



Clinical and Microbiological Effects of Probiotics in Experimental Induced Periodontitis

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in
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Declaration

I, Simona Marieta Gatej, certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Preface

This thesis is submitted in the 'Combined conventional publication format' in accordance with "Specifications for thesis 2018" of the University of Adelaide. It contains an introduction, a detailed literature review and three experimental chapters followed by conclusion and appendices.

The resulting research conducted during the three and a half years of this PhD program has resulted in publication of two articles in reputed journals. Additionally one other journal article is under preparation. Furthermore, the research findings of this PhD study have been presented at seven national and international conferences. A complete list of publications and conference presentations is provided in the following pages.

List of publications and conference presentations

Peer review journal articles published

1. **S. Gatej**, N. Gully, R. Gibson, P.M. Bartold, “Probiotics and Periodontitis – A literature Review”, *Journal of the International Academy of Periodontology*, 2017, 42-50
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Abstract

Objectives: This study investigated the role of *Lactobacillus rhamnosus* GG (LGG) on bone loss and local and systemic inflammation in an *in vivo* mouse model of induced periodontitis. Changes in the gastrointestinal physiology and the influence of different probiotic administration methods were also investigated.

Methods: 36 mice were allocated into six groups ($n = 6$ per group). Experimental periodontitis was induced in three of the groups by oral inoculation with *Porphyromonas gingivalis* and *Fusobacterium nucleatum* over a period of 44 days. The probiotic LGG was administered via two different methods (oral inoculation and oral gavage) prior to, and during, disease induction. The antimicrobial activity of LGG on the pathogens used was tested. Alveolar bone levels were assessed using *in vivo* micro-computed tomography. Gingival and intestinal tissue changes were evaluated using histological analysis. Systemic and intestinal inflammation were assessed by measuring the level of the pro-inflammatory markers IL-6 and LIX in tissue and blood serum using multiplex assays and immunohistochemistry. The phylogenetic structure and diversity of the intestinal microbiota were analysed by sequencing the 16S rRNA genes of the caecal content. Statistical significance was accepted when for $p < 0.05$.

Results: Pre-treatment with LGG either via oral gavage or oral inoculation significantly reduced bone loss ($p < 0.0001$), gingival inflammation ($p < 0.0001$) and TRAP positive cells ($p = 0.0020 - 0.0176$) for the probiotic treated groups when compared with controls. Analysis of the pro-inflammatory marker LIX expression in serum demonstrated a significant increase in systemic inflammation for the disease mice when compared with controls. LGG demonstrated no antimicrobial activity

against *P. gingivalis* and *F. nucleatum* . There were significant changes in the histology of the gastrointestinal tract of disease mice when compared with controls ($p < 0.05$). Additionally, disease mice presented a significant increase in the expression of the inflammatory marker IL-6 in gut tissue when compared with controls. Mice pre-treated with LGG via gavage had significantly reduced tissue inflammation scores in the duodenum and significantly lower levels of IL-6 in the ileum when compared with disease. Oral inoculation with *P. gingivalis* and *F. nucleatum* led to a significant change in the bacterial composition of the caecal microbiome of the control group versus disease ($p < 0.05$). LGG therapy prevented gut microbiome changes induced by *P. gingivalis* and *F. nucleatum*, regardless of the probiotic mode of administration.

Conclusions: Administration of *P. gingivalis* and *F. nucleatum* induced significant changes in intestinal and systemic inflammation and significant changes in the intestinal microbiome. Therapy with LGG effectively suppressed bone loss and local inflammation for all probiotic treated groups when compared with disease irrespective of the mode of administration. Additionally, pre-treatment with LGG exerted a protective effect against intestinal and systemic inflammation and had a significant influence on the composition of the gut microbiome, promoting beneficial bacteria in the intestines of treated mice. Clinically, in the future, LGG may offer a low-risk, easy to use treatment option for the management of periodontitis.

CHAPTER 1

Introduction

Chapter 1. Introduction

1.1 Periodontitis

For nearly three decades now, periodontitis has been defined as a chronic inflammatory condition of both the supporting soft and hard tissues of the teeth resulting in progressive destruction of periodontal ligament and alveolar bone (1). It has also been acknowledged that, in periodontitis, specific microorganisms are necessary but their presence alone is not sufficient for the development of the disease (2). Indeed, as recently pointed out, the host immune system and an inappropriate inflammatory response, together with genetic and modifying environmental factors play a pivotal role in the pathogenesis and treatment of this complex condition (3).

In 2015, the American Association of Periodontology (AAP) recognised the 1999 classification of chronic periodontitis as slight (or mild), moderate and severe (or advanced) (Table 1) and established guidelines for the severity of this disease (4). According to the report, diagnosis of periodontitis is based on the presence of two or more of the following clinical and radiographic parameters: 1. one or more sites of inflammation (bleeding on probing), 2. increased probing depth or clinical attachment loss and 3. radiographic bone loss. The report also defined features of chronic and aggressive periodontitis, the two main forms of destructive periodontal disease, and stated that correct diagnosis of these conditions had significant implications on their therapy and long-term prognosis (4). According to a World Health Organization

report from 2012, 15–20% of adults aged 35-44 years suffer from severe periodontal disease resulting in tooth loss (5). In Australia, the most recent data come from the 2004-2006 National Survey of Oral Health and show that one in five Australian adults suffer from moderate to severe periodontitis that may result in tooth loss (6).

Table 1. Severity of periodontitis as per American Association of Periodontology clinical interpretation of the 1999 Classification of Periodontal Diseases and Conditions adapted from Geurs *et al.* (4)

	Slight (Mild)	Moderate	Severe (Advanced)
Probing depth	> 3 and < 5 mm	≥ 5 and < 7 mm	≥ 7 mm
Bleeding on probing	Present	Present	Present
Radiographic bone loss	≥ 2 and ≤ 3 mm	> 3 and ≤ 5 mm	> 5 mm
Clinical attachment loss	1-2 mm	3-4 mm	≥ 5 mm

Periodontitis is a complex condition and associations have been reported with various systemic conditions such as rheumatoid arthritis (7), diabetes mellitus (8), cardiovascular disease (9), obesity (10), chronic pulmonary disease (11) and chronic kidney disease (12). Therefore, considering the health impact of periodontitis on the individual, there is a clear need for new prevention and periodontal treatment strategies.

The precise aetiology of periodontitis is complex, multifactorial and not completely understood, and some of the specific factors that determine a person’s susceptibility to this disease are still to be elucidated (13). Presence of bacterial biofilm

accumulation on tooth surfaces at the gingival margin initially results in gingivitis (Figure 1b), an inflammation of the soft tissues which can be completely reversed under normal physiological conditions by using adequate oral hygiene practices(14). In individuals susceptible to periodontitis, soft tissues inflammation is accompanied by significant changes in the ecology of the oral microflora towards **dysbiosis**, together with destruction of gingivae, periodontal ligament and alveolar bone (15) (Figure 1c). (Terms highlighted in bold throughout the text are included in Table 2).



Figure 1. a. Healthy periodontal tissues. b. Early gingival inflammation (arrow: dark red, oedematous gingivae). c. Clinical appearance of chronic periodontitis (arrow: scalloped, receding, inflamed gingivae). (14) © John Wiley & Sons, reproduced with permission (License no 4311130321878)

Disease initiation and progression are influenced by several environmental risk factors including yet not limited to smoking and uncontrolled type-2 diabetes (16) and host-specific risk factors determined by the individual's genetic makeup (17). Current treatments for periodontitis, more specifically, subgingival debridement together with the use of antimicrobial mouthwashes, lead to a temporary reduction in the level and proportion of certain pathogenic bacteria and associated inflammation that has not proven sufficient to control the disease (18, 19). Systemic broad-spectrum antibiotics such as doxycycline, amoxicillin or metronidazole are sometimes used in conjunction with subgingival debridement for the treatment of chronic and aggressive periodontitis (20-22). The rationale for use of these particular adjunctive antibiotics is

based on the change they induce in the microbial composition of the subgingival biofilm, together with reduced bleeding, clinical attachment loss and probing depths (23). Treatment compliance may be affected by adverse gastrointestinal side-effects such as antibiotic-associated diarrhoea, nausea, vomiting (24) and intestinal dysbiosis (25). Additionally, the benefits of antibiotic use on the patient's periodontal condition need to be considered in the light of increased bacterial resistance due to the frequency of antibiotic use (26).

Table 2. Glossary of terms

Glossary of terms
<p>Metagenomics Analysis of genetic information recovered from a host sample</p> <p>Microbiota A complex community of microorganisms present in a certain environment. It includes prokaryotes such as bacteria and archaea, and eukaryotes such as fungi, protozoa and viruses</p> <p>Microbiome All genetic material within a microbiota</p> <p>Symbiosis Long-term biological relationship between two or more species</p> <p>Dysbiosis Alteration in the diversity, composition or function of the microbiome (such as types and number of bacteria), associated with a diseased state</p> <p>Probiotics Live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host</p>

A recent randomised, double blind, placebo controlled clinical trial examined the relevance and long term impact of adjunctive systemic antibiotics on the progression

of periodontitis (27). Over a period of 27.5 months and with 506 participating patients from different dental centres, the use of metronidazole plus amoxicillin as an adjunctive treatment resulted in a small, although statistically significant, additional reduction in further attachment loss when compared with placebo (27). The authors concluded that antibiotics should be restricted for use with specific patient groups and conditions, such as in aggressive and severe forms of periodontitis, whilst also taking into account the overall risk for periodontal disease and the modification of behavioural risk factors such as smoking (27). In conclusion, the potential benefits of using systemic antibiotics as adjuncts in the treatment of periodontitis and the impact of their side effects on individual health (gut dysbiosis) and public health (increase in antibiotic resistance) need to be carefully considered (Figure 2) (28).

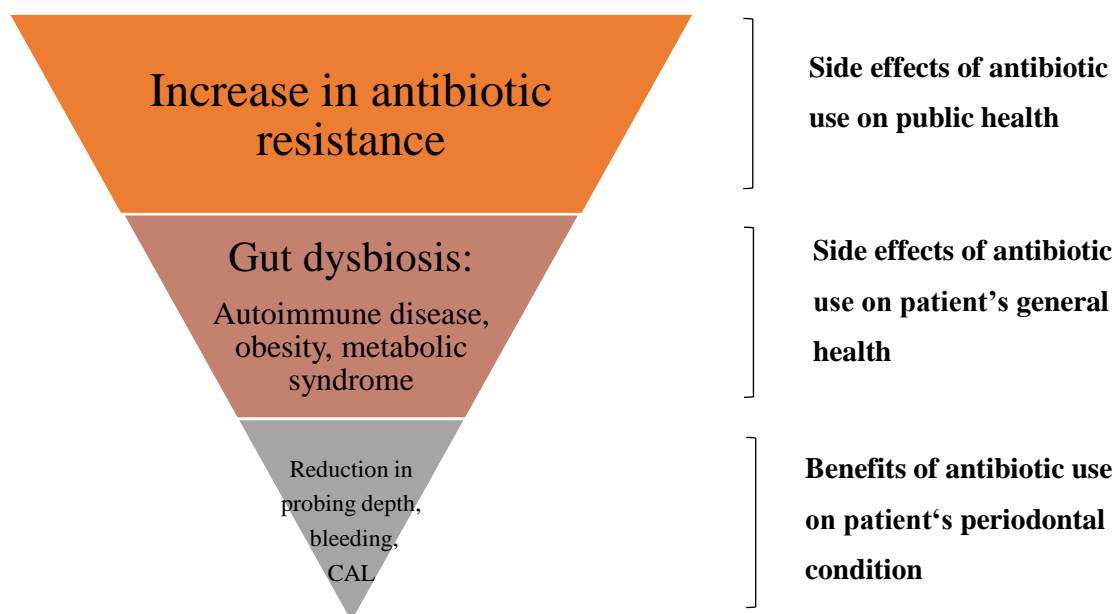


Figure 2. Benefits and side effects of antibiotic use in periodontitis on patient's periodontal condition and general health, and on public health. Abbreviations: CAL – clinical attachment loss

In this context, manipulation of the commensal pathogenic bacteria by increasing the number of beneficial microorganisms may be considered as an alternative strategy to antibiotic treatment and achievement of long term periodontal health (29).

1.1.1 The role of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in progression of periodontal disease

Recent developments in **metagenomics** clearly demonstrated both oral and gastrointestinal **microbiomes** are the most diverse human microbial environments (30). The oral cavity harbour 13 phyla with more than 700 species of aerobic, facultative anaerobic and anaerobic bacteria (31). Of all the Gram-positive or Gram-negative bacterial species commonly isolated from the oral cavity, six phyla contain 96% of the taxa (31) (Table 3). Changes in bacterial composition and structure of the diverse oral environment have been associated with transition from symbiotic to dysbiotic bacterial communities (32). *Fusobacterium nucleatum* and *Porphyromonas gingivalis* represent two oral microbiome bacterial species that are linked with progression of periodontal disease (33). *F. nucleatum* is an obligate anaerobe Gram-negative bacterium belonging to the *Fusobacteriaceae* family, ubiquitous in the human oral cavity, isolated from both healthy and diseased sites (34). *F. nucleatum* has a remarkable ability to increase chances of survival in a biofilm environment for anaerobic and facultative-anaerobic bacteria by co-aggregating with them (35). In the oral cavity, co-aggregation between species enables bacteria to withstand the salivary flow and establish a metabolic relationship (36).

Table 3. Six oral bacteria phyla contain 96% of the taxa (31)

Gram-positive bacteria	Bacterial Phylum	Bacterial Genus	Commonly isolated bacterial species
	Firmicutes	<i>Streptococcus</i>	<i>S. salivarius</i> , <i>S. mutans</i>
		<i>Staphylococcus</i>	<i>S. aureus</i>
		<i>Enterococcus</i>	<i>E. faecalis</i>
		<i>Lactobacillus</i>	<i>L. casei</i> , <i>L. salivarius</i>
		<i>Bacillus</i>	<i>B. cereus</i>
	Actinobacteria	<i>Actinomyces</i>	<i>A. israeli</i>
Gram-negative bacteria	Fusobacteria	<i>Fusobacterium</i>	<i>F. nucleatum</i>
	Bacteroidetes	<i>Tannerella</i>	<i>T. forsythia</i>
		<i>Porphyromonas</i>	<i>P. gingivalis</i>
		<i>Prevotella</i>	<i>P. intermedia</i>
	Spirochaetes	<i>Treponema</i>	<i>T. denticola</i>
	Proteobacteria	<i>Aggregatibacter</i>	<i>A. actinomycetemcomitans</i>

F. nucleatum is thought to be a key bacterium in the formation of mature oral biofilm, binding via interspecies adherence with early Gram-positive colonisers such as *streptococci* and *Actinomyces* (37, 38). Following establishment in the oral biofilm, *F. nucleatum* lowers the redox potential (Eh) of the environment, facilitating adherence and persistence of certain late anaerobic Gram-negative colonisers such as *Porphyromonas* and *Aggregatibacter* (39, 40). A recent metatranscriptome analysis supported the polymicrobial synergy and dysbiosis model for periodontitis pathogenesis and asked for further studies to determine the specific role of *F. nucleatum* in periodontitis (38). Oral infection with 10^9 *F. nucleatum* in 100 μ l of PBS in Balb/c mice induced inflammation and infiltration of macrophages in gingival tissue and promoted alveolar bone resorption (41). It has been previously demonstrated

that dual infection with *F. nucleatum* and *P. gingivalis* in mice aggravates alveolar bone loss and inflammation compared with animals treated with either bacterium alone (42).

P. gingivalis belongs to the phylum Bacteroidetes and is an anaerobic Gram-negative bacterium inhabiting the oral cavity (43). *P. gingivalis* is associated with *Treponema denticola* and *Tannerella forsythia*, forming the 'red complex', traditionally considered to play important roles in pathogenesis of periodontitis based on their bacterial virulence and association with affected sites (1, 44, 45). Recent advances in metagenomics demonstrate that the roles of the 'red complex' bacteria in disease progression are important. However, with emergence of recent research, these roles may need to be re-evaluated to include other virulence expressing organisms such as *Neisseria spp.*, *Corynebacterium matruchotii*, *Rothia dentocariosa*, *Veillonella parvula* and *Actinomyces spp* previously not linked with the pathogenesis of periodontitis yet which may be of potential significance (46).

According to Killian *et al.*, *P. gingivalis* occurs in relatively low abundance in the oral biofilm (33). Nevertheless, *P. gingivalis* presence has been associated with a shift in microbial balance from a symbiotic to a dysbiotic state, this bacterium being considered one of the keystone pathogens associated with pathogenesis and progression of periodontitis (46). In addition, germ free mice inoculated with *P. gingivalis* (10^9 CFU/ml) alone did not develop bone loss, leading to the conclusion that very low colonization levels of this bacterium changes the amount and composition of the oral **microbiota** and that a commensal microbiota is required for bone loss to develop (47). The colonisation of keystone pathogens such as *P. gingivalis* is facilitated by defects in the immune status of the host including neutrophil recruitment and results in an enhancement of further dysbiotic bacterial

communities that amplifies the destructive inflammatory response resulting in the destruction of periodontal tissue (48). The tissue breakdown products (e.g. degraded collagen, heme-compounds) provide nutrients for the dysbiotic microbiota thus contributing to the chronic, pathogenic cycle (49) (Figure 3).

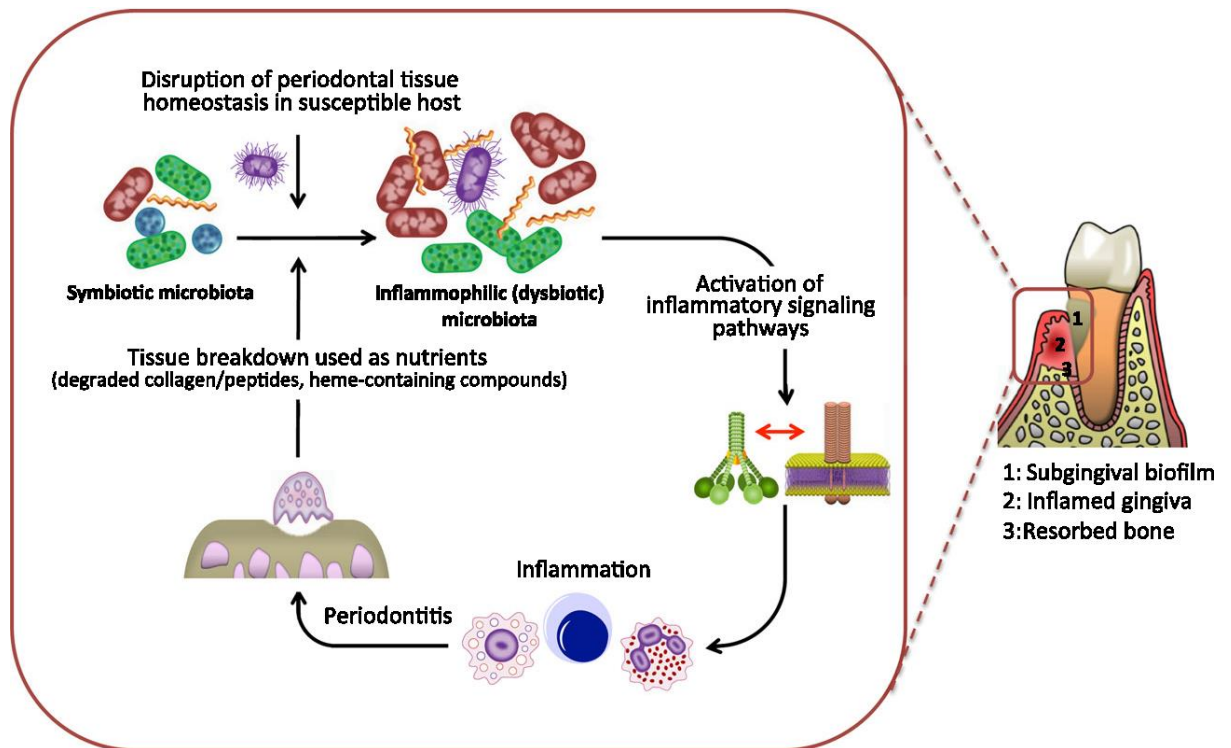


Figure 3. Inflammation and dysbiosis in periodontitis (49)

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P. gingivalis participates in modulating the host's response from **symbiosis** into dysbiosis by expressing a variety of virulence factors such as lipopolysaccharide (LPS), gingipains, fimbriae and capsules with roles in the destruction of periodontal tissues including alveolar bone (50). Of these virulence factors, the proteolytic enzymes gingipains, participate in stimulating or inhibiting the host's innate immune responses and the expression of inflammatory mediators (51, 52). Gingipains also participate in degradation of type I collagen and fibrinogen (53, 54) and abundantly

supply periodontal pockets with iron and haem required by periodontal pathogens (55).

