCIMB 5221 films were stored at room temperature under aerobic and sterile conditions during the course of the experiment. At each time point, over the course of 200 days, the bacterial cell counts of the CMC-*L. fermentum* NCIMB 5221 films, in terms of cfu/g (dry weight) of CMC film, were determined. Viability was determined by dissolving the film in a known volume of 0.85%

(w/v) NaCl followed by 10-fold serial dilutions and plating on MRS-agar plates which were incubated at 37°C and 5% CO<sub>2</sub> for 48 h. The experiment was performed with n = 3 different CMC-OTF-*L. fermentum* NCIMB 5221 samples at each time point.

#### **8.3.9 Method for determining probiotic antioxidant activity following storage**

Enzymatic activity following CMC-*L. fermentum* NCIMB 5221 storage for 150 days incorporated in a 5 mg/mL, 10 g (wet weight) CMC-OTF was determined. As described in a previous study by our group, *L. fermentum* NCIMB 5221 can be characterized by its antioxidant activity due to FAE when incubated in MRS-ethyl ferulate (EFA), at an EFA concentration of 1.5 mM [352]. Following 0 and 150 days of storage, CMC-*L. fermentum* NCIMB 5221 was incubated in MRS-EFA at a ratio of 15 mg CMC film: 10 mL MRS-EFA and incubated at 37°C on a rotary shaker set at 75 rpm. At each time points (0,4,8,16,24 and 48h), the total antioxidant production was measured using a QuantiChrom™ Antioxidant Assay Kit, a spectrophotometric assay based on the reduction of Cu<sup>2+</sup> to Cu<sup>+</sup>. The protocol provided with the assay kit was followed. A standard curve was generated for Trolox, a standard provided with the kit, at concentrations of 0, 300, 600 and 1000 µM plotted against absorbance at 570 nm ( $R^2 = 0.9970$ ) read using a UV spectrophotometer Victor<sup>3</sup>V 1420 Multilabel Counter (Perkin Elmer, Boston, MA). Viability using cfu on MRS-agar was also determined at each time point. Un-inoculated MRS-EFA broth was used as a blank and all samples were treated in triplicate.

#### **8.3.10 Statistical analysis**

Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was carried out using SPSS Version 17.0 (Statistical Product and Service

Solutions, IBM Corporation, New York, NY, USA). Comparison of the means was performed using the general linear model and Tukey's post-hoc analysis. Statistical significance was set at  $p < 0.05$  and p-values less than 0.01 were considered highly significant.

## **8.4 Results**

### **8.4.1 CMC film preparation and characterisation**

CMC films were prepared at different concentrations and wet mass. It was concluded that 5 g (wet weight) CMC was too little to form a film of 100 mm of diameter and 40 g (wet weight) CMC took too long to dry, causing complete probiotic cell death. Drying time was determined by observation, at the point at which the CMC film was easily removable from the PD. This was determined to be following 12 h of drying at room temperature. The dry weight of the CMC films was determined following film formation. As expected, the data demonstrates a significant increase in dry weight as the CMC concentration increases and as wet weight increases. For instance, the 1.25 mg/mL CMC had a dry weight of  $0.14 \pm 0.01$  g and  $0.27 \pm 0.01$  g for the 10 g and 20 g wet weight, respectively. For the 10.00 mg/mL CMC, a dry weight of  $1.00 \pm 0.02$  g and  $1.96 \pm 0.02$  g were measured for the 10 g and 20 g (wet weight), respectively. From the weight wet and dry weight values, the water remaining in the film following drying was determined. The CMC film thickness was also measured and significantly increased as the CMC concentration increased, as film dry weight increased and as film wet weight increased. The thickness ranged from  $16.00 \pm 2.45$   $\mu\text{m}$  for the 1.25 mg/mL, 10 g (wet

weight) CMC film to  $262.00 \pm 30.23 \mu\text{m}$  for the 10.00 mg/mL, 20 g (wet weight) CMC film. Film dry weight, water remaining and thickness results are tabulated in **Table 8.2**.

**Table 8.2:** Characterisation of Carboxymethyl cellulose (CMC) oral thin films for thickness, dry weight and water remaining in the films. Characterization was done for 10 g and 20 g (wet weight) CMC films of 1.25, 2.5, 5, 7.5, 10 mg/mL CMC concentrations. Data is presented as mean  $\pm$  SEM with n = 3.

CMC concentration (mg/mL)	Wet weight (g)	Dry weight (g)	Water remaining (g)	Thickness ( $\mu\text{m}$ )
1.25	10.0	$0.14 \pm 0.01$	$0.015 \pm 0.010$	$16.00 \pm 2.45$
	20.0	$0.27 \pm 0.01$	$0.020 \pm 0.010$	$22.00 \pm 2.00$
2.50	10.0	$0.31 \pm 0.02$	$0.060 \pm 0.020$	$28.00 \pm 3.74$
	20.0	$0.55 \pm 0.03$	$0.050 \pm 0.035$	$72.00 \pm 9.16$
5.00	10.0	$0.79 \pm 0.03$	$0.285 \pm 0.035$	$66.00 \pm 5.09$
	20.0	$1.21 \pm 0.08$	$0.210 \pm 0.080$	$42.00 \pm 9.69$
7.50	10.0	$0.72 \pm 0.01$	$-0.030 \pm 0.000$	$94.00 \pm 4.00$
	20.0	$1.59 \pm 0.02$	$0.0950 \pm 0.025$	$218.00 \pm 11.14$
10.00	10.0	$1.00 \pm 0.02$	$0.000 \pm 0.020$	$188.00 \pm 36.25$
	20.0	$1.96 \pm 0.02$	$-0.0450 \pm 0.025$	$262.00 \pm 30.23$

#### 8.4.2 The CMC film incorporation efficiency of viable *L. fermentum* NCIMB

##### 5221

To determine the CMC film incorporation efficiency of viable *L. fermentum* NCIMB 5221, the bacterial cell viability before and after film formation was determined. *L. fermentum* NCIMB 5221 was added to the CMC solution at a concentration of  $2.02 \times 10^{10} \pm 8.58 \times 10^8$  cfu/film. The final dry film contained  $6.75 \times 10^8 \pm 4.6 \times 10^7$  cfu/film.

Results in **Table 8.3** show that the loss of viability for *L. fermentum* NCIMB 5221 following CMC film formation was  $1.96 \times 10^{10} \pm 8.97 \times 10^8$  cfu/film.

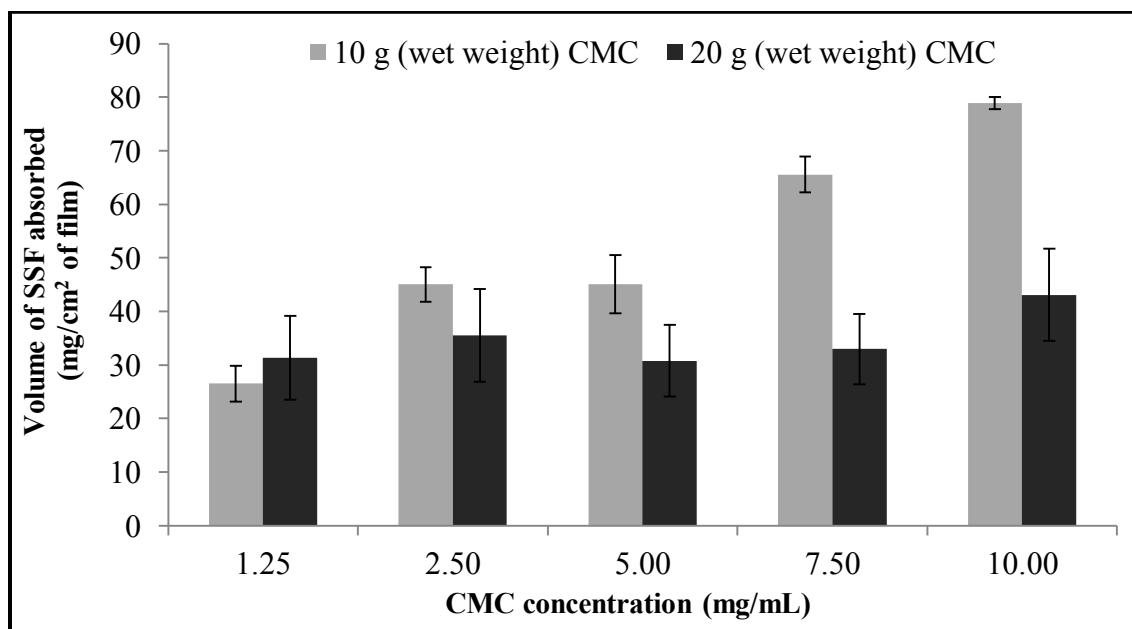


**Table 8.3:** The loss of viability of *L. fermentum* NCIMB 5221 after CMC film formation. Each film was of 100 mm diameter. Data is presented as mean  $\pm$  SEM with n = 3

<b>Initial bacterial concentration added to CMC film (cfu/film)</b>	$2.02 \times 10^{10} \pm 8.58 \times 10^8$
<b>Bacteria remaining following CMC film formation (cfu/film)</b>	$6.75 \times 10^8 \pm 4.6 \times 10^7$
<b>Loss in bacterial cell viability during film formation (cfu/film)</b>	$1.96 \times 10^{10} \pm 8.97 \times 10^8$