Future research into *P. gingivalis* virulence factors and mechanisms of action may be of significant value in understanding the role of these bacteria in the progression of periodontal disease (56).

1.1.2 *P. gingivalis* and *F. nucleatum* link with systemic inflammation and gut dysbiosis

There has been growing evidence in recent years regarding the ability of *P. gingivalis* and *F. nucleatum* to alter the microbial balance towards dysbiosis and systemic inflammation (44, 57). Oral administration of a combination of *P. gingivalis*, *F. nucleatum* and *Prevotella intermedia* (10^9 CFU/ml each) in five week old wild type female mice resulted in significant dysbiosis of the oral microbiome and slight changes in gut microbiome together with significant changes in the local and systemic immune response when compared with controls (58). Specifically, mice with induced periodontitis on a normal diet presented a significant increase in genus *Lactococcus* in the periodontal microbiota (58). In contrast, when mice were on a high fat diet, induced periodontitis significantly increased genera *Bacteroides*, *Clostridium* and *Ruminococcus* (58). Furthermore, changes in the periodontal microbiota were associated with an increase in insulin resistance (58). In a separate pre-clinical study, oral administration of *P. gingivalis* (10^{10} CFU/ml) twice a week for five weeks resulted in altered proportions between Bacteroidetes and Firmicutes in the ileal microbiome with a significant increase in the order Bacteroidales (59).

These changes coincided with increases in IL-6 serum levels and insulin resistance which further lead to inflammatory changes in adipose tissue and liver (59).

Nakajima *et al.* (2015) demonstrated that in mice, a single oral administration of 10^9 CFU/ml of *P. gingivalis* (strain W83) significantly increased the proportion of phylum Bacteroidetes and decreased the proportion of phylum Firmicutes in the gut, and increased serum endotoxin levels (60). Infected mice presented higher quantities of bacterial DNA in their liver (60). In addition, intestinal gene expression of proteins involved in intestinal permeability, such as tjp-1 and occludin, was downregulated (60). These results suggest that intestinal microbiota changes induced by oral administration of *P. gingivalis* precede systemic inflammatory changes and may provide a mechanistic link in the associations between periodontitis and systemic disease (60).

1.1.3 Bone loss in periodontitis – is there a connection with the gut?

Periodontitis is the result of an imbalance between bone resorption and bone remodelling and advances in the knowledge of bone loss mechanisms in general have led to a progress in understanding pathology of bone loss in periodontitis (61) (Fig 4). The bone resorption and remodelling processes represent a complex interplay involving bone resorbing osteoclasts and bone forming osteoblasts (62). Pro-inflammatory mediators including TNF- α , IL-1, IL-17, RANKL, OPG, PGE2 play an important role in regulating this process (63). The host inflammatory response against certain pathogens increases production of pro-inflammatory cytokines

creating a state of chronic systemic inflammation which contributes to bone resorption (64).

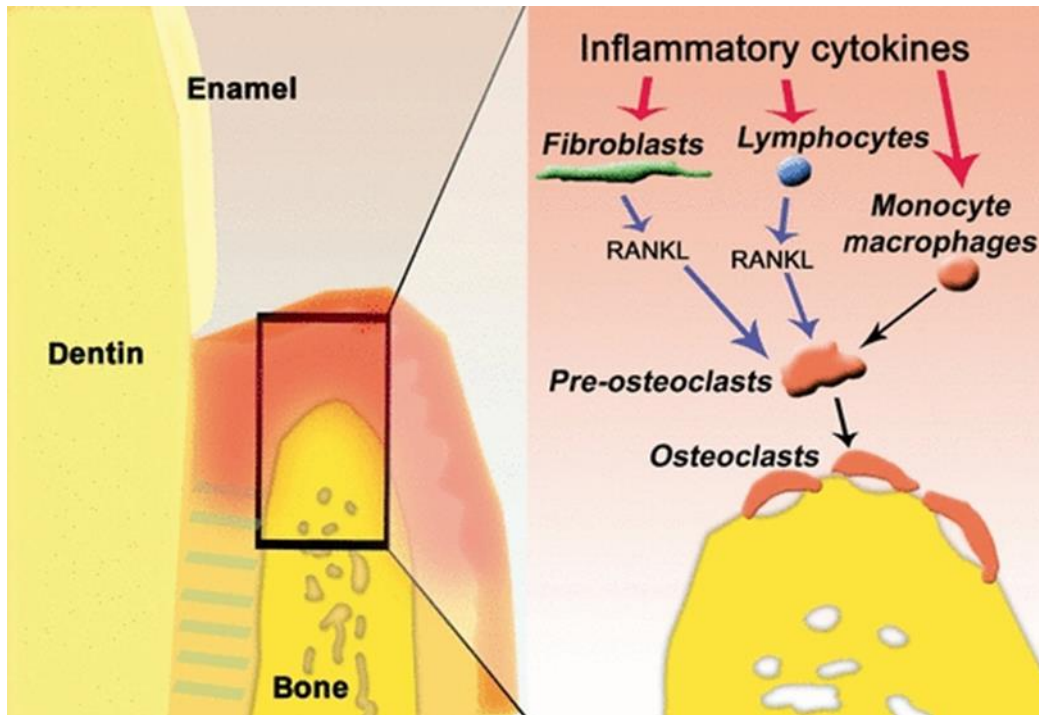


Figure 4. Mechanisms of bone destruction in periodontitis (61)

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The gastrointestinal tract regulates calcium absorption and thus participates in bone health (65). The associations between the intestinal microbiome and bone metabolism have been studied using germ free (GF) and conventionally (CONV) raised mice and have established that intestinal microbiota played a significant role in regulating bone density and physiology (66) (Table 4).

Absence of intestinal microbiota in GF mice leads to an increase in bone mass, decrease in osteoclasts and osteoclast precursor cells, and decrease in pro-inflammatory cytokines IL-6 and TNF- α in bone when compared with CONV mice

(66). Mouse models also demonstrated that exposing animals to low doses of antibiotics resulted in larger bones with increased mineral content (67).

Table 4 Studies demonstrating interactions between gut and bone

Study	Type of participants	Results
Balesaria <i>et al.</i> , 2009 (66)	Patients aged 24-80 years	Human duodenal calcium transport proteins respond to vitamin D metabolites involved in calcium absorption
Oostalaner <i>et al.</i> , 2012 (70)	CD patients	Osteoclastogenesis is increased in patients with CD due to interactions between osteoclasts precursors and T cells
Sjogren <i>et al.</i> , 2012 (67)	Female mice	Absence of gut microbiota in GF mice leads to increase in bone mass and decrease in osteoclasts, osteoclast precursor cells and inflammatory cytokines IL-6 and TNF- α in bone when compared with CONV mice
Cox <i>et al.</i> , 2014 (68)	Male and female mice	Exposing mice to low doses of antibiotics from birth resulted in larger bones with increased mineral content when compared with controls

CD – Crohn’s disease, GF – germ-free mice, CONV – conventionally raised mice

Clinical studies have demonstrated that chronic inflammatory diseases in which the inflammatory site is distant from the bone, such as Crohn’s disease, may also cause bone loss independent of calcium absorption (68, 69). A recent review discussing the epidemiologic and biologic evidence for periodontal manifestations of inflammatory bowel disease, especially Crohn’s disease, was unable to make any definite conclusions regarding the effect of periodontal treatment on the evolution of inflammatory bowel disease (IBD) and more clinical trials were needed (70).

Nevertheless, clinical studies included in this comprehensive review reported higher values for mean clinical attachment loss and deeper periodontal pockets in IBD patients (71, 72). Current research demonstrates that the intestinal microbiome may play an important part in bone health, with the mechanisms of the interactions between intestinal inflammation and bone loss still to be determined (73).

1.2 Probiotics

1.2.1 The human gut microbiome

The human microbiota includes bacteria, archaea, fungi, protozoa and viruses (74). The ratio between bacterial and human cells in the body has recently been suggested to be close to 1:1 (75). Of all body microbiota, microbes residing in the gastrointestinal (GI) tract, together with their genes, are referred to as the gut microbiome and their roles in metabolism, physiology and immunity are of vital importance (76). Particularly, interactions between gut microbiota and the host mucosal immune system are paramount for maturation and modulation of the immune system (77). Advances in metagenomics and related methods have shown that bacterial composition varies in different sites of the GI tract and the main gut bacterial species belong to the Firmicutes and Bacteroidetes phyla with Actinobacteria, Proteobacteria, and Verrucomicrobia phyla also present (78-80). Adult gut microbiome composition varies between individuals and can be altered by several factors including age, diet and medical interventions (81).

1.2.2 Gut microbiome dysbiosis link with disease

The gut microbiome encompasses the largest numbers and the most diverse species of bacteria with important roles in health and disease (82). The relationship between commensal bacteria and the host is paramount for maintaining homeostasis of a healthy individual (83). Some of the well-known roles played by gut bacteria include providing a barrier against pathogenic organisms, synthesising vitamins B and K and fermenting non-digestible dietary fibres into short chain fatty acids (SCFA) (84). New clinical evidence is accumulating regarding the complex role of the microbiome in systemic disease proved by clinical interventions using probiotics, prebiotics and antibiotics (85).

Gut dysbiosis represents an alteration of the composition of the gut microbiome (86). It has been correlated with a multitude of systemic conditions such as inflammatory bowel disease, irritable bowel syndrome and colon cancer (87), type 2 diabetes, obesity and atopy (88, 89), anxiety, depression, autism spectrum disorders and memory abilities (90). However, when considering connecting gut dysbiosis with a diseased state, it is currently difficult to distinguish between cause and effect since the gut microbiota can influence the host and the diseased state can influence the microbiota (74).

1.2.3 Biology and mechanisms of probiotics

The definition of **probiotics** as "live micro-organisms which, when administered in

adequate amounts, confer a health benefit on the host" (91) has recently been reinforced as relevant (92). Most probiotics are ubiquitous to the GI, genitourinary and oral tracts where they play paramount roles in metabolism, physiology and immunity (93). The majority of probiotics belong to the Firmicutes, Actinobacteria and Proteobacteria phyla (94) with the following genera commonly isolated:

Lactobacillus (94), *Enterococcus* (95), *Streptococcus* (96), *Bacillus* (97), *Pediococcus* (98), *Leuconostoc* (99), *Bifidobacterium* (94), *Streptomyces* (100), and *Escherichia* (101) (Table 5).

Table 5 The most commonly isolated probiotic bacterial taxa

Bacterial Phylum	Bacterial Genus	Commonly isolated bacterial species
Firmicutes	<i>Lactobacillus</i>	<i>L. casei</i> , <i>L. salivarius</i> , <i>L. acidophilus</i> , <i>L. reuteri</i> , <i>L. rhamnosus</i> , <i>L. plantarum</i> , <i>L. fermentum</i> , <i>L. brevis</i> , <i>L. gasseri</i> , <i>L. paracasei</i>
	<i>Enterococcus</i>	<i>E. faecium</i>
	<i>Streptococcus</i>	<i>S. thermophilus</i>
	<i>Bacillus</i>	<i>B. coagulans</i>
	<i>Pediococcus</i>	<i>P. acidlactici</i>
	<i>Leuconostoc</i>	<i>L. mesenteroides</i>
Actinobacteria	<i>Bifidobacterium</i>	<i>B. bifidum</i> , <i>B. breve</i> , <i>B. longum</i> , <i>B. infantis</i> , <i>B. animalis</i> , <i>B. adolescentis</i> , <i>B. lactis</i>
	<i>Streptomyces</i>	<i>Streptomyces</i> spp
Proteobacteria	<i>Escherichia</i>	<i>E. coli nissle</i>

Mechanisms of probiotics are currently under study and, although they seem to depend on the strain, dose and mode of delivery (93), three general modes of action have been suggested (Figure 5). Probiotics may inhibit pathogenic bacteria by decreasing the luminal pH and thus restricting the pathogens' adhesion capabilities, by competing with pathogens for nutrients, and by secreting antimicrobial substances such as bacteriocins, lactic acid and hydrogen peroxide (102).

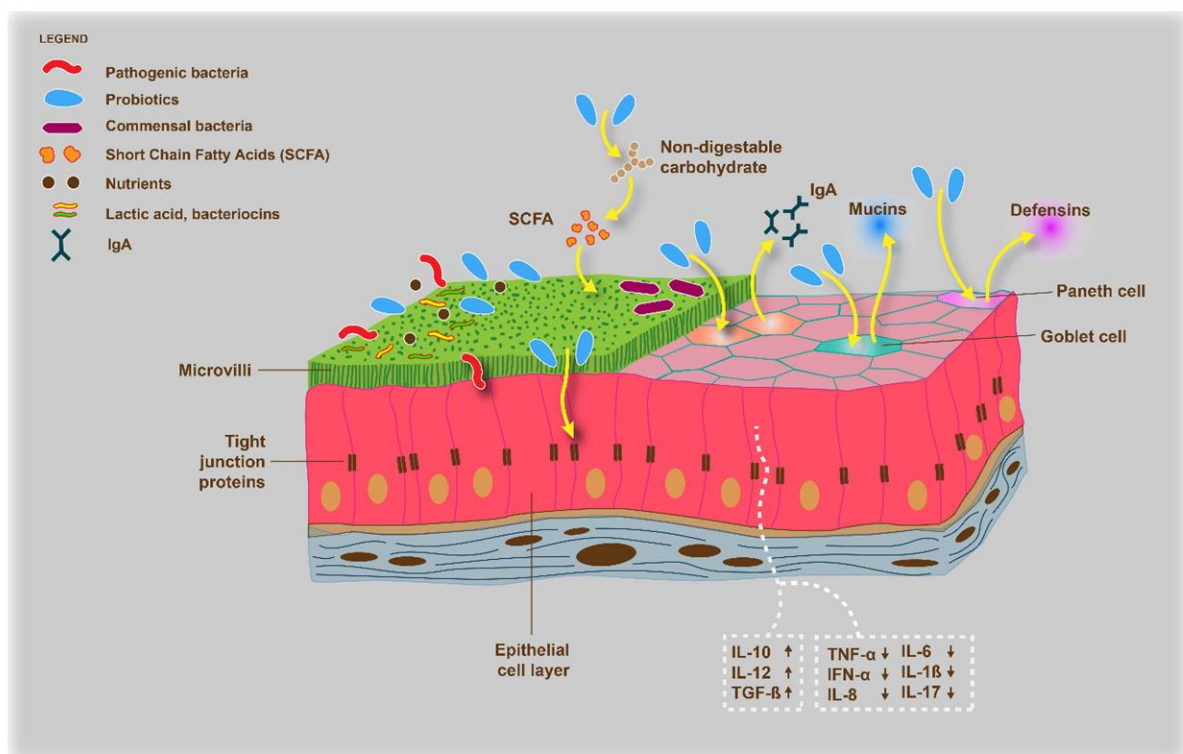


Figure 5. Proposed mechanisms of probiotic action include restriction of the pathogens' adhesion capabilities, competition with pathogens for nutrients and secretion of antimicrobial substances

Probiotics may improve the epithelial barrier function by modulating signalling pathways, increasing expression of tight junction proteins occludin, ZO and cadherin, producing short chain fatty acids and enhancing production of mucins and defensins

(103). Probiotics may also modulate the immune system by increasing secretion of IgA, increasing production of anti-inflammatory cytokines IL-10, IL-12, TGF- β , and decreasing production of pro-inflammatory cytokines TNF- α , INF- γ , IL-8, IL-17, IL-1 β , IL-6 (104).

Temporary colonisation of probiotics may have an effect on the host with or without major changes to the resident microbiome (105). For example, *Lactobacillus rhamnosus* GG (LGG) administered to 76 volunteers for four weeks, temporarily colonised the gastrointestinal tract and lowered the activity of β -glucuronidase, an intestinal enzyme that catalyses the breakdown of complex carbohydrates, by approximately 80 % in all participants (106). When administered to mice for three days prior to radiation exposure, LGG (5×10^7 CFU/ml) significantly reduced radiation-induced epithelial injury and improved crypt survival, protecting the intestine without significantly altering the bacterial composition (107). This study also demonstrated that administration of a small number of this probiotics (LGG, 5×10^7 CFU/ml) has the ability to exert an influence in areas populated by large numbers of other bacteria (107). A large number of systematic reviews of probiotic action also highlight the probiotics ability to exert their effects at sites distant from the site of administration. For instance, *Bifidobacterium* or *Lactobacillus* species with doses between 10^9 and 10^{10} CFU/ml administered orally or via gastric gavage for two weeks in animals and four weeks in humans exerted significant effects on the central nervous system, improving memory abilities and psychiatric disorder-related behaviours such as anxiety, depression and autism (90). Probiotic action can also be shared by a larger number of members of a certain taxonomic group although different probiotic strains can exert their effect through completely different mechanisms (108). Future studies are needed for a better understanding of the

probiotics and their contribution to health, together with appropriate probiotic strain selection for disease prevention (105)

1.2.4 Probiotics and bone loss prevention

Recent pre-clinical and clinical studies in the area of probiotics and bone loss prevention reveal the implication of the intestinal microbiota in regulating bone health (109, 110). Current research indicates probiotics may act on bone indirectly by either changing the gut microbiome, modifying the intestinal barrier function or modulating the immune system (111). For example, McCabe *et al.* showed that treating healthy mice with 300 µl of *Lactobacillus reuteri* 10⁹ CFU/ml three times per day for four weeks enhanced bone density in male mice but not female mice whilst suppressing levels of the pro-inflammatory cytokine TNF-α in the jejunum and ileum (109). Li *et al.* reported that administering 10⁹ CFU/ml of *Lactobacillus rhamnosus* GG at 3.5 day intervals for four weeks protected female mice against bone loss by increasing the gut barrier integrity and decreasing production of the osteoclastogenic cytokines IL-17, TNF, RANKL, IFNγ, and IL-4 (112). In eight week old female mice, consumption of 1.5 x 10⁸ CFU/ml of the probiotic *Bacillus subtilis* daily for 14 days before the introduction of orthodontic tooth movement significantly decreased osteoclasts numbers in periodontal tissues of teeth under mechanical loading (113). Similarly, other probiotic studies addressing bone loss prevention in animal models of experimental periodontitis have yielded encouraging results (114). In male mice, topical administration of *Lactobacillus brevis* CD2 (8 x 10⁵ CFU in 1 mm² lyopatch) for a week resulted in significantly decreased alveolar bone loss, together with lower

expression of gingival inflammatory cytokines TNF- α , IL-1 β , IL-6 and IL-17 and lower counts of anaerobic bacteria for treated mice when compared with placebo-treated mice (114). A more detailed discussion regarding probiotic-periodontitis studies published between 1980 and August 2015 is presented in chapter 2 of this thesis. More recently, 1.5×10^8 CFU/ml of *Bacillus subtilis* and *Bacillus licheniformis* administered orally for 44 days in rats with ligature induced periodontitis significantly protected against alveolar bone loss and local and systemic inflammation (115). Significant suppression of bone loss and gingival inflammation were also observed in 6-8 weeks old female Balb/c mice with periodontitis when using a daily dose of 200 μ l of 2.9×10^9 CFU/ml of *Lactobacillus rhamnosus* GG for 36 days (116). A key finding of this study was that probiotics exerted their effects irrespective of their mode of administration (oral gavage or via oral inoculation) (116). Another probiotic, *Lactobacillus gasseri* SBT2055 demonstrated protective effects on alveolar bone loss and gingival inflammation when 1×10^9 CFU/200 μ L were gavaged into 8-week old Balb/c female mice with periodontitis (117). Oliveria *et al.* (2017) investigated the effects of a probiotic from the *Bifidobacterium* genus (1.9×10^9 CFU/ml *Bifidobacterium animalis* subsp. *lactis*) with promising results (119). In 3-months old male Wistar mice, administration of this probiotic resulted in significantly greater expression of osteoprotegerin and β -defensins as well as lower levels of interleukin-1b and receptor activator of nuclear factor-kappa B ligand in periodontal tissues for the probiotic treated group when compared with the periodontitis group (118).

Given the limited numbers of these investigations, there is a clear need for future animal and clinical studies in elucidating the role of probiotics in bone prevention and their mechanisms of action.

1.3 Hypothesis

The hypothesis of my study was that probiotic treatment with *Lactobacillus rhamnosus* GG (LGG) prior to the induction of experimental periodontitis in mice will reduce or inhibit local and systemic inflammation and alveolar bone loss.

1.4 Aims

The aim of my research project was to investigate the administration of probiotics as a preventive measure using an established mouse model of experimentally induced periodontal bone loss. The project specifically determined:

- (i) the effect of LGG on alveolar bone loss and local and systemic inflammation,
- (ii) changes in the gut physiology and microbiome as a result of experimentally induced periodontal disease and probiotic treatment and
- (iii) the influence of different methods of probiotic administration on the observed bone and gut changes.

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CHAPTER 2

Probiotics and periodontitis – a literature review


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Chapter 2 Probiotic and periodontitis – a literature review

[Gatej *et al.*, (2017) *Journal of the International Academy of Periodontology*. 19(2): 42–50]

2.1 Abstract

Objective: This review was designed to explore the use of probiotics in prevention or treatment of periodontitis.

Methods: A search was performed using MEDLINE and bibliographies from previous reviews in order to identify any randomised controlled animal and human probiotic interventions in periodontitis.

Results: Five studies using probiotics in animal models of periodontitis and eight clinical studies using probiotics in patients with chronic periodontitis were analysed. The analysis of the animal models showed reduction in periodontal pathogens and bleeding on probing when probiotics were used in adjunction to mechanical debridement and significant increase in alveolar bone levels and bone density in the probiotic groups when compared with placebo. Some of the results of the clinical studies indicated decreased clinical parameters (gingival inflammation, bleeding on probing, plaque index) and decreased pro-inflammatory markers levels in saliva or gingival crevicular fluid in treated periodontitis patients when compared with controls or placebo. Other results included decreased periodontal pocket depth and clinical attachment loss for scaling and root planing plus probiotic treatment versus scaling and root planing alone or placebo and also reduction in *Porphyromonas gingivalis* numbers and the total viable count and proportion of obligate anaerobic bacteria.

Conclusions: Within the limitations of this review, the results are encouraging,

supporting the notion that there is a place for probiotics in the treatment of periodontitis. Future independent studies are needed to investigate specific probiotic strains, doses, delivery methods, treatment schedules, mechanisms of action, safety and how to maintain the results of the probiotic interventions.

2.2 Introduction

Periodontitis is a common chronic inflammatory condition affecting the dentition of the adult population (1). A key factor in the development of this disease is an increased bacterial challenge, specifically the presence of elevated numbers of certain potentially pathogenic commensal bacterial species resulting from altered environmental conditions arising from the host's inflammatory response (2).

Subgingival debridement, surgical interventions and in some cases selective use of antibiotics and antiseptics are current approaches used to reduce the pathogenic bacteria. While these treatments result in a temporary reduction of the bacterial load and associated inflammation, they are often not sufficient to control the disease (3, 4). Therefore, other adjunctive strategies need to be investigated. The administration of beneficial bacteria -probiotics- with antimicrobial and anti-inflammatory properties is one of several novel approaches being considered as an adjunct treatment for the management of periodontitis and may offer a low risk, inexpensive and easy-to-use treatment option.

2.3 Periodontitis

Periodontitis is a chronic inflammatory condition affecting both the hard and soft tissues surrounding the teeth, caused by a combination of specific bacteria and inflammatory host responses and resulting in the destruction of the connective tissue of the gingivae, periodontal ligament and alveolar bone (1). It is one of the two most prevalent oral health burdens world-wide. A World Health Organization report from 2012 found 15–20% of middle-aged adults suffered from severe periodontal disease resulting in tooth loss (5). Periodontitis is also considered a risk factor for cardiovascular disease, pulmonary disease, type II diabetes, rheumatoid arthritis and adverse pregnancy outcomes (6-9).

The precise etiology of periodontitis is complex, multifactorial and not completely understood. Generally, it is believed to be a biofilm-induced disease with the host's immune system playing a central role (2, 10-13). The disease is associated with an imbalance in the host's local microbiome with elevated numbers and proportions of bacterial species designed as 'pathogens' and reduced proportions of bacteria associated with health (14-16).

Current treatments for periodontal disease including subgingival debridement, surgical interventions and selective use of antibiotics and antiseptics, aim to reduce the pathogenic load (4). Although these numbers of pathogens are reduced considerably, the shift is only temporary as re-colonization occurs within months (17, 18). The use of antibiotics as part of the treatment brings with it the important global issue of antibiotics resistance that has the potential to render many antibiotics useless. It also brings along a long list of possible side-effects, notably, antibiotic-associated diarrhea

(19).

Considering all of these factors, increasing the proportion of beneficial bacteria with inflammation modulating properties presents as an option to address the bacterial imbalance and may be considered as a preventive or treatment option in achieving periodontal health.

2.4 Probiotics

2.4.1 History, sources, definition

The microorganisms that live inside and on humans outnumber the body's cells tenfold with the majority of them being bacteria, with some archaea and eukaryotes also being present (20). In periodontal health, communities of bacteria live in symbiosis with the host, playing a role in its immune function and health status. A disturbance in the microbial balance (a process labelled as 'dysbiosis') has been associated with several medical conditions (21). Obesity, metabolic diseases, gastrointestinal diseases, autoimmune diseases, allergies and cancer have all been partly associated with an increased number of harmful bacteria and a decreased number of beneficial bacteria (22).

Beneficial bacteria are present in preserved food and beverages around the world: Korean *kimchi*, Indonesian tempeh, Indian chutney, Japanese miso, sauerkraut, kefir, yogurt and cheese. The preservation process called 'lacto-fermentation' is an anaerobic process in which lactic acid bacteria, predominantly Lactobacilli, convert carbohydrates into lactic acid which acts as a preservative (23). Consuming fermented

foods is an ancient practice dating back as far as 5400 BC, whilst recommendations for gastrointestinal illness date back to 76 AD (24). The first scientist to lay down the foundations for the concept of beneficial bacteria was the Ukrainian born Nobel laureate bacteriologist Ilya Ilyich Mechnikov, known as “the father of modern immunology”. He proposed a theory that aging is caused by toxic bacteria in the gut and attributed the longevity of peasants from the Balkan area to their consumption of large quantities of sour milk that contained Lactobacilli (25).

Other scientists continued Metchnikov’s work and in 1965 the term “probiotics,” meaning “for life”, was introduced (26). The current definition for probiotics is given by the World Health Organisation which defines probiotics as live microorganisms, most often bacteria (sometimes fungi), which, when consumed, confer beneficial effects to the host (27). Probiotics are bacterial strains usually isolated from human commensal microbiota and adequately characterized for strain identity, content, stability, and proven health effects. The most commonly used species of probiotics belong to the *Lactobacillus*, *Bifidobacterium*, *Escherichia*, *Enterococcus* and *Bacillus* genera and are all ubiquitous residents of the human skin, gastrointestinal tract, respiratory tract and vagina (28). Lactobacilli are Gram-positive, rod-shaped, facultative-anaerobes. Some of the most commonly known members that have been isolated and studied are *Lactobacillus acidophilus*, *L. reuteri*, *L. bulgaricus*, *L. rhamnosus*, *L. salivarius*, *L. casei*. Bifidobacteria are Gram-positive, anaerobic bacteria, with some of the most commonly known members being *Bifidobacterium bifidum*, *B. breve*, *B. longum*, and *B. infantis* (29).

2.4.2 Function and mechanisms

Probiotics fulfil many useful functions thus having a major health impact. They produce lactic acid with anti-bacterial effect, hydrogen peroxide with antiseptic effect, and anti-viral and anti-fungal agents that suppress pathogens. Probiotics are important for immune system development and regulation, maintenance of a healthy lining of the GI tract, food digestion, synthesis of amino acids, proteins and different vitamins, absorption of calcium, iron and vitamin D (30-32). In order to exert all these effects, probiotics need to be able to survive the gastrointestinal passage resisting acid and bile and to preserve their stability during manufacturing and storage (33).

Additional studies have shown that a combination of different probiotic species and/or strains (e.g. *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12) can enhance their effects in a synergistic manner (34, 35)

The precise mechanisms of how probiotics exert their effects are not known yet and may depend on a variety of factors: the condition being treated, the strain and the concentration of the probiotics used and the stage when they are introduced, the presence of prebiotics or enteric bacteria (36).

The effects of probiotics can originate from three local or systemic main modes of action (37-39):

1. Indirectly, probiotics compete with pathogens for essential nutrients; they can also restrict the pathogens' adhesion capabilities by changing the environmental pH.
2. Directly, probiotics are involved in the production of antimicrobial substances (lactic acid, hydrogen peroxide, bacteriocins) that can kill or inhibit the growth of periodontal pathogens.

3. Probiotics can act on the host by modulating the host's innate and adaptive immune response (reducing the production of pro-inflammatory cytokines: IL-6, IL-1 β , TNF α and increasing production of anti-inflammatory cytokines: IL-10) and by improving the intestinal barrier integrity (40).

It has not been established yet if colonization of the oral cavity by probiotics is necessary in order for them to exert their effects in the mouth, and the process of colonization of the oral cavity itself remains unclear, with studies using biased methods of detecting bacteria (41).

2.4.3 Lactobacillus rhamnosus GG

One of the most studied probiotic microorganisms is *Lactobacillus rhamnosus GG* (LGG). It was originally isolated from healthy human intestines in 1983 (42). LGG survives the low pH of the stomach and the bile acids of the duodenum. It has pili facilitating adherence to the inner lining of the digestive system thus colonising the intestine (43). One study investigating the colonisation of LGG in the oral cavity concluded that this is improbable in majority of cases but possible in some (44).

Lactobacillus rhamnosus GG has been extensively investigated in gastrointestinal studies and it is now used in dairy products in many countries. This probiotic does not ferment sucrose or lactose and has been shown to significantly reduce the risk of caries (45, 46). It has also been demonstrated to have anti-inflammatory properties *in vivo* (47).

Considering its non-cariogenic and anti-inflammatory properties, LGG may prove to be a good candidate for future probiotic-periodontitis studies.

2.4.4 Probiotics in other fields of medicine

Traditionally, probiotics have been used in gastroenterology. Evidence-based reviews indicate that certain strains of probiotics contribute to the microbial balance of the gastrointestinal tract - supporting the immune system and reducing inflammation (31). Clinical trials have assessed the effects of probiotics in antibiotic-associated diarrhoea, gastroenteritis, irritable bowel syndrome, inflammatory bowel disease, Crohn's disease, obesity, rheumatoid arthritis and allergies (33, 48). There are also laboratory studies that have shown promising results in treatment of childhood autism and colon cancer (49, 50).

2.4.5 Probiotics and oral health

There have been many studies published investigating the potential health benefits of probiotics on systemic health, but investigations regarding their use in oral health are limited by comparison. These vary a lot in terms of probiotics strains used, concentrations, and vehicles for the application (cheese, lozenges, milk, kefir, ice cream, gum, drops, powder, and mouthwash) (51).

Probiotics have been evaluated in caries control and have demonstrated the capacity to reduce *Streptococcus mutans* levels in saliva (46) . A recent meta-analysis indicated that probiotics could have a positive effect in caries prevention (52) . There are also probiotic evaluations in oral conditions e.g. candidiasis, chemotherapy-induced mucositis or halitosis (53, 54).

2.4.6 Probiotics and periodontitis

In periodontal disease, some studies investigated the role of probiotics in gingivitis and reported a significant decrease in terms of plaque and gingival indices, bleeding on probing and gingival inflammation in the probiotic groups (54).

A search was performed using MEDLINE in order to identify any randomised controlled animal and human probiotic intervention studies in periodontitis. The search considered those works published between 1980 and August 2015 and aimed at evaluating the effects of probiotics in periodontitis using the words “periodontal disease”, “periodontitis” and “probiotics”. Additional hand searches were performed and included bibliographies from previous reviews on the topic of oral probiotics (51, 53-56). Only articles published in English were selected. Five studies using probiotics in animal models of periodontitis and eight clinical studies using probiotics in patients with chronic periodontitis were identified.

2.4.6.1 Probiotics in animal models of periodontitis

Table 1 highlights four animal studies where periodontal pockets were artificially created or a ligature induced periodontitis model was used in either rats or beagle dogs.

Five mm periodontal bony defects were surgically created four months prior to the experiment in a split mouth, double blind, randomised trial in beagle dogs (57). Pellets

containing a mixture of *Streptococcus salivarius*, *S. sanguinis* and *S. mitis* were applied to the root surface after scaling and root planing (i.e. to a suppressed oral microbiota). The authors concluded that the use of probiotics significantly delayed and reduced inflammation (bleeding on probing) in the probiotic group when compared with scaled and root planed pockets alone. There was also reduction in total anaerobic bacteria and delay in recolonization of pockets by the pathogens when compared with the control group and the reduced levels were maintained 12 weeks after the treatment in the probiotic group but not the control group (51, 57). The limitations of the study were the absence of a placebo control group, the inter-subject variation, the small sample size (eight dogs) and the intra-oral translocation.

Another study using the same model in eight beagle dogs found that there was a significant increase in bone levels for the periodontal pockets treated with probiotics for 12 weeks in comparison with the control group (58). Bone density in the probiotic group also improved significantly. The previously mentioned limitations apply, together with the use of conventional radiographic films that introduces potential measurement accuracy errors.

In a randomised controlled study, 32 rats with ligature induced experimental periodontal disease were administered *Bacillus subtilis* for forty four days (59). The probiotic intervention generated reduced attachment loss and alveolar bone loss and protected the small intestine from reactive changes induced by ligature-induced periodontitis. There are a few shortcomings to this study. Like all ligature induced periodontitis models, the mechanical lesions could aggravate the periodontal destruction (60). In addition, the mode of probiotic administration, via drinking water, makes it difficult to quantify the amount ingested by each animal.

In another ligature induced periodontitis study, a 44-day experiment using the probiotic *B. subtilis* and restrain stress concluded that probiotics supplementation may reduce tissue breakdown in unstressed rats and that immunomodulatory effects of probiotics in intestinal and periodontal tissues were influenced by stress (61). All the limitations of the Messori *et al.* study (59) apply here as well.

The animal studies showed an effect of probiotics on oral microbiota and a limited effect on periodontal parameters. Due to the limited data available and all the limitations discussed above, it is premature to draw a conclusion on the recommended methodology (probiotic strain, concentration, duration of treatment and mode of administration).

Table 1. Animal probiotics studies included in this review

Study	Type of participants,	Condition	Probiotic strains, vehicle, time	Results
Teughels <i>et al.</i> , 2007 (Teughels <i>et al.</i> , 2007b)	Beagle dogs, 8	Artificially created periodontal pockets	<i>Streptococcus salivarius</i> , <i>S. sanguinis</i> , <i>S. mitis</i> , pellets, 12 weeks	Reduction in periodontal pathogens and BOP when probiotics were used in adjunction to mechanical debridement
Nackaerts <i>et al.</i> , 2008	Beagle dogs, 8	Artificially created periodontal pockets	<i>S. salivarius</i> , <i>S. sanguinis</i> , <i>S. mitis</i> , pellets, 12 weeks	Significant increase in bone levels and bone density in probiotic group when compared with placebo
Messori <i>et al.</i> , 2013	Wistar rats, 32	Ligature-induced periodontitis	Product based on <i>Bacillus subtilis</i> , in water, 44 days	Mean values of AL and ABL were significantly higher in the induced periodontitis group compared with the treatment group
Foureaux <i>et al.</i> , 2014	Wistar rats, 64	Ligature-induced periodontitis associated with restraint stress	Product based on <i>B. subtilis</i> , in water, 44 days	Bone loss was prevented in the probiotic treated induced periodontitis unstressed group

2.4.6.2 Probiotics in clinical studies in patients with chronic periodontitis

Studies using probiotics in patients with chronic periodontitis present a high degree of heterogeneity in the probiotic strains, dosages, vehicles of administration, modes of administration and duration. Table 2 presents eight clinical studies with variations in terms of the severity of disease, sample size and administration of oral hygiene instructions.

L. brevis lozenges were used in a 2007 double blind 4-day study in 21 male and female adults with no systemic diseases and with moderate-severe chronic periodontitis to assess anti-inflammatory effects of this probiotic (62). The authors concluded that all clinical parameters (gingival index (GI), plaque index (PI), calculus and temperature sensitivity) decreased in the probiotic group, in association with salivary levels of prostaglandin E₂ (PGE₂), metalloproteinase (MMP) and interferon γ (INF- γ).