#### 8.4.3 Hygroscopicity of CMC films in simulated salivary conditions

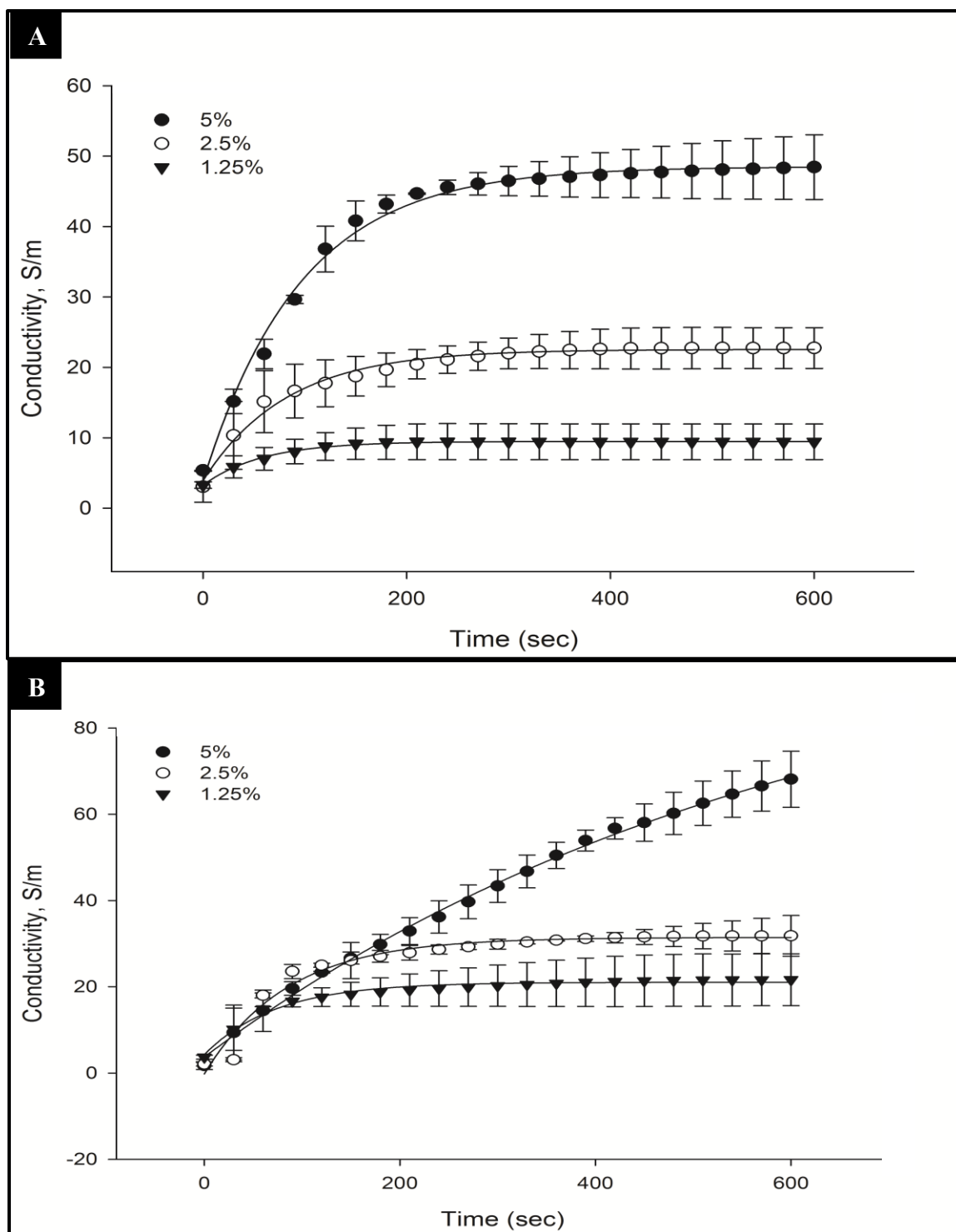
The SSF absorption volume of the CMC films of different concentrations and wet weight was measured following submersion in SSF, as an indicator of dissolving time, as shown in **Figure 8.2**. As the CMC concentration increased for the 10 g (wet weight) CMC film, the SSF absorption volume increased linearly from  $26.5 \pm 3.31$  mg/cm<sup>2</sup> of film, for the 1.25 mg/mL film, to  $78.9 \pm 1.10$  mg/cm<sup>2</sup> of film for the 10 mg/mL film. The SSF absorption was highly variable across the different concentrations for the 20 g (wet weight) films.



**Figure 8.2** Characterization of Carboxymethyl cellulose (CMC) oral thin films for hygroscopicity. 10 g and 20 g (wet weight) CMCs of 1.25, 2.5, 5, 7.5 and 10 mg/mL of CMC concentrations were tested for simulated saliva absorption capacity. Data is presented as mean  $\pm$  SEM, with n = 3.

#### 8.4.4 CMC film dissolving time

The dissolution time of CMC films of different concentrations and wet weights was determined through conductivity measurements using dielectric spectroscopy. For the 10 g and 20 g (wet weight) samples there exists a clear correlation between percent CMC and conductivity, shown in **Figure 8.3**. Higher CMC percentage was shown to be directly proportional to the conductivity of the resulting dissolved polymer solution. Moreover, the 20 g (wet weight) samples of the same CMC percentage displayed higher conductivities than their 10 g counterparts. These results confirm the higher ionic content associated with polymer solutions containing dissolved CMC films of higher CMC percentage and wet weight. This may be translated into an effective method of measuring and determining the CMC polymer content and dissolution rate within a sample.



**Figure 8.3** Determining the dissolution time of (A) 10 g and (B) 20 g (wet weight) carboxymethyl cellulose (CMC) oral thin films of 8 mm diameter and of 1.25, 2.5 and 5 mg/mL of CMC concentrations. Dissolving time measured by the conductivity of the solution in which CMC is dissolved, measured by dielectric spectroscopy. Data is presented as mean  $\pm$  SEM, with  $n = 5$

The dissolution rate of the CMC films was determined through exponential curve fitting of the conductivity measurements over time. The relationship between conductivity and time was plotted according to the Guggenheim equation [353]:

$$G(t) = G_s (1 - e^{-kt})$$

where  $G(t)$  is the conductivity of the solution expressed in siemens/meter,  $t$  is the dissolution time in sec,  $G_s$  is the saturated conductivity, and  $k$  is the rate constant of dissolution specific to the dissolved CMC film concentration and wet weight. The dissolving times obtained by this method are presented in **Table 8.4**, as well as a list of the dissolution constants for the 1.25, 2.5, and 5 mg/mL CMC films of 10 and 20 g (wet weight). The lowest dissolution time was for the 1.25 mg/mL 10 g (wet weight) CMC at approximately 135 sec. The highest dissolution time was for the 5.0 mg/mL 20 g (wet weight) CMC at > 600 sec. The 5 mg/mL 10 g (wet weight) CMC had a dissolution time of 420 sec. In addition, as expected, the saturated conductivities of the CMC films increased with CMC percentage and the values are about two-fold comparing the 20 g (wet weight) to their 10 g counterparts. Furthermore, the rate constant of polymer dissolution also decreased with increasing CMC percentage for both wet weights indicating a slower rate of polymer degradation for films containing higher CMC concentrations.

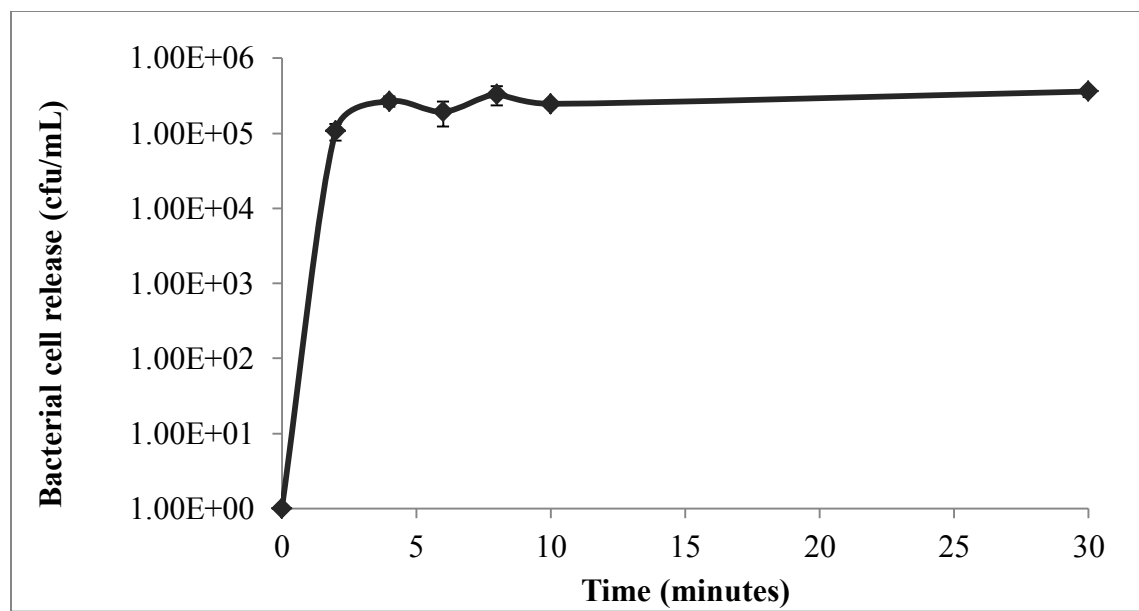
**Table 8.4:** The dissolution time of 10 g and 20 g (wet weight) carboxymethyl cellulose (CMC) films of 1.25, 2.5 and 5 mg/mL CMC concentrations, as determined by dielectric spectroscopy. The CMC dissolution time for each concentration was determined as the time after which the conductivity of the solution did not vary.  $G_s$  is the saturated conductivity, and  $k$  is the rate constant of dissolution specific to the dissolved CMC film concentration and wet weight.