Table 2. Clinical probiotics-chronic periodontitis studies included in this review

Study	Type of participants, number, age	Probiotic strains, vehicle, time	Results
Riccia <i>et al.</i> , 2007	Adults, 29, 24-51	<i>Lactobacillus brevis</i> , lozenges, 4 days	Decreased clinical parameters in treated periodontitis patients when compared with controls (gingival inflammation, BOP, plaque, calculus, temperature sensitivity); Decreased levels of PGE ₂ , MMP and INF- γ in saliva samples of treated periodontitis patients
Shimauchi <i>et al.</i> , 2008	Adults, 66, 32-61	<i>L. salivarius</i> , tablets, 8 weeks	Current smokers in the probiotic group showed a significantly greater improvement of plaque index and probing pocket depth from baseline when compared with those in the placebo group

Vivekananda <i>et al.</i> , 2010	Adults, 30, 34-50	<i>L. reuteri</i> , lozenges, 42 days	PPD, CAL, GI, GBI and PPD significantly reduced in the SRP plus probiotic group compared with SRP alone or placebo
Teughels <i>et al.</i> , 2013	Adults, 30, older than 35	<i>L. reuteri</i> , lozenges, 12 weeks	Significantly more pocket depth reduction and attachment gain in the moderate and deep pockets and also reduction in <i>P.gingivalis</i> numbers in the test group when compared with controls
Vicario <i>et al.</i> , 2013	Adults, 20, 44-65	<i>L. reuteri</i> , tablets, 30 days	Improved short-term clinical outcomes (PI, BOP, and PPD) in non-smoking patients with initial-to- moderate chronic periodontitis
Szkaradkiewicz <i>et al.</i> , 2014	Adults, 38, 31-46	<i>L. reuteri</i> , tablets, 2 weeks	Significant improvement in SBI, periodontal probing depth and clinical attachment level and also decreased levels of pro-inflammatory cytokines TNF- γ , IL-1 β , IL-17 in treated patients when compared with the control group
Ince <i>et al.</i> , 2015	Adults, 30, 35-50	<i>L. reuteri</i> , lozenges, 3 weeks	Significant differences in PI, GI, BOP and PPD and significant mean values of attachment gain in favour of the test group compared with controls. Significant decreased levels of MMP-8 and increased levels of TIMP-1 were found in GCF for the test group up to day 180
Tekce <i>et al.</i> , 2015	Adults, 30, 35-50	<i>L. reuteri</i> , lozenges, 3 weeks	1 year follow up study from the previous Ince <i>et al.</i> ; 2015. PI, GI and BOP significantly lower in the test group compared with controls; difference in the total viable count and the proportion of obligate anaerobes were decreased in the test group up to day 180

PGE₂, prostaglandin E₂; MMP, metalloproteinase; TIMP-1, tissue inhibitor of metalloproteinase; INF- α , interferon α ; PI, plaque index; BOP, bleeding on probing; PPD, pocket probing depth; CAL, clinical attachment loss; GI, gingival index; GBI, gingival bleeding index; SRP, scaling and root planing; SBI, sulcus bleeding index

The anti-inflammatory effects of *L. brevis* were attributed to its capacity to prevent the production of nitric oxide and hence the release of PGE₂ and the activation of MMPs (62). No placebo group was used in this study, no data were provided on the periodontal disease and data for bleeding on probing (BOP) were unclear.

L. salivarius tablets were administered three times daily for eight weeks in a double blind, placebo controlled, randomised clinical study that included 66 adult patients with mild to moderate chronic periodontitis (63). The authors found significantly decreased PI, GI and pocket depth in probiotic treated smokers when compared with placebo. No significant difference was detected in BOP between the probiotic and the placebo groups. The study also looked at salivary lactoferrin levels as a measure for the host's immune response and found that these were decreased significantly in the test group smokers. The study does not report on the lactoferrin levels of the non-smokers group alone. The Hawthorn effect regarding altered oral hygiene regimens due to observation was taken into account. The patients who volunteered were workers at the company that manufactured the probiotic tablets and funded the study (63).

The use of *L. reuteri* lozenges in 30 adult patients with mild to moderate chronic periodontitis combined with scaling and root planing (SRP) significantly reduced GI, clinical attachment loss (CAL), gingival bleeding index (GBI) and periodontal pocket depth (PPD) and was more effective than either treatment alone (64). Patients receiving only probiotics without SRP also showed significant clinical improvement when compared with placebo. The administration of probiotics started 21 days after SRP, twice a day for three weeks. The authors of this double blind, randomised, placebo controlled clinical trial presented probiotics as an adjunct or alternative to periodontal treatment when SRP might be contraindicated. The private laboratory making the probiotic funded the test products and the publication of the study (64).

L. reuteri lozenges were also used for 12 weeks in a randomised, placebo controlled clinical trial in 30 adults with moderate to severe previously untreated chronic periodontitis (65). The group found that there was more pocket depth reduction and attachment gain, together with reduction in *Porphyromonas gingivalis* numbers in the

probiotic group when compared with controls, concluding that *L. reuteri* can be a useful adjunct to SRP. The private laboratory that manufactured the probiotic partially supported this study (65).

Two strains of *L. reuteri* tablets were used for 30 days in a double blind, placebo-controlled, randomised clinical trial in non-smoking patients with initial to moderate chronic periodontitis (66). The probiotics used significantly improved short-term clinical outcomes (BOP, PI and PPD). No mechanical intervention was performed. The subject size used in this study was quite small (20 patients) and the study period short. No statistically significant changes could be shown in the control group (66).

In 2014, an experiment using *L. reuteri* tablets for two weeks in 38 adult patients with moderate chronic periodontitis found significant improvement in sulcus bleeding index (SBI), PPD and CAL in treated patients when compared with controls (67). The gingival crevicular fluid (GCF), levels of pro-inflammatory cytokines TNF- α , IL-1 β and IL-17 were decreased in the treated group (67).

The effects of *L. reuteri* on clinical and biochemical parameters, adjunctive to initial periodontal therapy were evaluated in a randomised, parallel, controlled, double-masked clinical trial of 30 adult patients with initial to moderate chronic periodontitis over a one year period (68, 69). Significant differences were found in PI, GI, BOP and PPD in favour of the test group, together with significant mean values of attachment gain. The GCF levels of proteolytic enzyme metalloproteinase MMP-8 and the tissue inhibitor of metalloproteinase TIMP-1 were measured and followed for 360 days with significantly decreased levels of MMP-8 and increased levels of TIMP-1 detected up to day 180. Both forms of MMPs (active and latent) were measured and the active forms seemed to be found at sites with progressive periodontitis. The total viable count

and the proportion of obligates anaerobes were also decreased up to day 180. The study was supported by a private laboratory. However, the authors of the study declared that the company was not involved in the data management (68, 69).

The high degree of heterogeneity of the human studies (different strains and concentrations, small sample size, different duration of treatment, durability of response, mode of administration and the role of environmental factors such as the pH of the delivery area) makes it difficult to draw a robust conclusion. Despite all these limitations, it seems that probiotics can still have an impact on the oral microbiota and a limited effect on periodontal parameters. This now needs to be investigated further.

Probiotic therapy is generally considered to be safe and complications rare (31) with a closer exploration needed in critically ill or immunocompromised patients (70)

2.5 Conclusions and recommendations for future research

Periodontitis is an inflammatory disease that has proven very difficult to treat. The results of the animal and clinical periodontitis studies included in this short literature review support the notion that there is a place for probiotics in the treatment of periodontitis and that probiotics may offer a low-risk, inexpensive, easy to use prevention or treatment option for the management of periodontal disease. In the future, more independent studies are needed to look into specific probiotic strains, doses, delivery methods, treatment schedule, mechanisms of action, safety and how to maintain the results of the probiotic interventions.

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CHAPTER 3

Competition between *Lactobacillus rhamnosus* GG and opportunistic pathogens *Porphyromonas gingivalis* and *Fusobacterium nucleatum in vitro*

CHAPTER 3: Competition between *Lactobacillus rhamnosus GG* and opportunistic pathogens *Porphyromonas gingivalis* and *Fusobacterium nucleatum in vitro*

3.1 Introduction

Periodontitis is a common polymicrobial chronic inflammatory condition affecting the tissue surrounding the teeth (1). *Porphyromonas gingivalis* and *Fusobacterium nucleatum* are two key bacteria believed to be involved in the aetiology of this disease (2). An increasing number of preclinical and clinical studies have demonstrated the benefit of probiotic treatment in inflammatory diseases with a polymicrobial aetiology (3). *Lactobacillus rhamnosus GG* (ATCC 53103) (LGG) is a naturally occurring Gram positive, facultative anaerobic bacteria which exerts a series of health benefits and has been extensively investigated in clinical trials (4-8). LGG, due to its non-cariogenic and anti-inflammatory properties, is thought to be a good candidate for probiotic-periodontitis studies (9). The molecular mechanisms through which LGG exerts its effects are yet to be elucidated and may include inhibition of pathogens, competition for nutritional resources and modulation of the immune system by regulating the intestinal epithelial barrier (10). Antimicrobial activity of LGG has been demonstrated against some bacterial species but studies investigating its antimicrobial activity against pathogens involved in the aetiology of periodontitis are limited (11).

The aim of this experiment was to investigate the antimicrobial ability of LGG against *P. gingivalis* and *F. nucleatum in vitro* and make suggestions regarding its potential use in experimental periodontitis studies.

3.2 Materials and methods

3.2.1 Bacterial strains

The probiotic LGG was obtained from the ATCC collection, Manassas, Virginia, USA (ATCC[®] Number 53103). It was cultured in de Man, Rogosa and Sharpe (MRS) (Oxoid, Basingstoke, UK) broth and on MRS agar plates at 37°C in an atmosphere of N₂/CO₂/H₂ (90:5:5) for 48 hours.

F. nucleatum (ATCC[®] Number 25586) and *P. gingivalis* (strain W50) were cultured on anaerobic blood agar plates (Oxoid) and stored for three days at 37°C in an atmosphere of N₂/CO₂/H₂ (90:5:5).

3.2.2 LGG - growth rate and cell density

The growth curves of LGG were established in order to correlate the optical density (OD) of the culture with the number of viable bacterial cells. Briefly, LGG was cultured in MRS broth at 37°C in an atmosphere of N₂/CO₂/H₂ (90:5:5). After 48 hours, colonies were Gram stained and 1 ml of the culture was placed into a sterile tube together with 45 ml of fresh broth. Optical density was measured using a

spectrophotometer (Shimadzu, Japan) at a wavelength of 600 nm every two hours initially and then every hour for a total of six hours.

Concentrations of live bacteria were confirmed by serial dilutions and plating for counting of colony forming units (CFU). Broth cultures were serially diluted at each time point using 0.85% saline. Aliquots of 0.1 ml of each of the 10^{-4} , 10^{-5} , 10^{-6} and 10^{-7} solutions were plated in duplicate on MRS agar plates and incubated at 37°C in an atmosphere of N₂/CO₂/H₂ (90:5:5) for 48 hours after which colonies were counted and recorded as colony forming units per millilitre (CFU/ml).

3.2.3 Diffusion assays

The anti-microbial activity of the probiotic LGG against *P. gingivalis* and *F. nucleatum* was examined using an agar diffusion assay.

In the first experiment, *P. gingivalis*, *F. nucleatum* and LGG were grown on agar plates at 37°C in an atmosphere of N₂/CO₂/H₂ (90:5:5). Bacterial colonies were used to inoculate 10 ml of heart infusion broth (Oxoid) for *P. gingivalis* and *F. nucleatum* and 10 ml of MRS broth for LGG. After three days growth, *P. gingivalis* and *F. nucleatum* were swabbed separately across three agar plates and three wells were created equilaterally in each plate. 1.5 ml of LGG culture was placed in four sterile Eppendorf tubes, centrifuged at 10000 g for five minutes and filtered through a 0.22 µm bacterial filter (Sarstedt, Germany). The cell free supernatant (CFS) was placed in two of the wells of the agar plates containing either *P. gingivalis* or *F. nucleatum*

while sterile broth was placed in the third well to act as a control. The plates were incubated for four days after which inhibition zones were observed. The tests were performed in duplicate.

The second experiment followed a protocol described by Khalaf *et al.* (12). One hundred μl of *P. gingivalis* (10^{10} CFU) and 100 μl of *F. nucleatum* (10^8 CFU) were spread onto separate agar plates and allowed to dry. Five ml of LGG broth (10^9 CFU) was centrifuged at 10000 g for five minutes and filtered (0.22 μm). After adjusting the pH to 7, 10 μl of the CFS were placed onto plates containing either *P. gingivalis* or *F. nucleatum*. The plates were incubated for four days after which inhibition zones were observed. All tests were performed in duplicate.

3.3. Results

3.3.1 Colonies were identified as LGG

After incubation, the plates were visually inspected. Colonies were identified as LGG based on colonial morphology (large, round, creamy white, opaque) and Gram stain appearance (small uniform Gram positive rods).

3.3.2 LGG growth rate

Bacterial growth curve of LGG correlated with its cell density (CFU/ml) and showed that in the relatively short time of five hours the concentration of LGG varied between $3 - 8 \times 10^8$ CFU/ml (optical density of 0.6 - 1 at 600 nm) (Figure 1 and Figure 2).

These values correspond with concentrations used in previous animal studies (13).

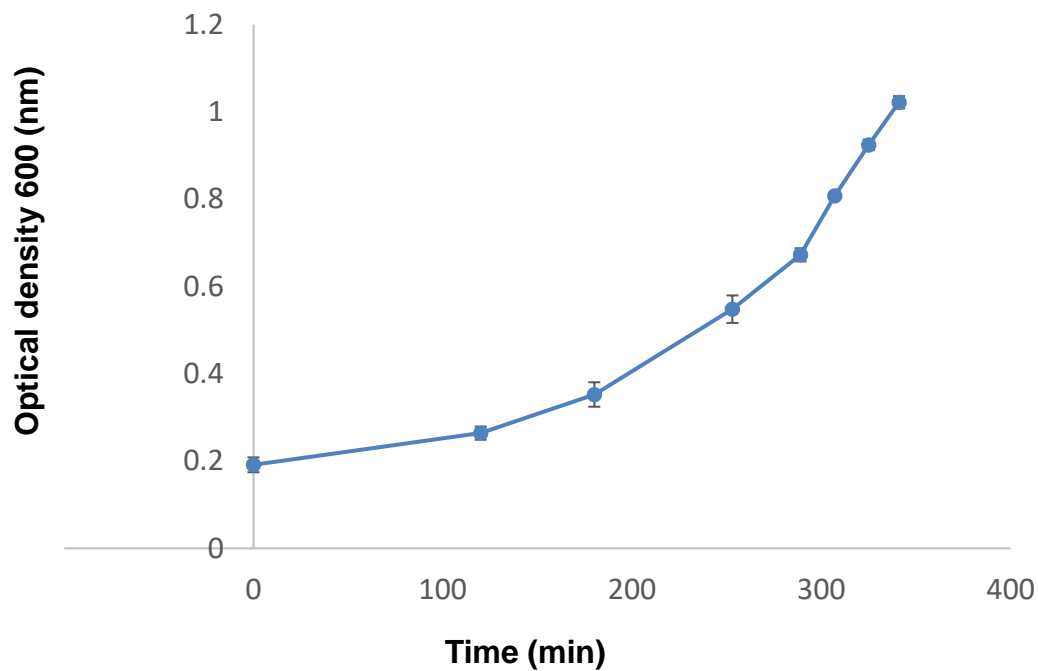


Figure 1: Bacterial growth curve for *Lactobacillus rhamnosus* GG; Optical density measured at a wavelength of 600 nm; Each point represent the average of two measurements \pm SEM.

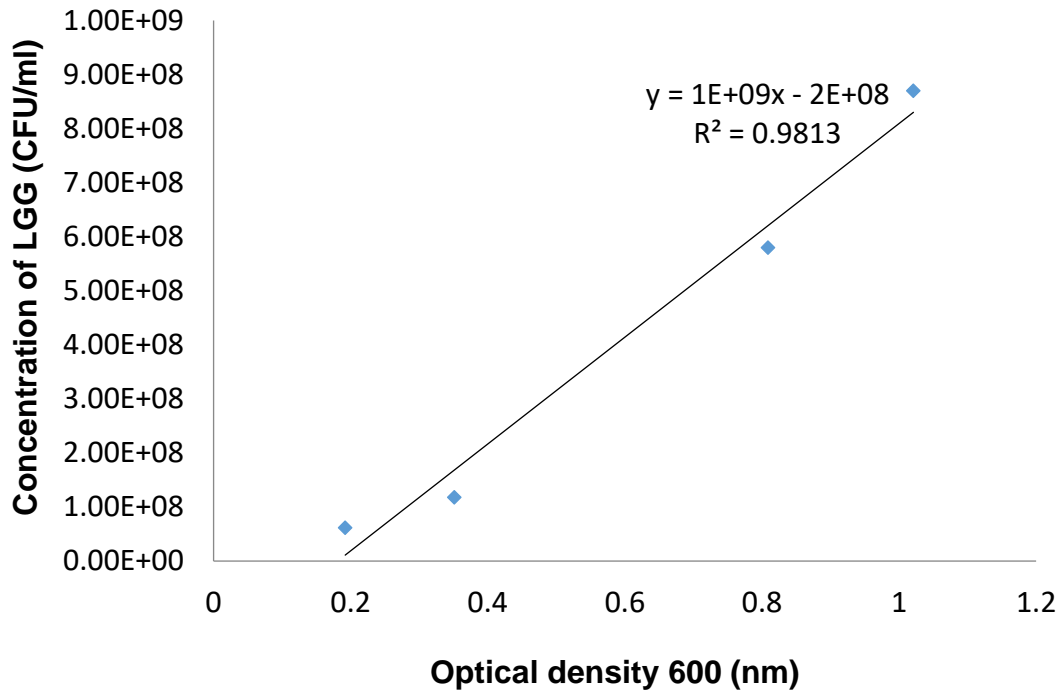


Figure 2: Concentration of LGG (CFU/ml) relative to optical density; Optical density measured at a wavelength of 600 nm.

3.3.3 LGG exerts no antimicrobial activity against *P. gingivalis* and *F. nucleatum in vitro*

Antimicrobial activity of LGG was investigated using two methods. After four days of incubation, *F. nucleatum* and *P. gingivalis* formed confluent lawns on the plates and no inhibition zones were observed using either method. Results using the first agar diffusion method are illustrated in Figures 3 and 4 and results using the second direct contact method are illustrated in Figures 5 and 6.