	$G_s$	$k$	Dissolving time (sec)
<b>10 g wet weight (mg/mL CMC)</b>			
5.00	48.2535	0.0115	420
2.50	22.2643	0.0158	270
1.25	9.4029	0.0261	135
<b>20 g wet weight (mg/mL CMC)</b>			
5.00	92.3592	0.0022	$\geq 600$
2.50	31.4496	0.0118	390
1.25	20.7825	0.0184	390

Results are presented as mean  $\pm$  SEM,  $n=4$ , where \*  $p < 0.05$ .

#### 8.4.5 Bacterial release from CMC films under simulated oral conditions

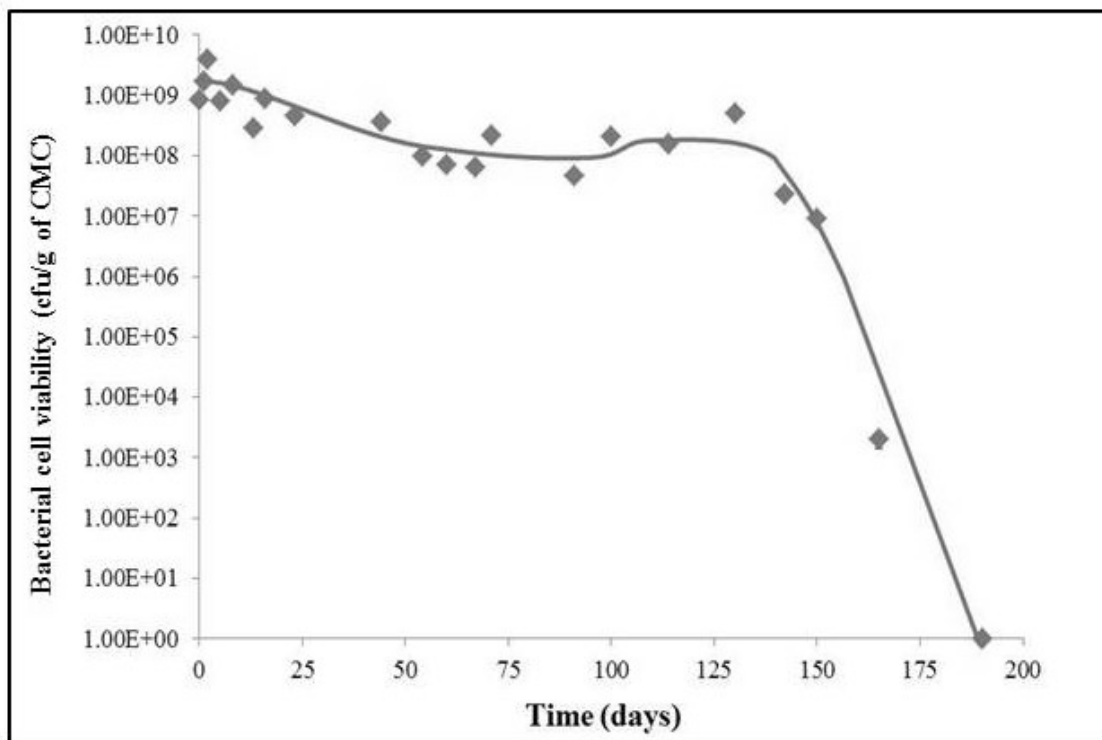
The release of *L. fermentum* NCIMB 5221 from a 5 mg/mL CMC film of 10 g (wet weight) in SSF was measured, shown in **Figure 8.4**. The original viability of the CMC film was  $8.60 \times 10^8 \pm 5.86 \times 10^7$  cfu/g of CMC. Following 2 min of incubation,  $1.07 \times 10^5 \pm 2.73 \times 10^4$  cfu/ml of SSF was released from the CMC film. Following 4 min of incubation,  $2.67 \times 10^5 \pm 4.18 \times 10^4$  cfu/ml of SSF was released from the CMC film. It was determined that the film was completely dissolved by 4 min, as the *L. fermentum* NCIMB 5221 count released in SSF did not increase significantly over 30 min into the experiment.



**Figure 8.4** The release of *L. fermentum* NCIMB 5221 in simulated salivary fluid from the 5 mg/mL carboxymethyl cellulose (CMC) film incubated under simulated oral conditions on a rotary shaker at 37°C. Probiotic release was measured using standard colony forming units with MRS agar plates. Data is presented as mean  $\pm$  SEM, with  $n = 3$

#### 8.4.6 Maintenance of probiotic viability incorporated in CMC films

The viability of *L. fermentum* NCIMB 5221 in a 5 mg/mL CMC film of 10 g (wet weight) was monitored over 200 days, with storage at room temperature under ambient humidity. The initial viability, following film formation, was  $8.60 \times 10^8 \pm 5.86 \times 10^7$  cfu/g of CMC. No significant difference was demonstrated in the viability of the dry film until day 142 where the viability of *L. fermentum* NCIMB 5221 decreased to  $2.37 \times 10^7 \pm 2.85 \times 10^6$  cfu/g of CMC, as shown in **Figure 8.5**. This translates to  $2.75 \pm 0.33\%$  of the original viability. Following 190 days of storage, no viable probiotic cells were detected.

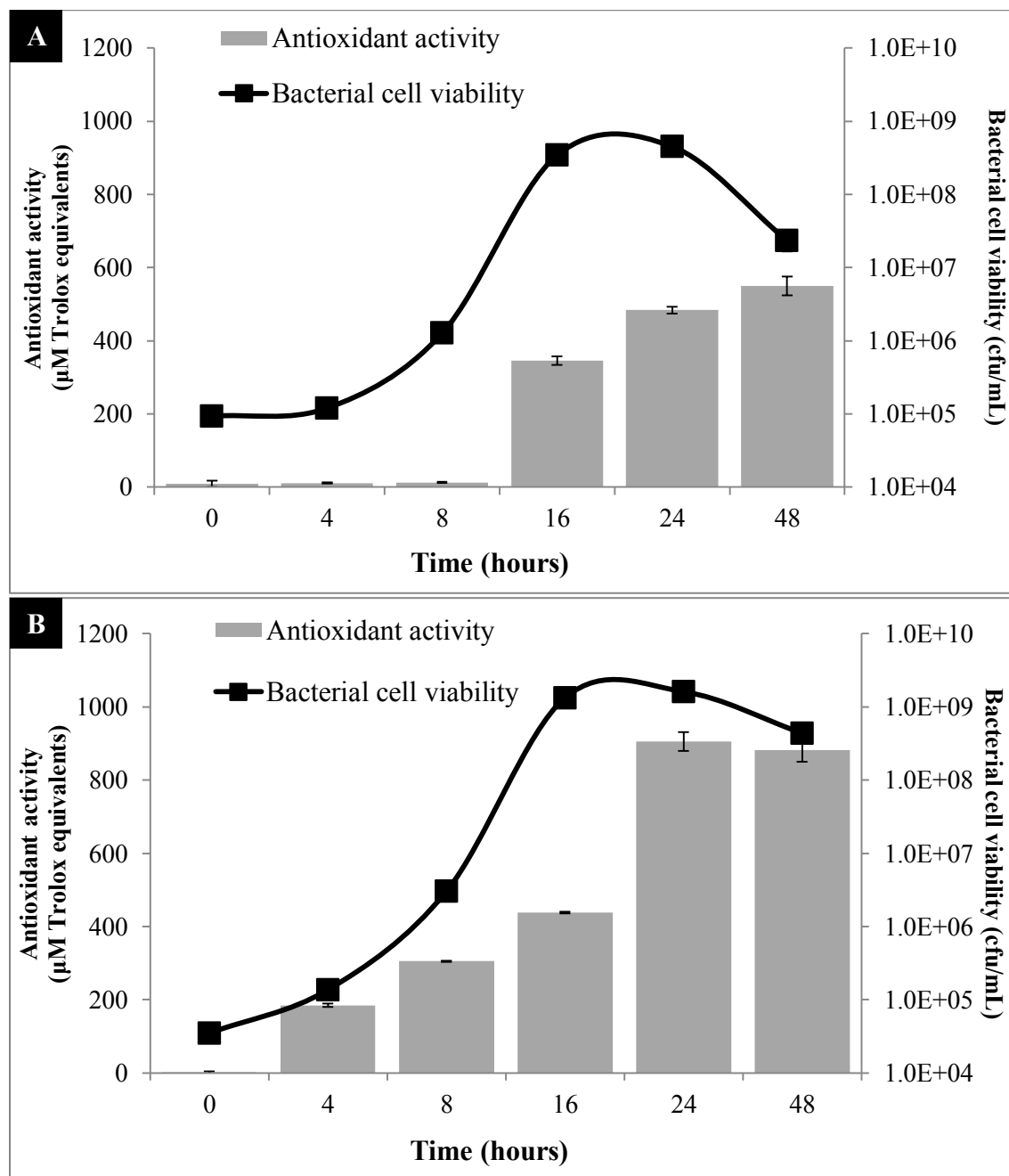


**Figure 8.5:** Determination of the viability of *L. fermentum* NCIMB 5221 embedded in 5 mg/mL carboxymethyl cellulose (CMC) oral thin films, when stored at room temperature under non-regulated humidity conditions. Probiotic viability was measured using the standard colony forming unit method with MRS agar plates. Data is presented as mean  $\pm$  SEM, with  $n=3$ .