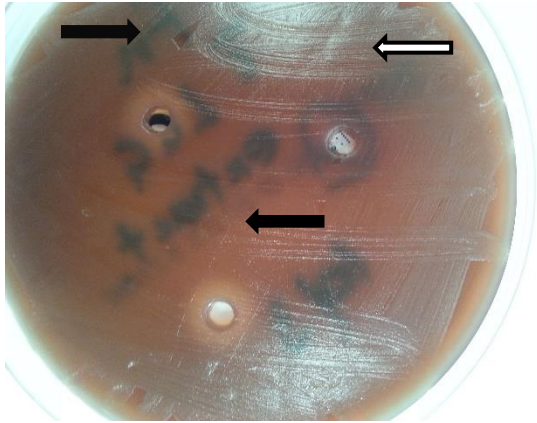


Figure 3: LGG developed no inhibition zones against *F. nucleatum*
 Black arrows - LGG CFS wells; White arrows - Negative control (sterile broth); CSF – cell free supernatant

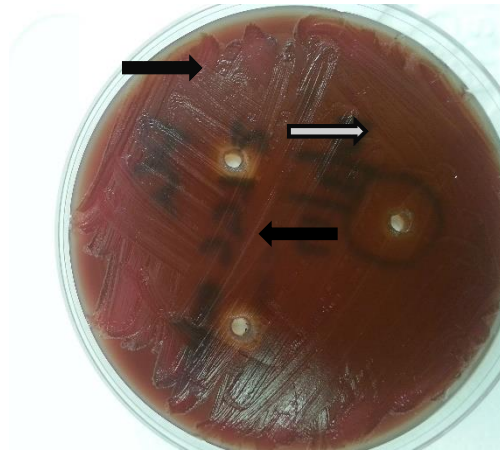


Figure 4: LGG developed no inhibition zones against *P. gingivalis*
 Black arrows - LGG CFS wells; White arrows - Negative control (sterile broth); CSF – cell free supernatant

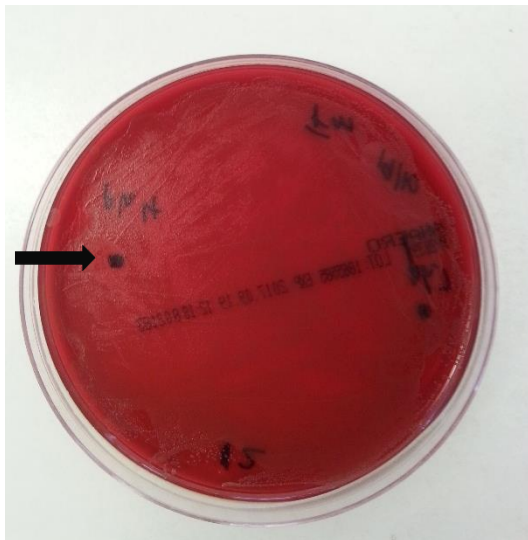


Figure 5: LGG developed no inhibition zones against *F. nucleatum* at pH 7
 Black arrows – LGG CFS; CSF – cell free supernatant

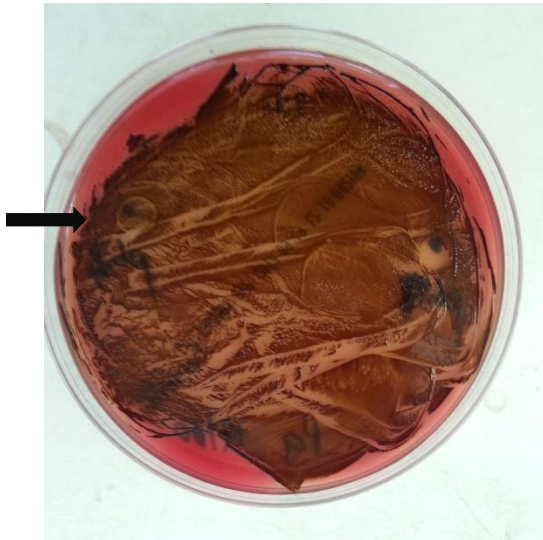


Figure 6: LGG developed no inhibition zones against *P. gingivalis* at pH 7
 Black arrows – LGG CFS; CSF – cell free supernatant

3.4 Discussion and conclusions

Although bacterial antagonism of some strains of lactobacilli against oral pathogens has been previously investigated (14, 15), there are limited studies available investigating competition between LGG, *P. gingivalis* and *F. nucleatum*.

Results from the current study found no antimicrobial activity for LGG against the two pathogens. Previously, human isolates of oral lactobacilli including *L. paracasei*, *L. casei*, *L. salivarius*, *L. plantarum*, *L. fermentum* and *L. rhamnosus* demonstrated an inhibitory effect against some Gram negative periodontal pathogens including *P. gingivalis* (14). The LGG used in the current study did not originate from an oral source and has been reported as being unable to colonise the oral cavity (8, 16).

The antimicrobial activity of lactobacilli is generally linked with their ability to produce lactic acid, hydrogen peroxide and bacteriocins (11). Lactobacilli antimicrobial activity is also species dependent: peptide bacteriocins from *L. plantarum* were found to prevent colonisation by *P. gingivalis in vitro* whilst bacteriocins from *L. brevis* had no antimicrobial effect on *P. gingivalis* (12). The lack of inhibition zones observed in the current study might suggest that the mechanisms of action did not involve LGG's antimicrobial ability. Future investigations should explore the potential antagonism between LGG, *P. gingivalis* and *F. nucleatum* in co-culture as well as experimenting with a wider variety of environmental conditions such as pH and temperature.

Other mechanisms of lactobacilli action including anti-inflammatory effects have been reported previously (17). An *in vitro* study proved that the probiotic strain *L. rhamnosus* (ATCC 9595) prevented *P. gingivalis* induced inflammation by

modulating TLR signaling (3) whilst animal studies showed that probiotic supplementation with *L. acidophilus*, *Bacillus subtilis*, *Enterococcus faecium* and *Bifidobacterium bifidum* improved local and systemic inflammation in rats with induced oral mucositis (18). In the gut, LGG can block uncontrolled inflammatory responses (19). These mechanisms may prove to be of interest in periodontal disease studies since it has been demonstrated that controlling inflammation in periodontitis can promote tissue regeneration and restore homeostasis (20).

In conclusion, the current study suggests the effect of LGG on *P. gingivalis* (W50) and *F. nucleatum* (ATCC 25586) was not the result of any observed antimicrobial property. Taking into account LGG's demonstrated anti-inflammatory properties and immunomodulatory effects, this probiotic may prove to be a good candidate for probiotic-periodontitis pre-clinical studies.

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CHAPTER 4

Probiotic *Lactobacillus rhamnosus* GG prevents alveolar bone loss in a mouse model of experimental periodontitis

This chapter was published in the Journal of Clinical Periodontology:

Simona M Gatej, Victor Marino, Richard Bright, Tracy R Fitzsimmons, Neville Gully, Peter Zilm, Rachel J Gibson, Suzanne Edwards, Peter M Bartold, “Probiotic *Lactobacillus rhamnosus* GG prevents alveolar bone loss in a mouse model of experimental periodontitis”, *Journal of Clinical Periodontology*, 2017, DOI 10.1111/jcpe.12838, page 1-9

Statement of Authorship

Title of Paper	Probiotic <i>Lactobacillus rhamnosus</i> GG prevents alveolar bone loss in a mouse model of experimental periodontitis
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Principal Author

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Contribution to the Paper	Under the guidance of P.M. Bartold, R. Gibson, N. Gully and P. Zilm I designed and developed this study. I was responsible for all experimental work, analysis of data, micro-CT scanning, multiplex analysis, histology, TRAP analysis and preparation of the manuscript. I am the corresponding author				
Overall percentage (%)	85 %				
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.				
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- i. the candidate's stated contribution to the publication is accurate (as detailed above);
- ii. permission is granted for the candidate to include the publication in the thesis; and
- iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Assisted with the design of the experiment, animal work, micro-CT scanning and reviewing the manuscript for publication				
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***Lactobacillus rhamnosus* GG prevents alveolar bone loss in a mouse model of experimental periodontitis**

[Gatej *et al.*, (2017) *Journal of Clinical Periodontology*. DOI 10.1111/jcpe.12838: 1–9]

4.1 Abstract

Aim: This study investigated the role of *Lactobacillus rhamnosus* GG on bone loss and local and systemic inflammation in an *in vivo* mouse model of experimental periodontitis.

Materials and methods: Experimental periodontitis was induced in mice by oral inoculation with *Porphyromonas gingivalis* and *Fusobacterium nucleatum* over a period of 44 days. The probiotic LGG was administered via oral inoculation or oral gavage prior to, and during disease induction. The antimicrobial activity of LGG on the inoculum was also tested. Alveolar bone levels and gingival tissue changes were assessed using *in vivo* micro-computed tomography and histological analysis. Serum levels of mouse homologues for IL-8 were measured using multiplex assays.

Results: Pre-treatment with probiotics either via oral gavage or via oral inoculation significantly reduced bone loss ($p < 0.0001$) and gingival inflammation ($p < 0.0001$) when compared with periodontitis group. Oral gavage treatment group had significantly less TRAP positive cells ($p < 0.02$) than periodontitis group. LGG showed no antimicrobial activity against *Porphyromonas gingivalis* and *Fusobacterium nucleatum*.

Conclusions: LGG effectively suppresses bone loss in a mouse model of induced periodontitis irrespective of the mode of administration.

Clinical Relevance

Scientific rationale for the study: Probiotic interventions in periodontitis have increased in recent years with more studies needed to elucidate their role in alveolar bone loss.

Principal findings: In the current study, the use of the probiotic *Lactobacillus rhamnosus* GG prior to the induction of experimental periodontitis prevented bone loss and reduced local inflammation for all probiotic treated groups, irrespective of the probiotic mode of administration.

Practical implications: *Lactobacillus rhamnosus* GG may prove to be a good candidate for human probiotic-periodontitis studies and may offer a low risk, easy to use option for adjunctive use in the management of periodontitis.

4.2 Introduction

Periodontitis (PD) is a chronic inflammatory condition affecting the tissues surrounding teeth. It is influenced by elevated numbers of specific bacteria that can become pathogenic due to changes in the local environment controlled by the host (1, 2). Alveolar bone loss is a distinctive feature of periodontitis and its prevention still represents a challenge for clinicians. There is a clear need for future adjunctive preventive bone loss therapies together with new treatment protocols targeting the host response (3). The administration of probiotics with inflammation modulating

properties is one of several new approaches being considered as an option to address the bacterial imbalance and prevent bone loss in periodontitis. This may offer a low risk, inexpensive and easy-to-use preventive or treatment option in achieving periodontal health (4).

Probiotics are defined by the World Health Organisation as live microorganisms, most often bacteria, which, when consumed, confer beneficial effects to the host (5). Probiotics play important roles in food digestion and nutrient absorption, synthesis of proteins and vitamins whilst also being important in the development and regulation of the immune system (6, 7) The use of probiotics in the management of periodontitis has previously been investigated; however, there is insufficient data regarding their role in controlling alveolar bone loss (4). Of the limited number of published studies available, most have investigated bone loss using conventional radiography or histomorphometric analyses that introduce potential measurement inaccuracies (8-10)

Lactobacillus rhamnosus GG (LGG) is a probiotic that has been extensively and safely used in gastrointestinal clinical applications (11). LGG has the ability to survive the low pH of the stomach and the bile acids of the duodenum and exerts anti-inflammatory properties *in vivo* (12).

The primary outcome of this study was to determine the effect of LGG on alveolar bone loss and the secondary outcome was to determine changes in local and systemic inflammation.

4.3 Materials and Methods

4.3.1 Ethics

This project was approved by the University of Adelaide Animal Ethics Committee (M-2015-116) and complied with National Health and Research Council (Australia) Code of Practice for Animal Care in Research and Training (2014).

4.3.2 *In vitro* evaluation of LGG antimicrobial activity on *P. gingivalis* and *F. nucleatum*

To investigate the potential antimicrobial activity of LGG, *P. gingivalis* (W50), *F. nucleatum* (ATCC® 25586) and LGG (ATCC® 53103) were grown on agar plates at 37°C in an atmosphere of N₂/CO₂/H₂ (90:5:5). Bacterial colonies were Gram stained and used to inoculate ten ml of heart infusion broth (Oxoid, Basingstoke UK) for *P. gingivalis* and *F. nucleatum* and ten ml of MRS broth (Becton, Dickinson and Company, Sparks, USA) for LGG. After three days *P. gingivalis* and *F. nucleatum* were swabbed uniformly across three agar plates and then three wells were created equilaterally in each plate. LGG broth was placed in four sterile Eppendorf tubes, centrifuged at 10000 g for five minutes and filtered (0.22 µm). The cell free supernatant was placed in two of the wells on the *P. gingivalis* and *F. nucleatum* agar plates while sterile broth was placed in the third well to act as a control.

The antimicrobial activity of LGG was also assessed following a protocol described by Khalaf *et al.* (13). Briefly, 100 µl of *P. gingivalis* (10¹⁰ CFU) and 100 µl of *F. nucleatum* (10⁸ CFU) were spread on agar plates and allowed to dry. Five ml of LGG broth (10⁹

CFU) was centrifuged at 10000 g for five minutes and filtered (0.22 µm). After adjusting the pH to 7, 10 µl of LGG's supernatant were placed onto the *P. gingivalis* or *F. nucleatum* layer. The plates were incubated for four days after which inhibition zones were observed.

4.3.3 Animals

Thirty-six 6-8 week old BALB/c female mice were obtained from the Laboratory Animal Services of the University of Adelaide and were housed in a PC2 animal holding facility (OGTR certification No 2067/2008). All mice were subjected to a period of five days of acclimatization and were kept in a room with a 12h light/dark cycle and temperature from 22-24 °C. The animals were housed with soft, sterile bedding, free of antibacterial products, were fed a powdered, sterile, non-granular food to prevent impaction of food around the gingiva and had access to sterile non-acidic water throughout the experiment. Mice were randomly assigned to six groups ($n =$ six animals/group).

Group 1: Control (no periodontitis, no treatment)

Group 2: PD (periodontitis, no treatment)

Group 3: LGG Gav + PD (treatment with LGG via oral gavage (Gav) and subsequent periodontitis)

Group 4: LGG Gav (treatment with LGG via oral gavage)

Group 5: LGG Oral + PD (treatment with LGG via oral inoculation (Oral) and subsequent periodontitis)

Group 6: LGG Oral (treatment with LGG via oral inoculation).

All mice were assessed daily for general health parameters. At the completion of the study (day 44), animals were killed by cervical dislocation under anesthesia with a final

solution of xylazine (20 mg/kg of body weight) and ketamine (100 mg/kg of body weight). Heads, livers, kidneys, spleens, stomachs and intestines were kept for analysis.

4.3.4 Probiotic treatment

The mouse model of experimental periodontitis used in this study has been described previously (14, 15). Briefly, all animals received kanamycin 1mg/ml (Sigma, St. Louis, MO, USA) in deionised water *ad libidum* for a period of seven days to reduce the native flora and support colonisation of the pathogenic bacteria. LGG is not susceptible to kanamycin (16, 17). After the cessation of the antibiotic (day 8), twenty-four mice received a daily dose of 200 µl of 2.9×10^9 CFU/ml of LGG (ATCC® Number 53103) in sterile 2% carboxymethyl cellulose (CMC) (Sigma, St Louis, MO, USA) in phosphate buffered saline (PBS). Twelve mice (groups LGG Oral and LGG Oral + PD) received oral inoculation of LGG with bacteria directly swabbed around the molars using a small brush. Another twelve mice (groups LGG Gav and LGG Gav + PD) received oral gavage of LGG with bacteria administered directly into the stomach using a bulb tipped gavage needle attached to a syringe. LGG treatment started three days prior to the induction of periodontitis and it continued daily for the whole duration of the experiment (day 44).

4.3.5 Induction of experimental periodontitis

Animals from groups PD, LGG Gav + PD and LGG Oral + PD were inoculated over two sessions with an inoculum containing *Porphyromonas gingivalis* (strain W50) and

Fusobacterium nucleatum (ATCC® 25586). One hundred microlitres of $2-4 \times 10^{10}$ CFU/ml of *P. gingivalis* and $4-6 \times 10^8$ CFU/ml of *F. nucleatum* in sterile 2% CMC in phosphate buffered saline (PBS) were swabbed onto the molars with the use of a microbrush. The inoculation protocol has been previously documented (15, 18).

4.3.6 Live-animal micro-computed tomography

Mice were scanned using a Skyscan 1076 High Resolution Micro-CT Scanner (Skyscan, Bruker, Belgium) to determine bone changes in the jaws. The scanning specifications and protocol have been previously published (18). Live scans were taken before probiotic treatment (day 3) and at the end of the experiment (day 44). Mice in the control group were scanned at the same time points to give an indication of alveolar bone changes that occur with normal mouse growth.

4.3.7 Micro-CT data processing

Scans were reconstructed using a cone-beam algorithm with the following settings: smoothing = 1, ring artefacts reduction = 10, beam hardening correction = 30% (NRecon software, Version 1.6.10.2, Skyscan, Bruker, Belgium). DataViewer software (Version 1.5.2.4, Skyscan) was used for 3D viewing of the images and realignment of the sagittal planes. The images were opened using CTAnalyser software (Version 1.15.4.0 +, Skyscan) as previously described (18). Changes in the alveolar bone height were assessed by measuring the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) distance (CEJ-ABC) between first and second maxillary

molars on three slices (left and right) for each mouse using sagittal sections of the jaw (**Figure 1**). Two independent, blinded operators performed the measurements.

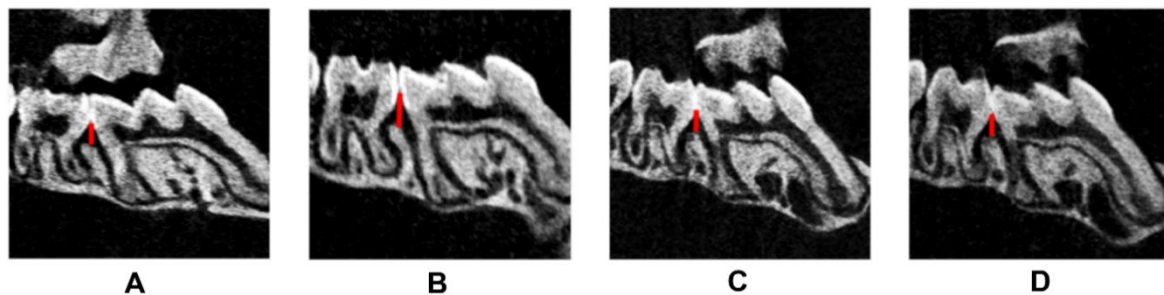


Figure 1. Representative micro CT appearance and analysis of periodontal bone loss between first and second maxillary molars. Control (A); PD (B); LGG Gav (C); LGG Gav + PD (D); Red bars represent CEJ-ABC distance measurements; Abbreviations: PD = Periodontitis, Gav = Gavage, CEJ-ABC = Cemento-enamel junction to alveolar bone crest.

4.3.8 Histological analysis

4.3.8.1 Haematoxylin and eosin staining

Heads were sectioned in half, processed and embedded in paraffin. Sections best representing the longitudinal cutting of the first and second molars from the maxillae were selected and stained with haematoxylin and eosin for analysis. Histological sections were imaged using the NanoZoomer Digital Pathology System (NDP Hamamatsu, Hamamatsu City, Japan) at x 40 magnification. Histological assessment and scoring were carried out by two independent, blinded operators using a four-point

scale system based on previous methods (19). Total numbers of inflammatory cells (lymphocytes, plasma cells, neutrophils or macrophages) were assessed within an area of four mm², which included the alveolar bone between the first and second molars. Normal tissue was scored as 0 (< 5% inflammatory cells), mild inflammation was scored as 1 (5–20% inflammatory cells), moderate inflammation was scored as 2 (20–50% inflammatory cells), and severe inflammation (> 50% inflammatory cells) was scored as 3. The number of multinucleated osteoclast cells (more than three nuclei) per square millimetre within the assessed area was also determined.

4.3.8.2 Tartrate-resistant acid phosphatase (TRAP) staining

TRAP staining was conducted on longitudinal cutting sections of the maxillae to detect the number of osteoclasts on the bone surface and pre-osteoclasts in the surrounding soft tissue using a method adapted from Udagawa *et al.* (20). Briefly, slides were TRAP stained (TRAP, Sigma-Aldrich, San Louis, MO) and left to incubate at 37 °C for 15 min before rinsing with PBS and counterstaining with haematoxylin. The number of TRAP-positive cells with three or more nuclei were counted by two blinded observers in a consistent region of interest (4 mm² that included the alveolar bone between the first and second molars) as previously described (21).