#### 8.4.7 Maintenance of probiotic antioxidant activity in CMC films

The FAE enzymatic activity of *L. fermentum* NCIMB 5221 in a 5 mg/mL CMC film of 10 g (wet weight) was monitored following 150 days of storage. The antioxidant activity of *L. fermentum* NCIMB 5221 as well as its viability throughout the assay is shown in **Figure 8.6**. A significant difference in antioxidant activity at 48 h is demonstrated between the fresh CMC film ( $882.14 \pm 32.46$   $\mu$ M Trolox equivalents) and the film stored for 150 days ( $549.52 \pm 26.08$   $\mu$ M Trolox equivalents). However, a clear increase in total antioxidant activity can be seen from 0 h ( $9.52 \pm 8.34$   $\mu$ M Trolox equivalents), with the highest antioxidant production following 48 h of incubation with

EFA, suggesting that the CMC-incorporated *L. fermentum* NCIMB 5221 maintained its antioxidant activity following 150 days of storage.



**Figure 8.6** CMC-probiotic OTF for the delivery of metabolically active *L. fermentum* NCIMB 5221 (A) at day 0 and (B) following 150 days of storage at room temperature. *L. fermentum* NCIMB 5221 was characterized by its antioxidant activity when incubated in MRS-EFA at an EFA concentration of 1.5 mM. At each time point, the antioxidant production, in terms of Trolox equivalents (µM) was measured using a QuantiChrom™ Antioxidant Assay Kit. Viability using standard colony forming units on MRS-agar was



determined at each time point. Un-inoculated MRS-EFA broth was used as a blank. Data is presented as the mean  $\pm$  SEM, with  $n = 3$ .

## 8.5 Discussion

Probiotic bacteria have proven beneficial for a number of health conditions including preventing allergy and reducing colonization by bacterial pathogens [354], preventing oral diseases [26,60], preventing metabolic syndrome [300,355], reducing the duration of acute diarrhea [356,357], preventing sepsis and severe acute pancreatitis [358], reducing post-operative infection rate [358,359], improving inflammatory bowel disease symptomatology [360] and reducing hyperlipidemia. Current probiotic formulations involve bacterial incorporation in foods such as yoghurt, capsules and chewing gums. These products have limited shelf-life and other important limitations. OTFs have proven beneficial for improving patient compliance and may also enhance product shelf-life. CMC is a readily available natural polymer with no documented side effects. This work proposes a novel CMC-OTF incorporated with probiotic bacterial cells. This formulation is proposed as a novel probiotic biotherapeutic for the treatment and prevention of oral health disorders, specifically dental caries and periodontitis.

We investigated CMC-OTFs incorporated with *L. fermentum* NCIMB 5221 a probiotic characterized for its FA production due to FAE activity [178]. FA is a naturally found phenolic acid characterized as a potent antioxidant able to neutralize free radicals, such as Reactive Oxygen Species (ROS) implicated in DNA damage, cancer and accelerated cell aging [292,361]. FA has also shown antitumor activity against breast cancer [362,363], liver cancer [364,365] and has proven effective at preventing cancer induced by the exposure to various carcinogenic compounds such as benzopyrene [366] and 4-nitroquinoline 1-oxide [367]. This antioxidant activity may prove beneficial for the treatment and prevention of periodontitis, a primarily inflammatory disorder of the

mouth. In addition, *L. fermentum* NCIMB 5221 has been shown to inhibit the cariogenic *S. mutans* and *C. albicans*, a microorganism responsible for oral candidiasis [24].

To develop an OTF, a number of variables are to be considered, including physical characteristics such as thickness, hygroscopicity and dissolving time. In terms of the probiotic, the CMC-OTF must allow for the maintenance of viability and enzymatic activity, preferably for an extended time period under non-controlled conditions. In the presented work, five different concentrations of CMC-1.25, 2.50, 5.00, 7.50 and 10.00 mg/mL of four different wet weights (5, 10, 20 and 40 g) were tested for their physical properties. Concentrations were originally chosen with respect to film drying and film size. Anything below 10 g would not cover a PD of 100 mm in diameter and CMC wet weight above 20 g needed too much time to dry, having detrimental effects on the viability of *L. fermentum* NCIMB 5221, with no detectable viable cells following film formation. The selected concentrations were further investigated to ensure suitable physical characteristics including hygroscopicity and dissolving time for oral delivery.

The first characterisation of the CMC films involved determining the thickness, with which it was concluded that as the CMC concentration increased, as film dry weight increased and as film wet weight increased, the thickness of the films increased, as expected. It was also determined that the films obtained were of relatively uniform thickness, as measurements of thickness were taken at various locations on the film. The 10 mg/mL OTFs, both 10 and 20 g wet weight were much thicker than all the other concentrations, suggesting a film that would take longer to dissolve under physiological conditions. Scanning electron microscopy was also performed to evaluate the pore size of the OTFs. However, the surface was too smooth and pore size could not be determined,

as there were no irregularities observed on the surface of the CMC films (data not presented). The hygroscopicity of the films was also determined; it was demonstrated that as the CMC concentration increased for the 10 g (wet weight) CMC, the SSF absorption volume increased linearly. On the other hand, the SSF absorption volume was highly variable across the different concentrations for the 20 g (wet weight) films. The inconsistency in the 20 g (wet weight) films in absorbing SSF may be explained by the increased thickness of the film, preventing even absorption of the solution.

The next characterization steps involved the determination of dissolving times of the OTFs under simulated oral conditions. The CMC films dissolving times were determined by dielectric spectroscopy. Following the thickness, swelling and dissolving time characterization of the different CMC film formulations, the 5 mg/mL 10 g (wet weight) was chosen as it presented the most uniform data and a constant and uniform dissolution time of approximately 7 min, appropriate for oral delivery of probiotic cells. The CMC films below 5 mg/mL concentration were thinner and dissolved faster. On the other hand, the 7.5 mg/mL and 10.0 mg/mL (as well as the 20 g (wet weight)) CMC films took too long to dissolve and dissolved un-uniformly, likely due to their uneven thickness. The 5.0 mg/mL CMC-film containing *L. fermentum* NCIMB 5221 was hence, used for all further studies.

The loss of viability of *L. fermentum* NCIMB 5221 upon film formation was determined. As expected, there was a significant loss in viability as the drying time is quite lengthy. Optimized drying apparatus may be required in the future to prevent this initial loss. Furthermore, future studies may investigate the use of a protectant, such as a

sugar or prebiotic, to further protect cellular integrity and maintenance of probiotic viability.

In addition, the probiotic bacterial release from the CMC-OTFs was determined under simulated oral conditions. The time for complete bacterial release was determined to be approximately 4 min, as compared to 7 min obtained for the dissolving time using the dielectric spectroscopy method. The discrepancy in dissolving time may be due to a number of factors, including, but not limited to, the use of a static (bacterial release method) vs. a continuous flow system (dielectric spectrophotometry method), the use of different CMC film area to dissolving solution volume ratios, and the presence of greater time points in the case of dielectric spectroscopy, as well as a higher sensitivity by the latter method.

The CMC-OTFs were shown to have desired properties for oral delivery, but investigations into the maintenance of probiotic viability and antioxidant activity were necessary. The CMC-OTFs containing *L. fermentum* NCIMB 5221 were shown to keep the probiotic viable for 150 days when stored under non-controlled conditions (room temperature, ambient humidity, light, etc.). It is important to note that the CMC-OTFs had a loss in mass as storage time increased, suggesting the further loss of water content. Hence, better care needs to be taken to ensure a constant water content and proper dehydration. Potentially, prolonged probiotic viability could be achieved by further controlling the storage conditions. In a recent study, Vesterlund *et al.* demonstrated a significant increase in the maintenance of bacterial viability in a dry food product when the food was efficiently dehydrated, with limited residual water activity [368]. Other techniques used to increase storage time of probiotic bacteria include lyophilization and

microencapsulation, both of which require complex and costly equipment and chemicals [369].

Previous research by our group investigated the antioxidant activity of the probiotic *L. fermentum* NCIMB 5221 using a fresh overnight bacterial culture [178]. In this study, following 150 days of storage in CMC-OTF, we demonstrate significant enzymatic activity, despite a loss when compared with the freshly formed CMC film. We hypothesize that the significant loss in viability observed between days 150 and 175 may be due to incomplete OTF dehydration or due to a loss of nutrients, as the bacteria may use the CMC as a carbohydrate source. These results suggest the successful maintenance of metabolic and enzymatic activity of the probiotic in the CMC-OTF formulation. Taken together, the incorporation of probiotic bacteria in CMC-OTFs is beneficial as it does not require any harsh chemicals nor manufacturing processes that could interfere with the viability and activity of the cells, or present toxicity upon oral delivery.

The development of the CMC-OTFs containing *L. fermentum* has great potential for the treatment and prevention of oral diseases [24,26,60], specifically dental caries and periodontitis. In addition, a CMC-OTF probiotic formulation has great potential as a biotherapeutic for the treatment and prevention of metabolic syndrome [300], hypercholesterolemia [300,303] and many other health disorders. This probiotic carrier system may be further optimized for the delivery of other probiotic strains and, potentially for the incorporation of a prebiotic. A prebiotic is a non-digestible food component that can stimulate the growth of beneficial bacteria. The incorporation of a probiotic and prebiotic in the CMC-OTFs would allow for the formation of a synbiotic, which may have a synergistic effect on human oral health [370]. The incorporation of a

prebiotic may also prove beneficial in terms of increasing the storage time of the embedded probiotic bacteria as well as the viability during CMC-OTF formation [371]. The molecular weight as well as other characteristics of the CMC may also be optimized to provide optimal OTF properties.

## **8.6 Conclusion**

In the presented research we have developed a novel CMC-OTF containing *L. fermentum* NCIMB 5221 suitable for oral delivery and the treatment and prevention of oral disorders. We characterized the CMC-OTFs for their physical properties, including thickness, dissolving time and hygroscopicity. We demonstrated that the CMC-OTFs can successfully maintain probiotic viability and antioxidant activity following long-term storage under non-controlled environmental conditions. The novel CMC-OTFs containing probiotic bacteria have great potential as oral delivery biotherapeutics for the treatment and prevention of oral diseases, as well as many other health disorders.

## **8.7 Acknowledgments**

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## **8.8 Conflict of interest**

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

## CHAPTER 9: GENERAL DISCUSSION

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Oral diseases are economic burden according to the World Health Organization, with dental caries and periodontal diseases being the most common oral diseases that affect patients worldwide [2]. However, the impact of oral diseases is often overlooked, even though they are not always limited to the oral cavity. It is well-established that dental caries and periodontal diseases lead to other health complication such as cellulites, osteomyelitis and infective endocarditis, as well as being associated with pre-term babies and diabetes [3-11]. Apart from dental caries and periodontal disease, oral candidiasis is on the rise, particularly in immunocompromised patients. Of more importance is the fact that the implications of oral candidiasis can be life threatening especially in patients infected with human immunodeficiency virus (HIV) [12,13].

The current therapeutics for these diseases focus mainly on prevention (listed in **Table 2.2, 2.3 and 2.4**). However, the onset of disease leads to the destruction of the supporting framework of the tooth, the tooth structures themselves and other oral tissues. The treatment approaches must then focus on the maintenance of the remaining tooth/oral tissue and restoration of lost structures [3-5,17]. These deleterious effects can be so widespread that they can lead to irreparable loss of these structures and dissemination of disease to much deeper and adjacent structures. It is important to note that oral disease, in particular dental caries and periodontal disease, is chronic in nature [372-374]. Moreover, the multifactorial pathogenesis of these diseases complicates the treatment prognosis. Briefly, the etiology of oral disease is initiated via salivary pellicle originating from glycoproteins present in the saliva which coats the tooth surfaces enabling the attachment of dental plaque [15,18]. Dental plaque houses a diverse microbial community within it and provides a surface that promotes the growth of opportunistic organisms [15,18]. As



for periodontal disease, the activated host immune system is responsible for increased levels of gingival crevicular fluid (GCF), an inflammatory exudate, increased presence of inflammatory cytokines and elevated leukocyte infiltration, which, together, contribute to periodontal disease progression [19]. It is important to note that bone remodeling is an important aspect of periodontal disease and is associated with an increase in bone resorption compared to bone formation [14,35]. In addition, dental caries and periodontal disease are positively associated with increased counts of certain bacteria such as *Streptococcus mutans* and the red complex (*Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*), respectively. Oral candidiasis, on the other hand, is caused by *Candida albicans*, an otherwise harmless organism, which manifests as an opportunistic infection in immunocompromised conditions, for example in HIV infections [16,375-377].

Keeping the pathogenesis of oral disease in mind, a natural therapeutic approach would be more beneficial as a safe long-term therapy. Probiotic bio-therapeutics provides one such approach that can prove beneficial to maintain oral health. Probiotics are “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host”, as defined by the WHO and the Food and Agriculture Organization of the United Nations [20]. Probiotic bacteria have been investigated for several diseases such as metabolic syndrome, skin inflammation, diarrhoea and colorectal cancer, to name a few [293,304,378]. Most importantly, several clinical trials have demonstrated that probiotic bacteria have the potential to lower the counts of oral pathogens, reduce oral inflammation and improve symptoms of oral candidiasis [21-24]. Interestingly, probiotic bacteria have also demonstrated the ability to produce both antimicrobial and anti-

inflammatory substances. Bacteriocins, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), nitric oxide (NO) and ferulic acid (FA) are just a few [178,293,379]. Based on these facts, we hypothesized that a novel probiotic oral thin film (OTF) could be formulated for the prevention and treatment of oral diseases that would limit growth of oral pathogens and reduce oral inflammation.

The initial goal was to screen probiotic bacteria with the potential to inhibit *S. mutans* and *C. albicans* responsible for dental caries and oral candidiasis, respectively. Previous research by Hasslof *et al.* demonstrated the inhibitory action of probiotic bacteria on oral pathogens [181]. We investigated the potential of probiotic by-products present in bacterial supernatant and live probiotic cells for their ability to inhibit the growth of both *S. mutans* and *C. albicans*. Our study demonstrated that live probiotic cells were able to inhibit both the pathogens. However, the cell-free supernatant could not inhibit any of the pathogens. This observation could be due to the fact that probiotic produced organic acids or bacteriocins that cause the inhibitory effects, may be unstable or degraded or are simply present in too low concentrations. In addition, the observed effects were dose and strain dependent [24]. This study leads us to investigate the mechanisms that would be responsible for the observed effect.

The next objective was to evaluate *S. mutans* inhibition by probiotic bacteria in simulated salivary conditions. These investigations have never been performed before. Our study demonstrated that all the tested probiotic bacteria were able to inhibit *S. mutans*. Quantitative analysis proved that the *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 702655 and *L. reuteri* NCIMB 702656 were best at inhibiting *S. mutans* NCIMB 702062. However, it was important to understand what was

causing these effects to choose an appropriate strain and optimize probiotic dose. Hence, the main objective was to investigate probiotic characteristics that would be responsible for the observed effects. The decrease of oral pH is associated with dental caries and we observed that *L. fermentum* NCIMB 5221, *L. fermentum* NCIMB 2797 and *L. acidophilus* ATCC 314 significantly modulated the pH of simulated saliva. Apart from oral pH, sucrose utilization by *S. mutans* contributes to caries formation aiding in lowering oral pH [14]. Our investigations demonstrated that probiotic *L. fermentum* NCIMB 5221 demonstrated high levels of sucrose consumption suggesting its role in competing for and reducing the amounts of nutrients available for *S. mutans* growth. Another interesting probiotic characteristic is their ability to form aggregates with pathogens, the phenomenon termed as co-aggregation. Pathogen co-aggregation with probiotic bacteria would aid in rapid clearance of pathogen from the mouth, limiting pathogen growth and progression of disease. In addition, a superior attachment of probiotic bacteria to oral surfaces compared to oral pathogens would be desirable characteristics to reduce the load of oral pathogens. Interestingly, we found that *L. fermentum* NCIMB 5221 also demonstrated the ability to co-aggregate with *S. mutans* and it successfully attached to gingival epithelial cells.

Another important aspect of oral disease is chronic inflammation that is present in periodontal diseases [373]. It is well-established that probiotic bacteria have the potential to secrete anti-oxidant molecules that can impart anti-inflammatory effects [178,293,307]. The investigated *L. reuteri* strains had superior anti-oxidant activity compared with all the other tested strains and would have the potential to reduce oral inflammation. We concluded that *L. reuteri* strains were superior *S. mutans* inhibitors and

*L. fermentum* NCIMB 5221 had other oral health promoting characteristics such as significantly buffered the salivary pH containing *S. mutans*, co-aggregated with *S. mutans*, demonstrated high levels of sucrose consumption and successfully attachment to gingival epithelial cells. Hence further investigations were performed to understand the inhibitory effects of *L. reuteri* strains and to study the anti-inflammatory effects of *L. fermentum* NCIMB 5221.