4.3.9 Serum collection

At the completion of the study (day 44), blood was collected through cardiac puncture and allowed to clot for 1 hour at room temperature. Serum was separated by centrifugation at 1000 g for 20 minutes and stored at -20 °C until required.

4.3.10 Multiplex analysis

Serum was analysed for the presence of mouse homologues of interleukin 8 (IL-8): keratinocyte chemoattractant (KC), lipopolysaccharide induced CXC (LIX) and macrophage inflammatory protein 2 (MIP2) using a magnetic Luminex Screening Assay according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). All standards and samples were assayed in duplicate using a Luminex 200 system (Luminex Corporation, Austin TX, USA). Concentrations were analysed using xPONENT version 3.1 software (Luminex Company).

4.3.11 Statistics

Data were analysed using the SAS 9.4 statistical software (SAS Institute Inc., Cary, NC, USA).

The power of this study was 85% for a sample size of six per group and a significance level of 0.05 based on the expected difference regarding the primary outcome. For analysis of bone resorption, body weights and TRAP data, a linear mixed-effects model was performed to account for clustering and repeated measurements on mouse together with a compound symmetry covariance structure (for bone) and an unstructured covariance structure (for weights). Six replicate measurements were carried out on each of the six mice per group to allow for a correlation of bone loss measurements within each mouse (measurements taken from the same mouse would likely be more similar than measurements taken from different mice). Post-hoc comparisons were used to analyse statistically significant differences between groups. All values shown are mean \pm standard error (SEM). A linear regression model was

performed to analyse local inflammation results and post-hoc comparisons were used to analyse statistically significant differences between groups. For systemic inflammation (LIX, MIP2 and KC), linear regression models with logarithmic transformation of the outcome were performed and post-hoc comparisons were used to analyse statistically significant differences between groups.

4.4 Results

4.4.1 Animals

Mice did not lose significant amounts of weight across the duration of the experiment. In addition, there were no other adverse events noted. One mouse died on day 26 but this was thought to be unrelated to the experiment.

4.4.2 LGG demonstrated no antimicrobial activity against *P gingivalis* and *F nucleatum in vitro*

Antimicrobial activity of LGG was investigated by using two methods. After four days of incubation no inhibition zones were observed using either method (Data not shown).

4.4.3 Effect of LGG administered orally on weight gain

Body weight was monitored weekly for eight weeks. Mice receiving LGG orally did not gain significant weight over the duration of the experiment: LGG Oral ($p = 0.085$), LGG Oral + PD ($p = 0.055$). All the other groups presented statistically significant weight gain: Control ($p = 0.0003$), LGG Gav ($p = 0.046$), LGG Gav + PD ($p = 0.038$) and PD ($p < 0.0001$). (Supplementary information).

4.4.4 Effect of LGG on bone resorption

Micro-CT scans of live animals at the end of the experiment and final histology analyses showed an established chronic inflammatory reaction in the disease (PD) group. Analysis of the micro-CT scans confirmed that periodontitis had been induced with statistically significant mean bone loss in the disease (PD) group ($p < 0.0001$) (**Figure 2**). Mice in this group, at the end of the experiment had mean bone loss 20% greater than the same mice at the start (exponentiated estimate=1.20, 95% CI: 1.12, 1.28). No significant mean bone loss ($p > 0.05$) between end and start of the experiment was observed for Control, LGG Gav, LGG Gav + PD, LGG Oral and LGG Oral + PD.

4.4.5 Effects of LGG on gingival and systemic inflammation

Semi-quantitative histological analysis of the periodontal tissues of the first and

second maxillary molar regions showed significantly more inflammatory cell infiltrate and osteoclast numbers in the PD group when compared with controls ($p < 0.0001$). All animals treated with probiotics had similar inflammatory scores when compared with controls ($p > 0.05$). However, when compared with the PD group, they presented with significantly less inflammation ($p < 0.001$) (**Figure 3**).

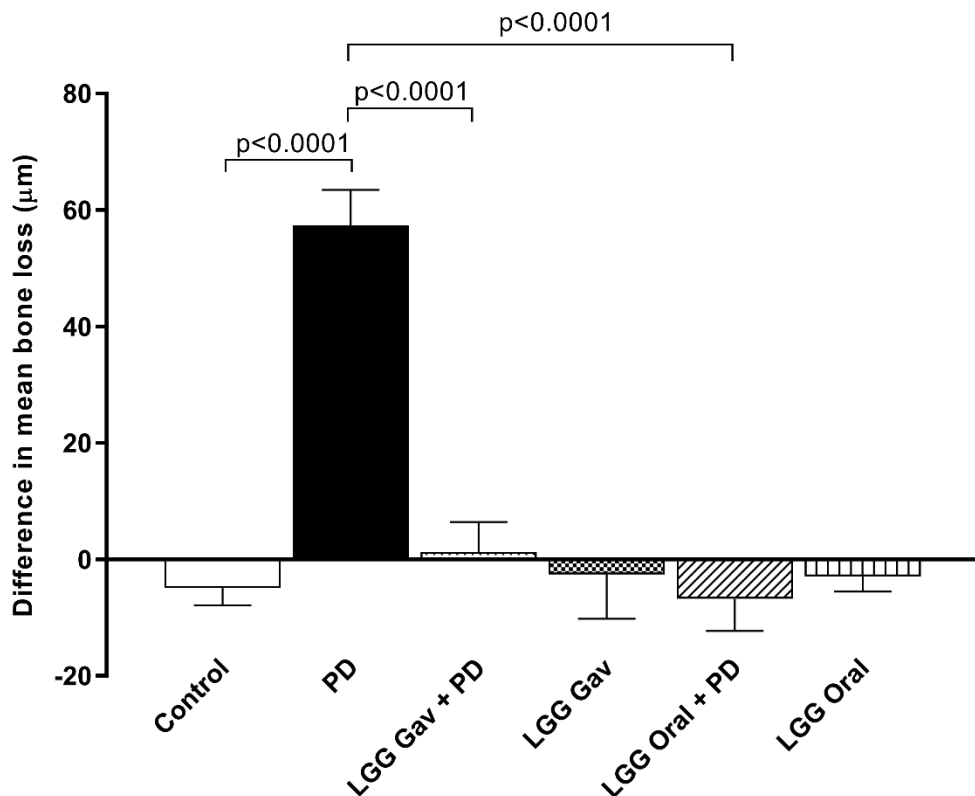


Figure 2. Analysis of periodontal bone loss. Control: mice ($n = 5$); PD: mice ($n = 6$) with induced periodontitis; LGG Gav + PD: mice ($n=6$) with PD and treated by oral gavage with LGG; LGG Gav: mice ($n=6$) treated by oral gavage with LGG; LGG Oral + PD: mice ($n=6$) with PD and treated by oral inoculation with LGG; LGG Oral: mice ($n=6$) treated by oral inoculation with LGG; Bars represent mean bone loss (μm) \pm SEM; Abbreviations: PD = Periodontitis, Gav = Gavage.

Analysis of TRAP staining (**Table 1, Figure 4 and 5**) indicated that mice in the PD group had a statistically significant greater number of TRAP positive cells compared with controls (7.24 ± 2.15 , $p = 0.0020$). LGG Oral, LGG Gav and LGG Gav + PD groups had statistically significant less TRAP positive cells than mice in the PD group ($p < 0.02$). The LGG Oral + PD group had less TRAPP positive cells than the PD group (3.99 ± 2.05), albeit not statistically significant ($p = 0.062$). Further comparisons presented in Table 1 were not significant.

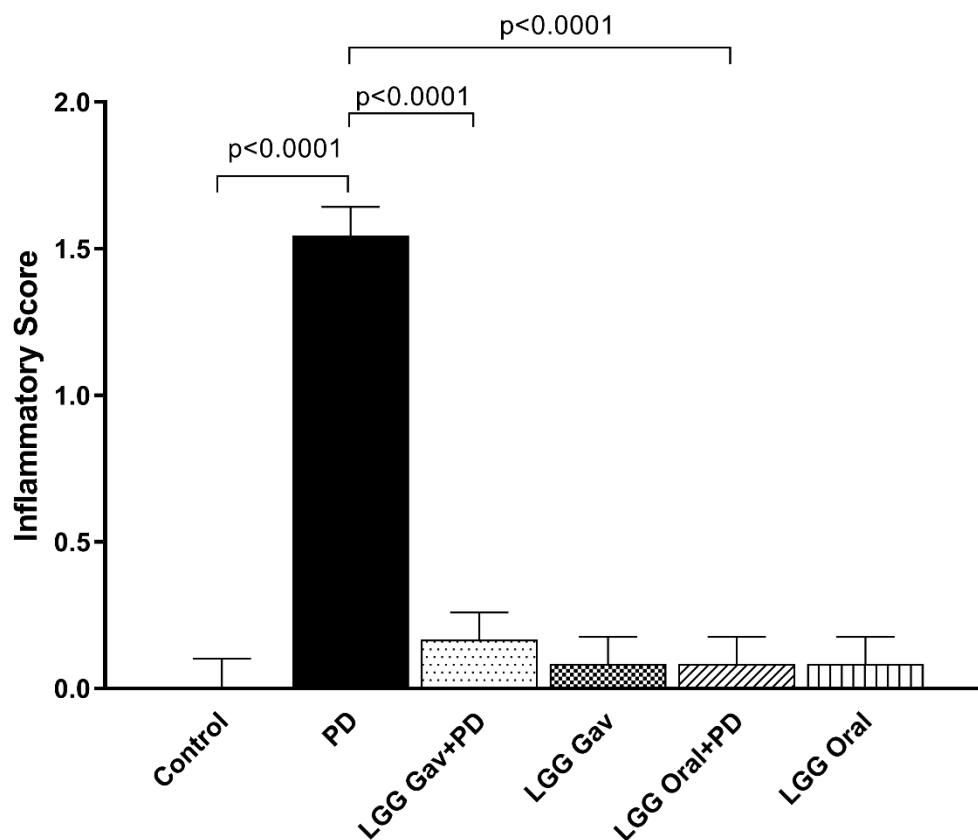


Figure 3. Histological analysis of maxillary periodontal tissues. Control: mice (n = 5); PD: mice (n = 6) with induced periodontitis; LGG Gav + PD: mice (n=6) with PD and treated by oral gavage with LGG; LGG Gav: mice (n=6) treated by oral gavage with LGG; LGG Oral + PD: mice (n=6) with PD and treated by oral inoculation with LGG; LGG Oral: mice (n=6) treated by oral inoculation with LGG; Bars represent mean inflammatory score \pm SEM; $p < 0.0001$; Abbreviations: PD = Periodontitis, GAV = Gavage.

Table 1. Linear regression results of TRAP versus Group. Groups in bold have a mean TRAP positive value significantly different to the PD group; $p < 0.05$; Abbreviations: PD = Periodontitis, GAV = Gavage, TRAP = Tartrate resistant acid phosphatase.

<i>Group – comparison value</i>	<i>Group – reference value</i>	<i>Estimate ± SEM (positive cells/mm²)</i>	<i>p value</i>
PD	LGG Gav + PD	5.86 ± 2.05	0.0076
PD	LGG Gav	6.00 ± 2.09	0.0072
PD	LGG Oral + PD	3.99 ± 2.05	0.0615
PD	LGG Oral	5.15 ± 2.05	0.0176
PD	Control	7.24 ± 2.15	0.0020
LGG Gav + PD	LGG Gav	0.14 ± 2.02	0.9453
LGG Gav + PD	LGG Oral + PD	-1.87 ± 1.98	0.3516
LGG Gav + PD	LGG Oral	-0.71 ± 1.98	0.7233
LGG Gav + PD	Control	1.37 ± 2.08	0.5133
LGG Gav	LGG Oral + PD	-2.01 ± 2.02	0.3256
LGG Gav	LGG Oral	-0.85 ± 2.02	0.6771
LGG Gav	Control	1.24 ± 2.11	0.5628
LGG Oral + PD	LGG Oral	1.17 ± 1.98	0.5605
LGG Oral + PD	Control	3.25 ± 2.08	0.1282
LGG Oral	Control	2.08 ± 2.08	0.3242

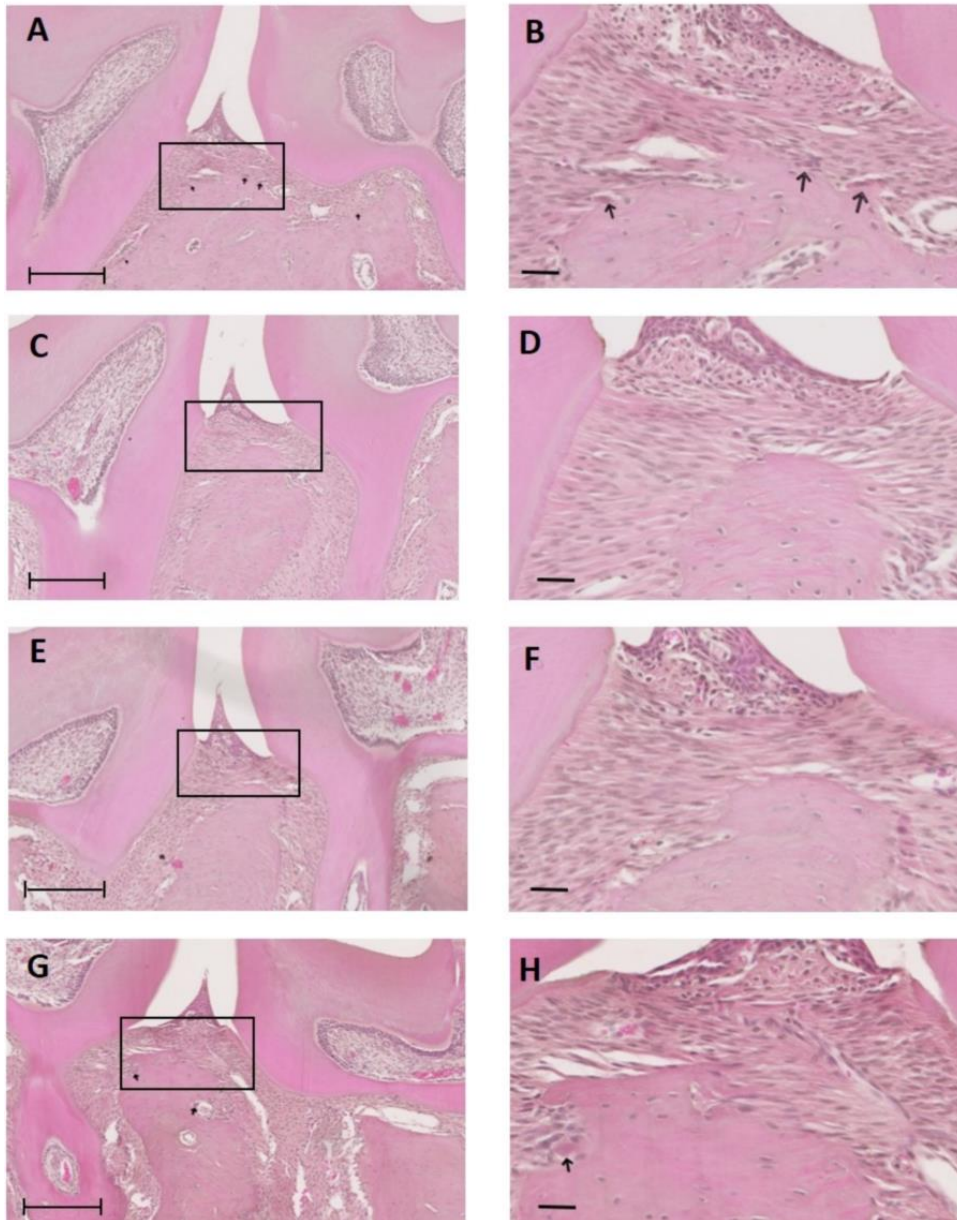


Figure 4. Representative images of standard hematoxylin and eosin staining of the gingival tissue near the first and second and third maxillary molars in: group PD (A and B); Control (C and D); LGG Gav + PD (E and F); LGG Oral + PD (G and H); Black arrows = multinucleated osteoclasts at resorption; Abbreviations: PD = Periodontitis, Gav = Gavage; Scale bars: 200 μ m (A, C, E and G) and 20 μ m (B, D, F and H).

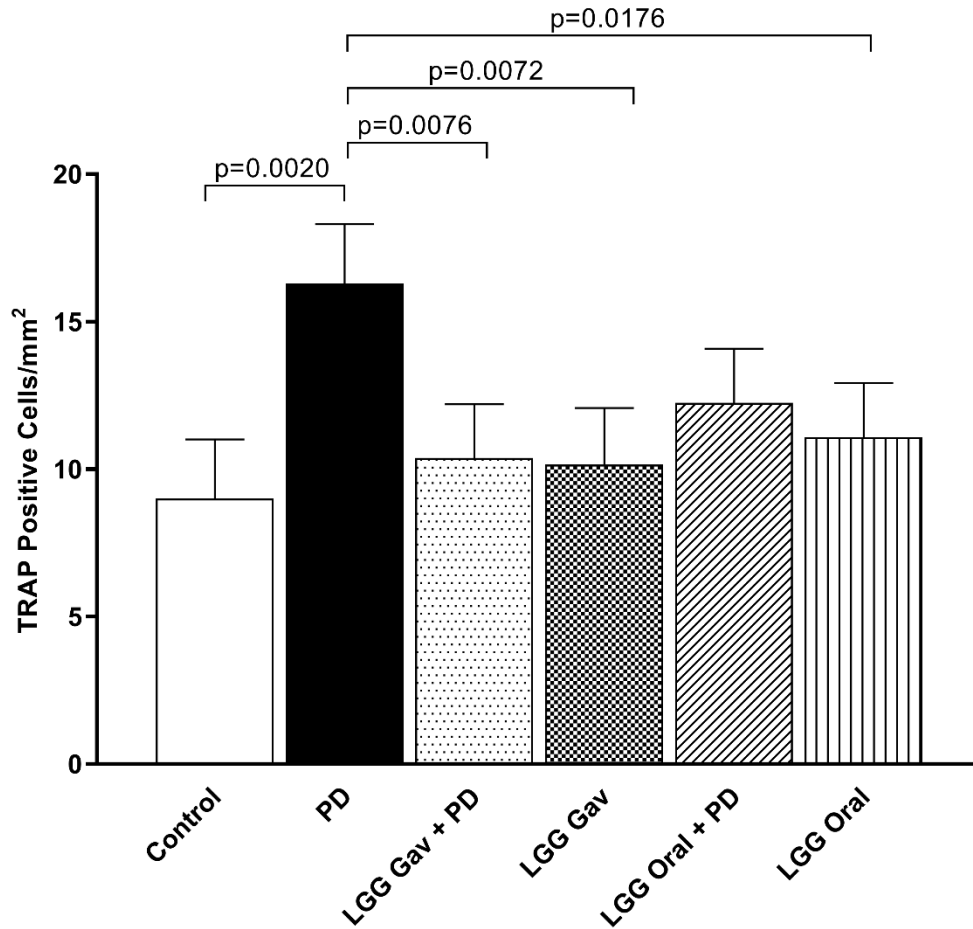


Figure 5. TRAP positive multinucleated cells/mm² in maxillary periodontal tissues. Control: mice (n = 5); PD: mice (n = 6) with induced periodontitis; LGG Gav + PD: mice (n=6) with PD and treated by oral gavage with LGG; LGG Gav: mice (n=6) treated by oral gavage with LGG; LGG Oral + PD: mice (n=6) with PD and treated by oral inoculation with LGG; LGG Oral: mice (n=6) treated by oral inoculation with LGG; Bars represent mean ± SEM; Abbreviations: PD = Periodontitis, GAV = Gavage, TRAP = Tartrate resistant acid phosphatase.