Following goal was to investigate the inhibition of cariogenic *S. mutans* via probiotic mediated hydrogen peroxide ( $H_2O_2$ ) production. It is well known that  $H_2O_2$  is an antimicrobial agent that has been used for wound disinfection and tooth whitening [139,251,257,258,263]. More interestingly, probiotic bacteria have been shown to produce  $H_2O_2$ . Previous research by Branton *et al.* demonstrated that *L. acidophilus*, has the capacity to produce  $H_2O_2$  [379]. Further,  $H_2O_2$  producing probiotic bacteria have demonstrated the ability to inhibit enteric, vaginosis-associated and uropathogenic pathogens [266]. In this study, we demonstrated that all the *L. reuteri* strains were able to produce  $H_2O_2$ . With more quantitative analysis, we observed that these strains were not only producing highest levels of  $H_2O_2$  within all the investigated *Lactobacillus* probiotic strains but were also the strains that were best at *S. mutans* inhibition. Similar observations were also made by Branton *et al.* [379]. However, to confirm that probiotic-produced  $H_2O_2$  was responsible for *S. mutans* inhibition, we added the enzyme catalase to the *L. reuteri* strains. Catalase has the ability to breakdown  $H_2O_2$  into simpler less toxic molecules. We observed that the catalase could reverse the inhibitory effects of *L. reuteri* strains, possibly due to probiotic produced  $H_2O_2$  breaking down into non-toxic compounds. This confirmed our hypothesis that  $H_2O_2$  was indeed one of the many

mechanisms by which probiotic bacteria demonstrate inhibitory effects on oral pathogens such as *S. mutans*.

Based on the previous results, the subsequent goal was to investigate *L. reuteri* NCIMB 701359 for its capability to limit *S. mutans* biofilm formation. We had previously demonstrated that *L. reuteri* NCIMB 701359 produced the most H<sub>2</sub>O<sub>2</sub> amongst all the investigated *L. reuteri* strains. In addition, it is important to note that *S. mutans* contributes to oral diseases by its ability to grow in planktonic form but thrives inside oral biofilms and in turn contributes to biofilm formation. Moreover, several *in vitro* models using glass slides, coverslips and hydroxyapatite beads have been to study oral biofilms [233,276,277]. The rationale behind our approach was to evaluate the reduction in *S. mutans* biofilm mass and *S. mutans* aggregates in the absence of probiotic bacterial biofilm contribution [198]. The work presented here is the first to study the effects of probiotic treatment on *S. mutans* biofilms simulated oral conditions. We demonstrated that not only did *L. reuteri* NCIMB 701359 inhibits planktonic *S. mutans* cultures but was also able to limit *S. mutans* biofilm formation. A decrease in biofilm mass, *S. mutans* counts in the biofilms and *S. mutans* aggregates were observed with *L. reuteri* NCIMB 701359 treatment.

Hence, we were able to establish that probiotic bacteria inhibited oral pathogens and possess several characteristics that could be responsible for their inhibitory potentials. With further investigations we also demonstrated that probiotic production of H<sub>2</sub>O<sub>2</sub> was responsible for such inhibitory effects and hydrogen peroxide producing *L. reuteri* NCIMB 701359 reduced *S. mutans* biofilm production. We then focused on the effects of probiotic bacteria on oral inflammation. As aforementioned, *L. fermentum*

NCIMB 5221 demonstrated the ability to utilize high levels of sucrose, co-aggregate with *S. mutans*, successfully attach to oral epithelial cells and have anti-oxidant activity. The fifth goal was to investigate *L. fermentum* NCIMB 5221 as a periodontal disease bio-therapeutic. As aforementioned, periodontal diseases are characterised as chronic inflammatory diseases that lead to the destruction of tooth supporting structures such as alveolar bone [374]. Moreover, previous research by our group has demonstrated that *L. fermentum* NCIMB 5221 is capable of producing Ferulic acid (FA) [178]. Of even more importance, was the fact that FA can scavenge reactive oxygen species, thereby acting as an anti-oxidant molecule [306]. Similarly, Jones *et al*, demonstrated that probiotic bacteria producing nitric oxide were able to alleviate inflammatory skin wounds [293]. The presented research demonstrated that *L. fermentum* NCIMB 5221 produced FA, NO and also exhibited anti-oxidant activity in simulated salivary conditions.

Further investigations revealed that *L. fermentum* NCIMB 5221 was able to modulate the host immune system, particularly, reduce pro-inflammatory cytokines IL-1 $\beta$  and IL-6 [316]. Similarly, *L. fermentum* NCIMB 5221 also increased cytokine IL12p-70 usually found in reduced amounts in periodontal diseases [323], suggesting their role in reducing oral inflammation. In addition, *L. fermentum* NCIMB 5221 also demonstrated the potential to enhance pre-osteoblast growth that could be beneficial for alveolar bone regeneration. Interestingly, investigations of *L. fermentum* NCIMB 5221 effects on F44 rats demonstrated a decrease in serum calcium, suggesting their role in modulating bone metabolism.

The final goal of the project was to develop a thin polymeric film for live probiotic delivery in the mouth. Currently several techniques and methods are used for

the oral delivery of probiotics, including incorporation in foods, capsules and gels [27-30]. However, these have important limitations. Oral delivery of probiotic bacteria, short storage time without loss of probiotic viability, limited probiotic residence time in the oral cavity, undesired effects on food consistency and patient non-compliance are just a few to name [344,345]. We developed a dissolvable carboxymethyl cellulose (CMC) OTF) for delivery of probiotic bacteria in the mouth. Following several optimizations for CMC film concentration, weight, hygroscopicity and dissolving time the 10 g 5 mg/mL CMC-OTF was chosen for probiotic delivery. *Lactobacillus fermentum* NCIMB 5221 was incorporated into the films. We demonstrated that the probiotic CMC-OTFs were able to maintain probiotic viability and antioxidant activity of the probiotic bacteria following 150 days of storage in non-controlled conditions [294]. Thus these investigations were able to demonstrate the potential of CMC-OTF probiotic bacteria for the prevention and treatment of oral diseases that would limit the growth of oral pathogens and reduce oral inflammation.

Results from current research appear optimistic that probiotic bacteria can be successful oral health bio-therapeutics. However, current research has several challenges that need to be addressed. Most studies focus on the inhibition of a specific pathogen like *S. mutans* for dental caries prevention and lack investigations to assess probiotic effects on the oral ecosystem as a whole. Studies such as the the Human Oral Microbiome Database (HOMD) that enumerate and characterise the oral microbiome are of utmost importance (<http://www.homd.org/>). In addition, it is important to remember that most oral diseases such as dental caries and periodontal diseases are polymicrobial. Therefore, looking at the reduction of a single species is not optimum for developing an effective

probiotic bio-therapeutic. Moreover, there is a vast variety of probiotic bacteria that can prove beneficial to prevent and treat oral diseases. There should be meticulous effort to screen hundreds of probiotic strains for limiting oral pathogens. Ideally, a single probiotic bacterial type should be able to inhibit all types of oral pathogens, which is of course a farfetched concept. It is likely that a successful approach may involve the use of a probiotic cocktail formulation, such as VSL#3 [322]. Moreover, it is well-established that the inhibitory effects of probiotic bacteria is both dose dependent and strain specific. Therefore, following probiotic strain selection, cocktail probiotic bacterial formulations should be tested for inhibiting a variety of oral pathogens. These studies could be time consuming and expensive to say the least.

Another important aspect of studying microbe-associated disease progression is the development of bioreactors that would mimic the mouth more closely for initial screening for oral health therapeutics. An initial screening of probiotic bacteria using such systems could be of great importance, particularly to study their effects on polymicrobial communities. Moreover, future research should not only focus on probiotic inhibition of pathogen growth but should also investigate their effects on metabolic activity of pathogenic microorganisms. Although there are a few clinical studies investigating probiotic bacteria, current studies have only been performed for short durations, whereas probiotic consumption is meant to be used as a long-term therapy. Moreover, the probiotic inhibition of oral pathogen in planktonic cultures does not imply that these pathogens would be inhibited when encased in biofilms. Therefore, the bioreactors should be designed to allow oral biofilm formation using multiple oral pathogens. These systems should also allow for a physical separation between the



pathogenic biofilms from the treatments containing probiotic bacteria that would provide better understanding. However, it is important to note that *Lactobacillus* strains have the ability to form biofilms, hence, selected probiotic *Lactobacillus* strains should be carefully selected and evaluated for any pathogenic trait they might impart [380]. Development of optimized *in vitro* models is crucial to the understanding of probiotic influence on oral diseases.

Although recent research is promising, future investigations should focus on elucidating the mechanisms responsible for the observed probiotic effects. In addition, most clinical studies observe the decline or growth of oral pathogens and assess clinical markers of disease such as GI, PI and BOP. Studies should also focus on investigating the pathways that cause oral inflammation and their relation with other systemic conditions such as diabetes that can impair, interfere and suppress repair mechanisms. Measurements of inflammatory markers both in serum and GCF can be helpful for investigating such pathways. A few human trials did look at GCF markers, however, studies using animal models presents with other challenges [23]. For example, collection of GCF from animals is extremely challenging [381].

One important aspect is the use of appropriate animal models for investigating oral diseases. Non-human primates particularly cynomolgus monkeys and beagle dogs are the most phylogenetically similar species to humans [382]. They are most commonly used for dental research with respect to dental caries and periodontitis studies. However, these are expensive, animals are not easy to maintain and ethical reasons limit the number of animals used for investigation. In addition, wild monkeys may carry many infectious diseases [382]. Dogs have been used for systemic candidiasis however, they could be

difficult models to be investigated for oral candidiasis as disease is usually induced by placing acrylic plates resembling dental prosthetics [383]. An interesting area of research, is the association of periodontitis with metabolic syndrome which encompasses diabetes mellitus (type 2) and cardiovascular disease [304]. Hence, the effect of probiotic treatment on animal models of metabolic syndrome would be very useful for developing a bio-therapeutic for treating both metabolic syndrome and periodontitis [130,384].

Small animals such as rats and hamsters have been used for dental caries studies [382]. Experimental caries can be induced by feeding a high-sucrose diet and oral pathogens. However, studying the oral microbiota presents important challenges, such as collecting bacterial samples from the oral cavity. Traditionally, samples such as tongue tips and cotton swabs have been collected, which are then grown using selective media [385,386]. However, differences in growth conditions such as temperature, aerobic/anaerobic atmosphere and growth media used for such samples should be monitored very carefully. Any differences in these conditions and between samples can alter the counts of micro-organisms in question. Moreover, sites of sample collection and duration of swabbing can present with some variability. More importantly, rodents such as rats and hamsters are poor animal models for periodontitis studies as their oral microbiota is different from humans and the progression of periodontal diseases is not similar [382]. Investigations to develop or find appropriate animal models are imperative to the understanding of oral disease and the influence of therapeutics.

Equally important is the need for the development of a delivery system that allows for a prolonged residence time of the delivered probiotic in the oral cavity. Previous and current research has focused on the use of chewing gums, mouth rinses,

probiotic drops, chocolate, and food products such as milk, cheese and yoghurt for probiotic delivery. **Table 2.7** provides a brief overview of the carrier systems previously investigated for probiotic delivery. Although these delivery systems have shown promising results, there remain a number of limitations to be addressed. For example, probiotics incorporated in food products, upon swallowing, result in rapid loss from the oral cavity. A loss of probiotics from the oral cavity means a loss of their efficiency in preventing and treating oral diseases. Developed delivery systems should, hence, focus at increasing the retention time of the probiotics in the oral cavity, increasing the efficiency of the probiotic therapeutic. Carrier systems, such as chewing gums may increase the retention time of probiotics in the oral cavity, however the retention time may need to be increased further for an optimal effect. Factors that need to be considered while developing a delivery system include the probiotic's targeted delivery while maintaining the viability and metabolic activity of the probiotic cells. A carrier system which would enable the release of probiotics in areas difficult to reach, such as the interdental spaces, pits and fissures of the teeth should be developed. Current research into carrier systems such as collagen scaffolds and hydrogels may prove promising for probiotic delivery [387,388]. A carrier system which is made up of a prebiotic may just prove to be an appropriate technology. Lastly, the shelf-life of a carrier system and the probiotic incorporated in the system should be investigated, as bacterial viability is directly correlated with the efficacy of any developed therapeutic.

The safety of all the probiotic strains should be investigated prior to any clinical studies, as the delivery of any microorganism can potentially prove harmful. The safety studies should involve dose optimization studies, where only the required number of

bacterial cells would be delivered, to avoid any bacterial overload. Reliable and reproducible methods for sampling and quantifying oral bacteria *in vivo* should be investigated. In terms of PD, quantifying inflammatory cytokines and bone resorption are among the most important investigations. Procedures which would enable accurate sampling and analysis of inflammatory cytokines are of prime importance. Lastly, well-designed and lengthier human trials should be performed in order to produce an appropriate probiotic therapeutic for combating oral diseases.

It is clear from this study that probiotic bacteria can inhibit oral pathogens, modulate host immune response that has an important role in inflammation and limit oral tissue destruction for example by bone regeneration. This project shows great potential of probiotic bio-therapeutic for the prevention and treatment of oral disease and opens up several avenues for future investigations for the same.

## CHAPTER 10: SUMMARY OF OBSERVATIONS, CLAIMED ORIGINAL CONTRIBUTIONS TO KNOWLEDGE AND CONCLUSIONS

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### 10.1 Summary of observations

This novel work demonstrates the potential of an oral thin film probiotic bacterial formulation, as a natural oral health bio-therapeutic for the prevention and treatment of oral diseases. The precise novel findings are as below:

1. Probiotic *Lactobacillus* bacteria were screened for *Streptococcus mutans* and *Candida albicans* inhibition. Live *Lactobacillus* bacteria were required to observe inhibition of both organisms, suggesting their potential role and the importance of screening probiotic bacteria for their use as oral health therapeutic.
2. Quantitative inhibition assay in simulated salivary conditions demonstrated that *Lactobacillus reuteri* NCIMB 701359, *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 702655 and *L. reuteri* NCIMB 702656 inhibited *S. mutans* to non-detectable levels ( $< 10$  cfu/ml).
3. A reduced salivary pH has been associated with oral disease pathogenesis. Investigations into probiotic pH buffering of simulated salivary fluid (SSF) demonstrated that *Lactobacillus fermentum* NCIMB 5221 ( $4.18 \pm 0.005$ ), *L. fermentum* NCIMB 2797 ( $3.93 \pm 0.012$ ) and *L. acidophilus* ATCC 314 ( $4.12 \pm 0.018$ ) buffered the pH of saliva containing *S. mutans* ( $3.72 \pm 0.017$ ) significantly ( $p < 0.001$ ).
4. Sucrose is the preferred nutrition source of *S. mutans*, the primary causative agent of dental caries. Investigations into probiotic competition for sucrose demonstrated high levels of sucrose utilization ( $137.51 \pm 0.31$  to  $144.53 \pm 0.01$  mM) by all the

investigated probiotic strains. *L. reuteri* NCIMB 701089 consumed the most sucrose ( $144.53 \pm 0.01$  mM).

5. Probiotic bacteria with the ability to form aggregates with oral pathogens such as *S. mutans*, would limit their attachment to oral surfaces, thereby allowing their rapid clearance from the oral cavity. Investigations into probiotic co-aggregation with *S. mutans* suggested that *L. fermentum* NCIMB 5221 demonstrated the highest co-aggregation of *S. mutans* ( $11.24 \pm 1.09$  %).
6. Superior attachment of probiotic bacteria to oral surfaces as compared to oral pathogens would limit their growth and biofilm formation. Investigations into probiotic attachment to epithelial keratinocytes demonstrated that. *L. fermentum* NCIMB 2797 ( $p = 0.002$ ), *L. fermentum* NCIMB 8829 ( $p = 0.001$ ) and *L. reuteri* NCIMB 11951 ( $p = 0.003$ ) demonstrated the highest bacterial adhesion compared to all the other tested probiotic strains and *S. mutans*.
7. Nitric oxide (NO) demonstrated both antimicrobial and anti-inflammatory properties, beneficial for both oral pathogen inhibition and reducing oral inflammation. Investigations into probiotic NO production suggested that *S. mutans* NCIMB 702062 produced significantly higher levels of NO ( $7.50 \pm 2.09$   $\mu$ M) following 24 h of incubation in SSF.
8. Probiotic secreted antioxidant molecules demonstrated potential to reduce inflammation which would prove beneficial for treating periodontal disease. Investigations into probiotic antioxidant activity demonstrated that all of the tested *L. reuteri* strains produced significantly higher levels of antioxidants. *L. reuteri* NCIMB 701359 demonstrated highest ( $p < 0.001$ ) antioxidant activity.

9. Probiotic bacteria have the potential of inhibiting oral pathogens such as *S. mutans* and secreting hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). The concentration of  $\text{H}_2\text{O}_2$  inhibitory for *S. mutans* was determined to be 1.3  $\mu\text{M}$ . Qualitative and quantitative  $\text{H}_2\text{O}_2$  production by probiotic bacteria demonstrated that all of the tested *L. reuteri* strains produced significantly higher levels of  $\text{H}_2\text{O}_2$  and their quantities were higher than *S. mutans*  $\text{H}_2\text{O}_2$  MIC. Addition of catalase, that can diminish the effects of  $\text{H}_2\text{O}_2$ , significantly reduced *S. mutans* inhibition ( $p < 0.001$ ) via  $\text{H}_2\text{O}_2$  producing *L. reuteri* strains. This study demonstrated that inhibition of *S. mutans* was due to probiotic  $\text{H}_2\text{O}_2$  production.
10. Cariogenic *S. mutans*, form oral biofilm and flourish in the formed biofilm, associated with dental caries etiology. *L. reuteri* NCIMB 701359 was the highest  $\text{H}_2\text{O}_2$  producers among all the tested probiotic strains ( $6379.45 \pm 31.28 \mu\text{M}$ ). *L. reuteri* NCIMB 701359 inhibition of *S. mutans* biofilm formation was evaluated for the development of a potential probiotic dental caries therapeutic. Results demonstrated significant reduction in biofilm mass ( $p = 0.03$ ), *S. mutans* viability ( $p = 0.02$ ) and density of *S. mutans* aggregates ( $p = 0.01$ ).
11. Chronic inflammation is a hallmark of periodontal disease. Hence, probiotic secretion of antioxidant molecules such as ferulic acid (FA) and NO would be beneficial for reducing oral inflammation. The probiotic *L. fermentum* NCIMB 5221 demonstrated antioxidant activity ( $5.57 \pm 0.37 \mu\text{M}$  Trolox equivalents) in SSF. Moreover, this strain also produced  $676.63 \pm 197.51 \mu\text{M}$  of FA and  $7.23 \pm 0.18 \mu\text{M}$  NO in SSF demonstrating anti-oxidant and anti-inflammatory properties in the simulated oral conditions.