Circulating levels of LIX in blood serum were 61% less in control mice when compared with PD mice ($p = 0.0188$) (**Figure 6**). All probiotic and probiotic treated groups had lower concentrations for LIX when compared with the periodontitis group, albeit not statistically significant. Differences in circulating serum levels of KC and MIP2 were not statistically significant between groups. (Data not shown).

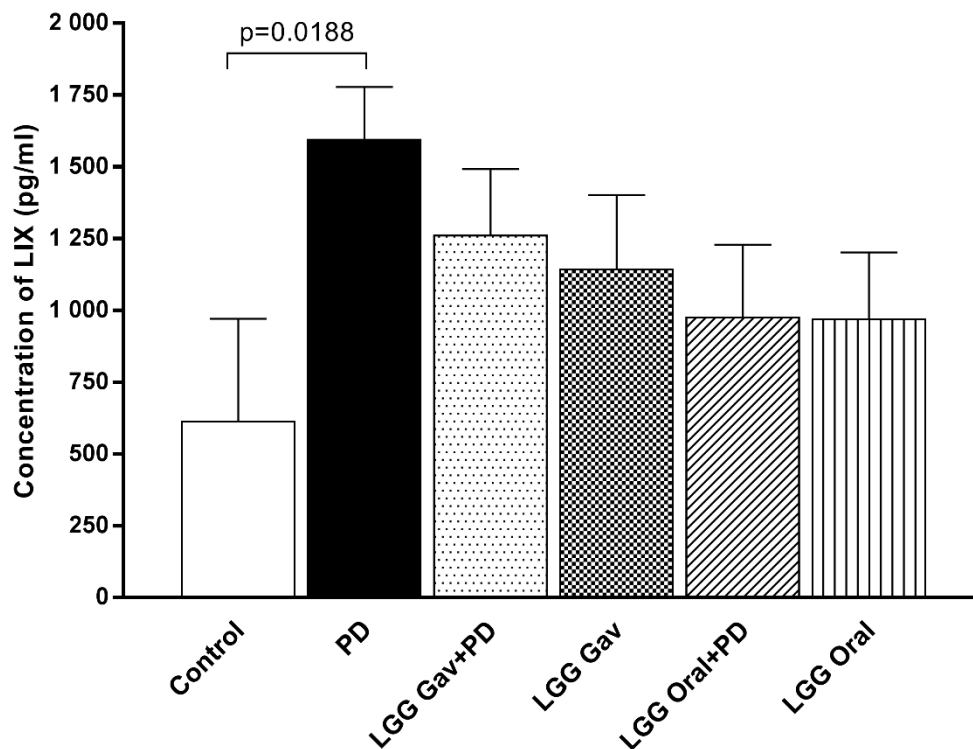


Figure 6. Serum LIX concentration (pg/ml) as an indicator of systemic inflammation. Control: mice (n = 5); PD: mice (n = 6) with induced periodontitis; LGG Gav + PD: mice (n=6) with PD and treated by oral gavage with LGG; LGG Gav: mice (n=6) treated by oral gavage with LGG; LGG Oral + PD: mice (n=6) with PD and treated by oral inoculation with LGG; LGG Oral: mice (n=6) treated by oral inoculation with LGG; Bars represent mean LIX concentration in serum (pg/ml) ± SEM; Abbreviations: PD = Periodontitis, Gav = Gavage, LIX - Lipopolysaccharide induced CXC.

4.5 Discussion

In the present study, the effects of the probiotic LGG were investigated in a mouse model of periodontitis. LGG was chosen due to its non-cariogenic (22) and anti-inflammatory properties (12). Previously, acid-producing lactobacilli and

bifidobacteria have been considered a risk for dental caries (23). This concept has been challenged with clinical studies suggesting some probiotic species are beneficial for oral health and LGG may reduce the risk of caries (24, 25). Treatment with LGG was conducted before the initiation of disease to assess its ability to prevent alveolar bone loss. Oral inoculation and oral gavage were used as different methods of probiotic administration.

The results of the present study demonstrated that use of LGG prior to the induction of periodontitis prevented alveolar bone loss and local inflammation in the probiotic treated groups when compared with the periodontitis group. The lack of inhibition zones suggested that the mechanisms of action might involve the anti-inflammatory properties of LGG rather than its antimicrobial ability. It was evident that LGG significantly inhibited alveolar bone resorption and gingival inflammation in the probiotic treated periodontitis groups, irrespective of the mode of administration (oral gavage or oral inoculation). This could suggest that LGG colonisation in the mouth was not a prerequisite for the inhibition of bone loss. Mice in the disease group (PD) with significant mean alveolar bone loss, increased presence of osteoclastic cells (TRAP) and inflammatory infiltrates are consistent with our previous studies (18, 19).

A key finding from the current study was that animals treated with probiotics prior to disease induction showed no evidence of bone resorption correlated with significantly reduced gingival inflammation. The oral gavage treatment group presented a significant reduction in TRAP positive cells.

Evidence supporting the use of probiotics in periodontal disease continues to increase. Previous studies have clearly shown that oral administration of probiotics improved periodontal pocket depth, bleeding on probing, attachment loss and

reduced the levels of periodontal pathogens in patients with chronic and advanced periodontitis (26, 27)

Further, these studies have demonstrated an effect of probiotics on the oral microbiome and some effects on periodontal parameters. However, more studies are required before a standardized protocol can be agreed upon in terms of the probiotic concentration, duration and frequency of treatment. In contrast to therapy studies, enquiries investigating bone loss prevention in periodontitis are scarce and tend to be pre-clinical in nature. Mice treated with locally administered probiotics have been shown to have significantly decreased bone loss and lower expression of inflammatory cytokines (10). Probiotics administered systemically via drinking water in a ligature-induced periodontitis model resulted in reduced alveolar bone loss and protected the intestine from reactive changes (9, 28, 29). Recently, probiotic therapy has been reported to decrease the number of TRAP positive cells for the probiotic treated groups when compared with controls in periodontal tissues surrounding teeth submitted to mechanical loading (30). LGG, the probiotic used in the current study, has been shown to suppress trabecular bone loss associated with estrogen deficiency in mice

(Li *et al.*, 2016). Li and colleagues demonstrated that in female mice, estrogen deficiency increased gut permeability and therefore increased susceptibility to infection leading to upregulation of Th17 cells and osteoclastogenic cytokines (RANKL, IL-17 and TNF) in the intestine via changes in the intestinal microbiota. The authors suggested the protective effect of LGG against bone loss was due to its ability to decrease gut permeability (31). It will be a matter for future investigations to determine whether this is the mechanism of action in our current study. Other probiotics (*L. reuteri*) have been shown to improve bone density in male mice (32)

and to prevent bone loss associated with estrogen deficiency in female mice possibly via alteration of the immune response due to changes in the intestinal microbiota (33). The mechanisms by which probiotics participate in bone homeostasis are not completely understood and depend on the strains of the probiotics used, concentrations, duration of treatment, mode of administration and clinical indication (34). In order to understand the mechanisms involved in the observed effects of probiotics in our study, systemic inflammation was analysed by monitoring the mouse homologues for IL-8: LIX, KC and MIP-2 that are known to activate and attract neutrophils and are involved in the amplification of the inflammatory cascade (35). Mice in the PD group presented significantly higher concentration of LIX when compared with controls. MIP2 and KC levels presented no differences between groups and this may be due to the LIX levels remaining elevated longer than KC or MIP2 or due to LIX having biological roles distinct from MIP2 or KC (36). LGG has been shown to mediate the effects of pro-inflammatory cytokines interferon- γ and TNF- α on the epithelial barrier integrity (37) and to improve intestinal epithelial barrier function via tight junction changes (38). These findings imply that LGG acts on the host's immune response by producing factors capable of modulating inflammation, an idea consistent with the proven fact that probiotics engage with the innate and acquired immune response (34). Future research will need to assess the levels of inflammatory cytokines in gut tissue (IL-1 β , IL-6) and gene expression of proteins related to gut barrier function in order to elucidate the underlying mechanisms of these interactions.

In conclusion, our findings indicated that therapy with LGG prior to inducing periodontitis suppressed the host response and resulted in significantly less bone loss and inflammation for all probiotic treated groups when compared with disease.

We conclude that LGG had a significant protective effect on alveolar bone loss irrespective of the mode of administration, which may be linked to changes not only in the oral microbiome but to changes in the gut microbiome. Whether the effects seen in this study were the result of the LGG's anti-inflammatory capacity it is yet to be determined.

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CHAPTER 5

Probiotic *Lactobacillus rhamnosus* GG protects against gut dysbiosis induced by *P. gingivalis* and *F. nucleatum* in mice

Chapter 5 Probiotic *Lactobacillus rhamnosus* GG protects against gut dysbiosis induced by *P. gingivalis* and *F. nucleatum* in mice

5.1 Abstract

Aims: This study investigated the impact of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* inoculation firstly on intestinal inflammation and secondly on the structure and diversity of the intestinal microbiome in an *in vivo* mouse model. The role of the probiotic *Lactobacillus rhamnosus* GG in altering these changes was also investigated.

Materials and methods: 36 mice were allocated into six groups. Experimental bone loss was induced in mice by oral inoculation with *P. gingivalis* and *F. nucleatum* over a period of 44 days. The probiotic LGG was administered via oral inoculation or oral gavage prior to and during disease induction. The probiotic treated groups were compared with animals with experimental bone loss and with controls. Intestinal tissue changes were assessed using histological analysis. Immunohistochemistry was used to assess IL-6 expression in gut tissue. Serum levels of C-reactive protein were measured using enzyme linked immuno-sorbent assays to determine systemic inflammation. The phylogenetic structure and diversity of the intestinal microbiota were analysed by sequencing the 16S rRNA genes of the caecal content. Statistical differences between groups were identified using a PERMANOVA pseudo F test for beta diversity, pairwise Kruskal-Wallis for alpha diversity, analysis of composition of microbes for detection of specific taxa associated with different treatments, pairwise Kruskal-Wallis followed by Dunn's multiple comparisons test for histology and IL-6 expression. Statistical significance was accepted when *p* value was less than 0.05.

Results: Inoculation with *P. gingivalis* and *F. nucleatum* induced inflammation throughout the gastrointestinal tract (duodenum $p = 0.0143$, jejunum $p = 0.0009$, ileum $p = 0.0017$, colon $p = 0.0442$), increased expression of IL-6 in the ileum ($p = 0.052$) and significantly altered the gut microbiome ($p < 0.05$) of experimental mice when compared with controls.

Mice treated with LGG had significantly reduced tissue inflammation in the duodenum ($p = 0.0437$) and significantly lowered levels of IL-6 in the ileum ($p = 0.048$) when compared with disease. LGG therapy prevented gut microbiome changes associated with *P. gingivalis* and *F. nucleatum* inoculation, irrespective of the probiotic mode of administration. *P. gingivalis* or *F. nucleatum* DNA were not detected in caecum or faecal samples. Serum levels of mouse CRP were not significantly different between groups.

Conclusions: *P. gingivalis* and *F. nucleatum* inoculation induced changes in intestinal inflammation and in the phylogenetic structure and diversity of the intestinal microbiome. Oral gavage with LGG prior to *P. gingivalis* and *F. nucleatum* inoculation exerted a protective effect against intestinal inflammation. Pre-treatment with LGG prevented gut microbiome changes associated with *P. gingivalis* and *F. nucleatum* inoculation, irrespective of the probiotic mode of administration.

5.2 Introduction

Periodontitis is a chronic inflammatory condition affecting the tissues surrounding teeth (1). Periodontitis is influenced by elevated numbers of specific bacteria which

may become pathogenic as a result of changes in the local environment controlled by the host (2, 3).

Porphyromonas gingivalis (*P. gingivalis*) and *Fusobacterium nucleatum* (*F. nucleatum*) represent two bacterial species of the human oral microbiome with roles in the pathogenesis of periodontal disease (4). Dual infection with *F. nucleatum* and *P. gingivalis* in mice aggravates alveolar bone loss and inflammation when compared with animals treated with either bacterium alone (5). Previous research has shown that in mice, oral administration of *P. gingivalis* (10^{10} CFU/ml) twice a week for five weeks induces insulin resistance, systemic inflammation and endotoxemia associated with changes in the gut microbiota of the ileum (6). A single oral administration of 10^9 CFU/ml of *P. gingivalis* (strain W83) significantly increased proportion of phylum Bacteroidetes and decreased the proportion of phylum Firmicutes in the gut, and increased serum endotoxin levels (7). The implications of repeated oral inoculations with *F. nucleatum* and *P. gingivalis* on the gut have been scarcely investigated. As such, further studies are needed to elucidate the effects of these bacteria on the gut physiology and microbiome (8).

The administration of probiotics with inflammation modulating properties is one of several new approaches being considered as an option to address the bacterial imbalance and prevent bone loss in periodontitis (9). Probiotics have been traditionally used as therapeutic and prophylactic strategies for gastrointestinal conditions such as inflammatory bowel disease, colitis, chemotherapy induced mucositis and diarrhoea (10) and more recently in the management of caries and periodontal disease (11). *Lactobacillus rhamnosus* GG (LGG) is a probiotic that has been extensively and safely used in gastrointestinal clinical applications (12). More recently, LGG was shown to effectively suppress bone loss in a mouse model of *P.*

gingivalis and *F. nucleatum* induced bone loss, irrespective of the mode of administration (13).

The primary aim of this study was to determine any changes in intestinal inflammation and in the structure and diversity of the intestinal microbiome induced by oral inoculation with *P. gingivalis* and *F. nucleatum*. The secondary aim was to determine the influence of LGG treatment on these changes.

5.3 Materials and methods

This project was approved by the University of Adelaide Animal Ethics Committee (M-2015-116) and complied with National Health and Research Council (Australia) Code of Practice for Animal Care in Research and Training (2014).

5.3.1 Preparation of bacterial inocula

P. gingivalis (strain W50) and *F. nucleatum* (ATCC 25586) were cultured on anaerobic blood agar (Oxoid, Thermo Fischer Scientific, Waltham, MA, USA) for 4 days in an anaerobic atmosphere consisting of a mixture of carbon dioxide, hydrogen and nitrogen in a ratio of 5:5:90. LGG (ATCC 53103) was cultured according to the manufacturer's recommendations on MRS agar plates (Oxoid, Thermo Fischer Scientific, Waltham, MA, USA) at 37°C for 2 days. It was then harvested using 2 ml of carboxymethyl cellulose (CMC) ((Sigma, St Louis, MO, USA)

per plate. Ten μ l of this solution was mixed with 990 μ l of PBS and the optical density measured and checked against the CFU/OD curve as described in Chapter 3 (Figure 2).

5.3.2 Murine periodontitis model

Thirty-six 6-8 week old BALB/c female mice were obtained from the Laboratory Animal Services of the University of Adelaide and were housed in a PC2 animal holding facility (OGTR certification No 2067/2008). All mice received individual tail tattoos for identification. They were randomly assigned to six groups (n = six animals/group) (Figure 1).

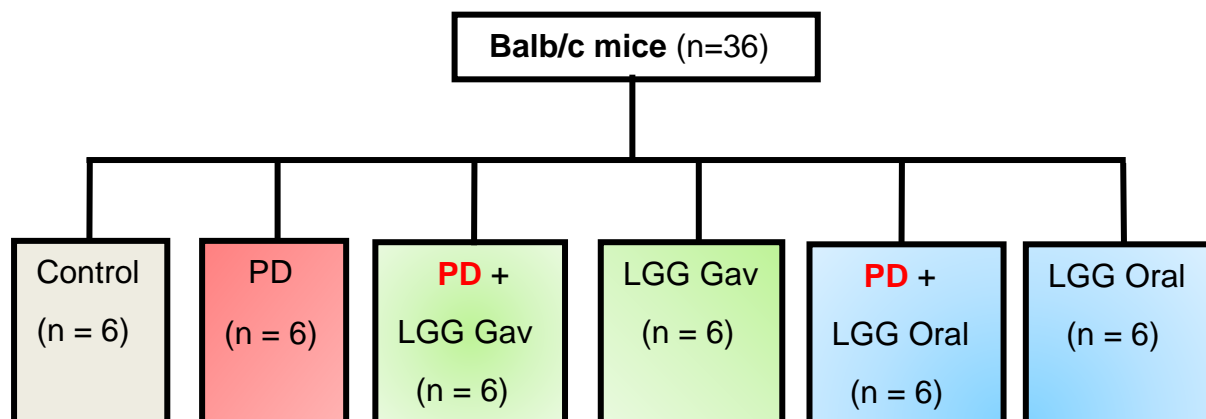


Figure 1: Study design. Control, n=6 (no periodontitis, no treatment), PD, n=6 (periodontitis, no treatment), PD + LGG Gav, n=6 (treatment with LGG via oral gavage (Gav) and subsequent periodontitis), LGG Gav, n=6 (treatment with LGG via oral gavage), PD + LGG Oral, n=6 (treatment with LGG via oral inoculation and subsequent periodontitis), LGG Oral, n=6 (treatment with LGG via oral inoculation). PD = Periodontitis, Gav = Gavage, Oral = Oral inoculation

All mice were acclimatised for 5 days and were kept in a room with a 12 h light/dark cycle and temperature from 22-24 °C. All animals received kanamycin 1 mg/ml (Sigma, St. Louis, MO, USA) in deionised water *ad libidum* for a period of one week prior to inoculation to reduce the native microbiota and support colonisation of the pathogenic bacteria. The animals were housed with soft, sterile bedding, free of antibacterial products and were fed a powdered, sterile, non-granular food to prevent impaction of food around the gingiva. All had access to sterile non-acidified, autoclaved water throughout the experiment. Mice were assessed daily for a number of general health parameters including dull/ruffled coat, change in temperament, reduced food/water intake and reluctance to move. The weight of the animals was recorded once a week.

Animals from group PD (n = 6), PD + LGG Gav (n = 6) and PD + LGG Oral (n = 6) were inoculated over two sessions with an inoculum containing *P. gingivalis* and *F. nucleatum* suspended in 2% (v/v) CMC. The inoculation protocol has been briefly described in Chapter 4. Two ml of CMC were pipetted onto each plate, spread with the aid of a sterile spreader and harvested. For each inoculation, one plate of each species were collected (two plates in total for the 18 experimental mice). The viable count of bacteria was determined to be 2-4 x10¹⁰ CFU for *P. gingivalis* and 4-6 x10⁸ CFU for *F. nucleatum*. One hundred µl of the bacterial solution (or CMC solution for control mice) were swabbed onto the molars with the use of a microbrush. The first inoculation sequence (4 inoculations over 8 days) began following probiotic administration (day 11) and took place 4-6 hours after probiotic treatment (Figure 2). After each inoculation, mice were kept without food and water for 1 hour. The first inoculation sequence was followed by twice a week inoculations for 2 weeks after which all mice received a second inoculation sequence (4 inoculations over 8 days).