12. *L. fermentum* NCIMB 5221 treatment on inflamed osteoblast like cells significantly reduced inflammatory cytokine IL-1 $\beta$  levels ( $0.029 \pm 0.001$  pg/ $\mu$ g,  $p = 0.00008$ ) as compared to non-treated ( $0.111 \pm 0.015$  pg/ $\mu$ g) at 24 h and IL-6 level ( $2.41 \pm 0.21$  pg/ $\mu$ g,  $p=0.004$ ) as compared to non-treated ( $3.256 \pm 0.13$  pg/ $\mu$ g) at 4 h. Interestingly, the IL-12p70 levels, found in decreased levels in disease, were elevated (non-significant) with probiotic treatment, suggesting their role in reducing oral inflammation.
13. *L. fermentum* NCIMB 5221 treatment on pre-osteoblast, significantly promoted their growth ( $\sim 140$  % compared to control at 72 h,  $p < 0.005$ ) suggesting its role in bone regeneration. Moreover, *L. fermentum* NCIMB 5221 treatment on F344 rats significantly reduced levels of serum calcium ( $02.87 \pm 0.036$  mmol/L,  $p = 0.02$ ) suggesting its role in bone metabolism.
14. Novel carboxymethyl cellulose (CMC)-probiotic-oral thin film (OTF) was developed to deliver probiotics for the treatment/prevention of oral disorders. CMC-OTF was characterized for CMC concentration (1.25–10 mg/mL), weight (5-40 g), thickness (16–262  $\mu$ m), hygroscopicity (30.8–78.9 mg/cm<sup>2</sup> film) and dissolving time (135-600 sec). 10 g 5 mg/mL CMC-OTF was selected for *L. fermentum* NCIMB 5221 incorporation. Long-term non-controlled storage of the probiotic CMC-OTFs in room temperature demonstrated no loss of bacterial viability and anti-oxidant activity for 150 days.



## 10.2 Conclusion

Oral diseases are considered economic burden and are often chronic conditions. With the limitations of current therapies, a long-term therapeutic approach might prove beneficial. Probiotic bacteria have great potential to combat oral diseases. Our investigations demonstrated that *Lactobacillus* probiotic bacteria successfully inhibited oral pathogens, namely *S. mutans* and *C. albicans*, primary causative organisms of dental caries and oral candidiasis, respectively. *L. reuteri* strains demonstrated superior inhibitory effects on oral pathogens while *L. fermentum* NCIMB 5221 demonstrated beneficial characteristics important in limiting dental caries, oral candidiasis and periodontal diseases. The specific characteristics were SSF pH buffering, co-aggregation with *S. mutans*, high levels of sucrose consumption and successful attachment to gingival epithelial cells. Additionally, *L. reuteri* strains demonstrated H<sub>2</sub>O<sub>2</sub> production and the highest probiotic H<sub>2</sub>O<sub>2</sub> producer successfully limited *S. mutans* biofilm, an important factor for dental caries etiology. Further investigations demonstrated *L. fermentum* NCIMB 5221 produced anti-oxidant molecules such as FA and NO, beneficial for limiting oral inflammation in periodontal diseases. *L. fermentum* NCIMB 5221 significantly reduced pro-inflammatory cytokines, secreted by inflamed osteoblasts and enhanced pre-osteoblast growth significantly, suggesting its role both in reducing oral inflammation and augmenting bone regeneration. *L. fermentum* NCIMB 5221 effects were also evident *in vivo*, by modulating serum calcium levels, suggesting its potential role in bone metabolism. Lastly, *L. fermentum* NCIMB 5221 was entrapped in a film made of carboxymethyl cellulose and were characterised for probiotic delivery in the mouth. This project enforces the potential of a probiotic bio-therapeutic, particularly via

*L. reuteri* NCIMB 701359 and *L. fermentum* NCIMB 5221, in the form of a dissolvable film, as long-term therapeutics for the prevention and treatment of oral diseases.

### **10.3 Claimed original contributions to knowledge**

1. Probiotic *Lactobacillus* bacteria were screened for the inhibition of *S. mutans* and *C. albicans*.
2. Results suggests that probiotic bacteria particularly, *L. reuteri* NCIMB 701359, *L. reuteri* NCIMB 701089, *L. reuteri* NCIMB 702655 and *L. reuteri* NCIMB 702656 inhibited *S. mutans* to non-detectable levels in simulated oral conditions.
3. Results suggests *L. fermentum* NCIMB 5221, *L. fermentum* NCIMB 2797 and *L. acidophilus* ATCC 314 significantly buffered the salivary pH containing *S. mutans*, suggesting their potential role in limiting the growth of acidogenic/aciduric pathogens such as *S. mutans*.
4. Probiotic bacteria demonstrated high levels of sucrose utilization suggesting their potential in nutrient competition with oral pathogens.
5. Probiotic *L. fermentum* NCIMB 5221 demonstrated highest co-aggregation of *S. mutans* limiting their attachment to oral surfaces aiding in rapid pathogen clearance from the mouth beneficial for dental caries.
6. Probiotic *L. fermentum* NCIMB 2797, *L. fermentum* NCIMB 8829 and *L. reuteri* NCIMB 11951 showed superior bacterial adhesion to oral epithelium compared to *S. mutans*, suggesting their potential in limiting oral pathogen growth and biofilm formation.

7. Probiotic *L. reuteri* NCIMB 701359 demonstrated superior antioxidant activity suggesting its potential in reducing inflammation beneficial for treating periodontal disease.
8. Probiotic bacteria producing H<sub>2</sub>O<sub>2</sub> showed successful inhibition of dental caries causing *S. mutans*.
9. Probiotic *L. reuteri* NCIMB 701359, the highest H<sub>2</sub>O<sub>2</sub> producer as compared to *L. reuteri* NCIMB 701389, *L. reuteri* NCIMB 702655, *L. reuteri* NCIMB 702656 and *L. reuteri* NCIMB 11951, also limited *S. mutans* biofilm formation, hence, limiting one of the major aetiologic factors for dental caries.
10. Results demonstrate that *L. fermentum* NCIMB 5221 produced FA and NO. These molecules showed potential to reduce inflammatory cytokines secreted by inflamed pre-osteoblasts and promoted pre-osteoblasts growth, suggesting their role in oral inflammation reduction and bone regeneration.
11. Results demonstrate that *L. fermentum* NCIMB 5221 also reduced serum calcium levels in F344 rats, suggesting role in bone metabolism.
12. A novel carboxymethyl cellulose-probiotic-oral thin film (CMC-OTF) was designed and characterised for their suitability to be used in dental caries, oral candidiasis and periodontal diseases.
13. Results indicate that CMC-OTF was suitable for probiotic delivery for the prevention and/ or treatment of dental caries, oral candidiasis and periodontal diseases.

## CHAPTER 11: RECOMMENDATIONS

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This thesis project demonstrates the potential of probiotic bio-therapeutics, particularly *Lactobacillus reuteri* NCIMB 701359 and *Lactobacillus fermentum* NCIMB 5221, for oral health. However, several investigations are needed to be performed before these bacteria can be recommended for clinical use.

Meticulous screening of probiotic bacteria is required that can not only limit one oral pathogen but can also limit numerous oral pathogens such as *S. mutans* and the red complex, simultaneously. Moreover, a cocktail of different probiotic strains might demonstrate broad spectrum anti-microbial effects. Development of bioreactors mimicking the oral ecosystem is imperative and essential for such studies.

Selected probiotic bacteria should be investigated for bacteriocin activity, anti-oxidant activity and production of anti-inflammatory molecules. In this thesis project we demonstrated that hydrogen peroxide production via probiotic bacteria was responsible for *S. mutans* inhibition. More probiotic strains should be screened for hydrogen peroxide production.

Elaborate mechanistic studies should be performed that would also investigate the molecular basis of such therapeutics. In terms of bone loss, molecular markers, namely Receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) could elucidate the beneficial properties of probiotic therapeutics.

In term of *in vivo* studies, the duration of treatment and amount of probiotic dose need to be estimated for future clinical applications. More importantly, techniques used for oral pathogens enumeration need to be established as a huge population of oral microbes are either not cultivable or are yet to be identified. Another major concern is

elaborated safety profiling. Lengthy *in vivo* animal and human trials are pivotal for such investigations. Techniques used for understanding bone architecture such as micro-computed tomography is pivotal for understanding probiotics effects in *in vivo* models. Bone and gingival histology examinations are also essential.

*In vivo* trials, to establish the effects of probiotic-CMC-OTF delivered orally, is essential. These studies would be challenging when performed using animal models, hence, further human clinical trials should be performed to explore their full potential.

Altogether, probiotic bacteria are safe and natural and demonstrate great potentials as oral health bio-therapeutics. Moreover, it is to be noted that these investigated strains are not only beneficial as oral therapeutics but are also beneficial for other health problems. Hence, probiotic bio-therapeutics can be used as a global approach for human health.

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