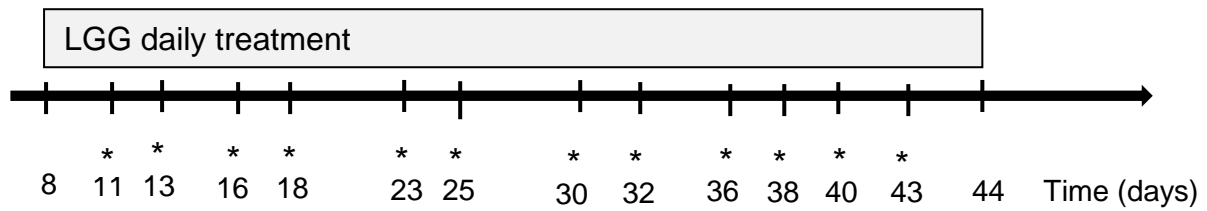


Figure 2: Timeline of treatment LGG treatment started three days prior to disease induction and it continued daily for the whole duration of the experiment (day 44); LGG = *Lactobacillus rhamnosus GG*; * = Inoculation with *P. gingivalis* and *F. nucleatum*

Animals from the control group received only oral CMC inoculations. After the cessation of the antibiotic, animals from group LGG Gav, PD + LGG Gav, LGG Oral, and PD + LGG Oral received a daily dose of 200 μ l of $2-9 \times 10^9$ CFU/ml of LGG in sterile 2% CMC in phosphate buffered saline (PBS). Groups LGG Oral (n = 6) and PD + LGG Oral (n = 6) received oral inoculation of LGG in which bacteria were directly swabbed around the molars using a small brush. Groups LGG Gav (n = 6) and PD + LGG Gav (n = 6) received oral gavage of LGG in which bacteria were administered directly into the stomach using a 24-gauge ball-tipped gavage plastic needle attached to a syringe. Probiotic treatment started three days prior to the induction of periodontitis and it continued daily for the whole duration of the experiment (day 44) (Figure 2). Each probiotic inoculation took place in the morning after which mice had unrestricted access to food and water straight after.

5.3.3 Post-mortem examination

5.3.3.1 Collection and processing of organs for histology

At the completion of the study (day 44) animals were killed by cervical dislocation

under anaesthesia with a solution of xylazine (20 mg/kg of body weight) and ketamine (100 mg/kg of body weight). Liver, spleen, kidneys and lungs were removed, fixed in a solution of 10% (v/v) PBS-buffered formalin for 48 h and in 70% ethanol for another 48 h prior to processing and embedding in paraffin.

The entire gastrointestinal tract was removed and stomach was detached. The gastrointestinal tract was cut at the distal end of the ileum, ahead of the ileocecal junction and caecum to separate the small and large intestine. It was flushed with 1 X PBS (pH 7.4) to remove digested content and faecal matter. Two samples of 0.5 cm length from each of the duodenum, jejunum, ileum and colon were collected in cassettes to be processed as described in section 5.3.4. Sections approximately 1 cm long from the duodenum, jejunum, ileum and colon were cut, snap frozen in liquid nitrogen and stored at -80°C for future analysis. The stomach was cut along the greater curvature. The content was removed and the stomach was placed in formalin for 48 h and in ethanol 70% for another 48 h then paraffin embedded.

5.3.3.2 Collection of caecum contents and faeces

The caecum was separated from the proximal end of the colon. Caecum content was removed, placed in sterile Eppendorf tubes, snap frozen and stored at -80°C. Faeces were collected from each group of mice at the completion of the study and stored at -80°C until required.

5.3.3.3 Collection of blood serum

Blood was collected via cheek puncture bleeds before treatment (day 8) and via cardiac puncture at the conclusion of the study (day 44) and allowed to clot for two hour at room temperature. Serum was separated by centrifugation at 1000 g for 20 minutes and stored at -80° C until required.

5.3.4 Histological analysis of gut samples

The 0.5 cm samples from the duodenum, jejunum, ileum and colon collected in cassettes were fixed in 10% buffered formalin for 24 h. Formalin was then replaced with 70% alcohol for 48 h after which the samples were processed and embedded in paraffin wax.

5.3.4.1 Embedding in paraffin wax

Cassettes were processed using a Leica TP 1020 processor (Leica Biosystems, Wetzlar, Germany). At the end of the process, cassettes were removed from the processor and they were embedded in paraffin wax using a Leica EG1140H paraffin embedding station. Two small regions from each of the duodenum, jejunum, ileum and colon as described in section 5.3.3.1 were embedded into paraffin wax blocks.

5.3.4.2 Sectioning, H&E staining and scanning of embedded gut sections

The embedded samples were sectioned using a Leica RM2235 Microtome (Leica Biosystems, Wetzlar, Germany) to 5 µm thickness. A total of six sections from each wax block were mounted on glass Superfrost® microscope slides (Menzel-Gläser, Braunschweig, Germany) and stained with haematoxylin and eosin (H&E) for visualisation. All slides were scanned using a Nanozoomer™ (Hamamatsu Photonics K.K., Hamamatsu City, Shizuoka Pref., Japan) at 40 X magnification and were analysed with Nanozoomer™ Digital Pathology View software (Hamamatsu Photonics K.K., Japan).

5.3.4.3 Gastrointestinal histopathological analysis

A total tissue injury score was generated based on the occurrence of eight histological criteria in the duodenum, jejunum and ileum and six criteria in the colon (14, 15). Two blinded operators scored the following parameters: villous fusion and villous atrophy (for duodenum, jejunum and ileum only), disruption of brush border, crypt loss, disruption of crypt cells, infiltration of neutrophils and lymphocytes, dilation of lymphatics or capillaries and oedema. Each parameter was scored as present = 1 or absent = 0.

5.3.5 Genomic DNA extraction from caecal and faecal samples

Caecal and faecal samples were removed from -80°C ultra-cold storage and thawed. For DNA extraction, a QIAmp® Fast DNA Tissue Kit (Qiagen, Hilden, Germany) was utilised in accordance to the manufacturer's protocol. Briefly, a master mix containing buffers and enzymes was placed in a tissue disruption tube tubes (Pathogen Lysis tubes, Qiagen, Hilden, Germany). A small tissue sample (5-25 mg) from each animal was added into each tissue disruption tube and processed. Caecum samples were processed for each animal whilst faeces samples were pooled from all animals in a group (to a total of 20-25 mg per tube). Samples were homogenised using the VortexGenie®2 (Scientific Industries Inc., Bohemia, New York, USA) and incubated for ten minutes at 56°C in a heating block, followed by centrifugation for 10 minutes at 10000 g. At this point, 5.3 mg lysozyme was added to each tube to improve the disruption of the Gram-positive bacterial cell wall and samples were incubated for one hour at 37°C, followed by buffer addition. A spin column was used to separate DNA from the rest of the solution. The DNA was used for PCR analysis (5.3.5.1 and 5.3.6) and 16S metagenomics sequencing (5.3.7).

5.3.5.1 Detection of Gram-positive bacteria using Polymerase Chain Reaction with Taq Polymerase

DNA extracted from faecal and caecum samples underwent testing for detection of Gram-positive bacteria (Firmicutes) before gene sequencing. To determine the

amount of DNA required for Polymerase Chain Reaction (PCR) detection, the concentration of genomic DNA was first analysed using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA) and then diluted with ATE buffer (Qiagen, Hilden, Germany) to a final concentration of 20ng/ml. Each PCR sample contained 1 µl of diluted DNA, 5 µl of 10X ThermoPol reaction buffer (New England Biolabs, Massachusetts, USA), 1µl of dNTPs, 0.5 µl of forward primer (Firm 934F, GeneWorks, Thebarton, SA, Australia), 0.5 µl of reverse primer (Firm 1060R, GeneWorks, Thebarton, SA, Australia), 1µl of Taq polymerase (New England Biolabs, Massachusetts, USA) and 41µl of sterile deionised water (Table 1).

Table 1: Primer Sequences for PCR Reactions; ^b = C or T

Primer Nomination	Primer Sequences (5' → 3')	Amplicon Length	Function
Firm 934F	GGAGYATGTGGTTTAATTCTGAAGCA ^b	126 bp	Detection of Firmicutes (Gram-positive)
Firm 1060	AGCTGACGACAACCATGCAC		

Table 2: Thermocycler Settings for PCR Reactions

Primer Pair	Initial Denaturation	Amplification cycles			Final Extension
		Denaturation Extension	Annealing		
934F/1060R (30 cycles)	3 min - 94°C	20 sec - 95°C	20 sec - 60°C	20 sec - 72°C	5 min - 72°C

PCR reactions took place in an automated thermocycler (Mastercycler Personal Eppendorf T100™ Thermal Cycler (BioRad, Hercules, CA, USA) with thermocycler settings as per Table 2. PCR products were visualised using a 1.5% agarose gel in

1x TAE buffer and 2µl Gel Red (Biotium, Fremont, CA, USA). Each 50 µl of PCR products were mixed with 5 µl of 6X Blue Gel Loading dye (New England Biolabs, Massachusetts, USA) prior to loading. 10 µl of the mix were loaded in each well and 10 µl of Quick-Load® 1kb DNA Ladder (New England Biolabs, Massachusetts, USA) were loaded into an adjacent line in order to estimate molecular size. PCR products were then visualised using trans-UV illumination by scanning with a ChemiDoc™MP System (BioRad, Hercules, CA, USA).

5.3.6 PCR Detection of *P. gingivalis* DNA in caecum and faecal samples

The PCR protocol for detection of *P. gingivalis* DNA in caecum and faecal samples was as described in 5.3.5.1 with thermocycler settings as per Table 3. PCR products were visualised using a 1.5% agarose gel in 1x TAE buffer and 2µl Gel Red (Biotium, Fremont, CA, USA). Each 50 µl of PCR products were mixed with 5 µl of 6X Blue Gel Loading dye (New England Biolabs, Massachusetts, USA) prior to loading. 10 µl of the mix were loaded in each well and 10 µl of Quick-Load® 1kb DNA Ladder (New England Biolabs, Massachusetts, USA) were loaded into an adjacent line in order to estimate molecular size. PCR products were then visualised using trans-UV illumination by scanning with a ChemiDoc™MP System (BioRad, Hercules, CA, USA).

Table 3: Thermocycler Settings for PCR Reactions

Primer Pair	Initial Denaturation	Amplification cycles			Final Extension
		Denaturation Extension	Annealing		
934F/1060R (30 cycles)	3 min - 94°C	20 sec - 95°C	20 sec - 60°C	20 sec - 72°C	5 min - 72°C

5.3.7 16S rRNA metagenomics gene sequencing

Genomic DNA from the mouse caecum content was extracted (see 5.3.5) and the concentration and quality was assessed using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). Aliquotes of 30 µl from each sample were placed into 0.6 ml sterile PCR tubes and sent to Flinders Genomics Facility (Flinders University, Adelaide, SA, Australia) where all samples underwent Illumina sequencing library preparation using Illumina MySeq System (Illumina, San Diego, CA, USA).

5.3.7.1 16S Library preparation

The library preparation used a two-step PCR protocol (Figure 3). Briefly, an initial PCR was performed using specific primers (Table 4) to amplify the variable V4 region of the 16S rRNA and attach sequence adapters. The amplified product was purified with AMPure XP Beads (Illumina, San Diego, CA, USA). Dual indices and Illumina sequencing adapters were added to the purified amplicons in the second

PCR stage using a Nextera XT Index Kit (Illumina, San Diego, CA, USA), followed by another clean-up process using AMPure XP Beads (Illumina, San Diego, CA, USA). Fluorometric quantification of the individual and mixed library was carried out using dyes that bind dsDNA. Normalisation was performed when the amplicon length and quality were tested using a 2001 Bioanalyzer (Illumina, San Diego, CA, USA). In preparation for sequencing, pooled libraries were diluted with a hybridisation buffer and heat denatured prior to MiSeq sequencing.

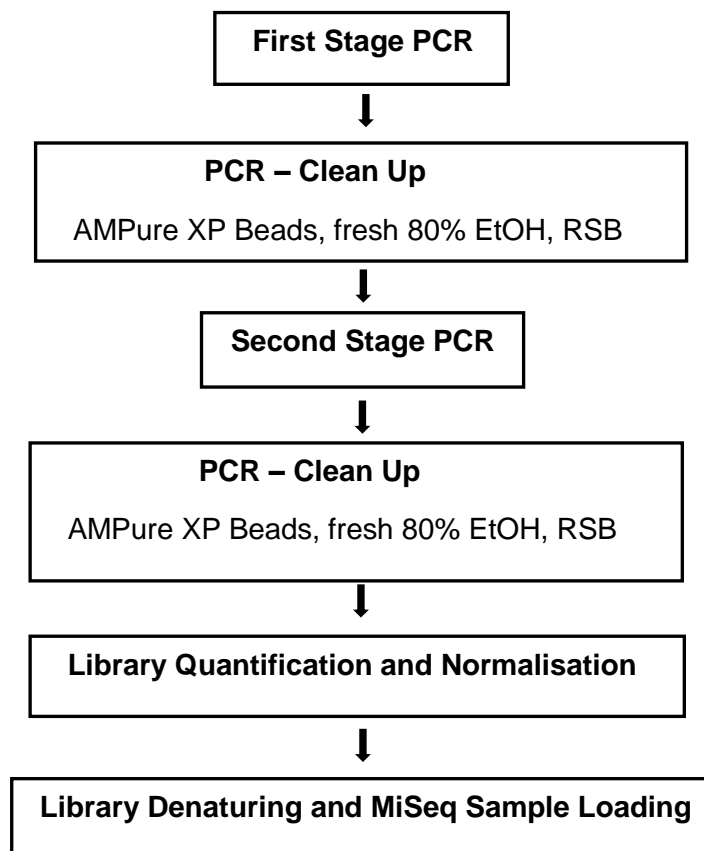


Figure 3: Steps in 16S Library Preparation for MiSeq Illumina Sequencing (MiSeq, Illumina, San Diego, CA, USA)

Table 4: Primer Sequences in MiSeq Library Preparation; Sequence adapters are underlined

Primer Nomination	Primer Sequences (5' → 3')
515F_Illumina	<u>TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG</u> GTGCCAGCMGCCGCGGTAA
806R_Illumina	<u>GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG</u> GGACTACHVGGGTWTCTAAT

5.3.7.2 Miseq sequencing

Miseq sequencing was performed by staff at Flinders Genomics Facility (Flinders University, Adelaide, SA, Australia). Samples were loaded in the MiSeq system (MiSeq, Illumina, San Diego, CA, USA) on flow cells and the sequenced using a 600 cycle Version 3 kit run as 2x300 paired-end reads. Briefly, the MiSeq system generated clusters of identical DNA strands via Illumina clonal bridge amplification that occurs simultaneously for all fragments on the flow cell. Sequencing of both forward and reverse strands was achieved by using the Next generation sequencing (NGS, Illumina, San Diego, CA, USA) method of paired-end sequencing-by-synthesis. Data generated underwent analysis (see 5.3.8)

5.3.8 Analysis of 16S metagenomics sequencing data

After sequencing, fastq files for the forward and reverse reads were created using the Illumina CASAVA pipeline (version 1.8.2). Overlapping forward and reverse reads were joined (based on a maximum of 5% nucleotide difference over a

minimum 5bp overlap) using BBmerge ([sourceforge.net/projects/ bbmap](https://sourceforge.net/projects/bbmap)). The resulting fastq file was imported into QIIME2 (QIIME 2 2017.12), a bioinformatics pipeline-based software for the analysis of metagenomic data (16). All further analysis of the amplicon datasets was conducted within the QIIME2 package. Sequences were trimmed at 250 bp (25th percentile with a Qscore over 35) and subsequently deblurred (17). The taxonomy of each sequence was identified using similarity to Greengenes database (version 13_8) with 99% similarity to full length 16S sequences (18). Taxonomic diversity measurements (alpha and beta-diversity) and statistical analyses were also performed and visualized in QIIME2. Core diversity analyses were completed by sub-sampling each sample to 9,500 sequences.

5.3.9 Immunohistochemistry of ileum samples

A previous study found significantly elevated expression of Il-6 in the small intestine in mice 48 hours after a single *P. gingivalis* oral administration (6). Subsequently, immunohistochemistry analysis using the avidin-biotin peroxidase method was carried out for pro inflammatory cytokine Interleukin 6 (IL-6) on formalin fixed, paraffin embedded sections of the ileum. Briefly, samples were dewaxed in histolene, rehydrated through graded ethanol and rinsed in phosphate buffered saline (PBS). Sections were blocked for 30 minutes with 3% normal horse serum. Subsequently they were incubated with the respective primary polyclonal goat antibody (1:2000, R&D Systems, Inc.) diluted in normal horse serum (Thermo Fisher Scientific, Waltham, MA, USA). This was followed by biotinylated secondary anti-

goat antibody (Vector Laboratories Inc., Burlingame, CA, USA) for 30 minutes. Streptavidin biotin complex was added onto the sections for one hour then developed with 20 mM 3,3'diaminobenzidine (DAB) (Sigma, St Louis, MO, USA). Slides were counter stained with hematoxylin (Thermo Fisher Scientific, Waltham, MA, USA), dehydrated and mounted. Il-6 stained tissue sections were scored by two independent, blinded operators based on the presence and intensity of staining in the epithelial cells, lamina propria and submucosa. Each section was scored as 0 for no staining, 1 for mild staining, 2 for moderate staining and 3 for intense staining based on the method as described by Warren *et al.* (19).

5.3.10 Enzyme-linked immunosorbent assay (ELISA) for concentration of C-reactive protein

Serum levels of murine C-reactive protein (CRP) was assessed as a surrogate measure of systemic inflammation. Serum was centrifuged at 13000 g for 10 minutes at room temperature before diluting 1:10,000. CRP levels were assessed in duplicate using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) and optical density (450nm) read on a PowerWave microplate reader (BioTek Instruments, Winooski, VT, USA). Standard curves were generated using KC4 software (BioTek Instruments, Winooski, VT, USA) and used to determine the concentration of mouse-CRP in each sample. All samples and standards were assessed in triplicate.

5.3.11 Statistics

The power of this study was 85% for a sample size of six per group and a significance level of 0.05. GraphPad Prism 6 (GraphPad Software Inc, La Jolla, California, USA) was used for statistical analysis of IL-6, mouse-CRP expression and histology. Differences between the six groups were analysed using the Kruskal–Wallis test, followed by Dunn’s multiple comparisons test. All values shown are mean \pm standard error of the mean (SEM). For metagenomics sequencing data analysis, statistical differences between groups were identified using a PERMANOVA pseudo F test for beta diversity, pairwise Kruskal-Wallis for alpha diversity, and analysis of composition of microbes (ANCOM) for detection of specific taxa associated with different treatments. A p-value of <0.05 was considered statistically significant.

5.4 Results

5.4.1 The effects of *P. gingivalis* and *F. nucleatum* administration

5.4.1.1 The effects of *P. gingivalis* and *F. nucleatum* administration on intestinal inflammation

There was a significant increase in the degree of inflammation present in all parts of the gastrointestinal tract of disease mice (PD) when compared with controls

(duodenum $p = 0.0143$, jejunum $p = 0.0009$, ileum $p = 0.0017$, colon $p = 0.0442$) (Figures 4, 5, 6, 7). This was characterised by an increased infiltration of macrophages, neutrophils, lymphocytes and plasma cells in the lamina propria (Figures 8 c and d, 9 b and c, 10 b, 11 b and d).

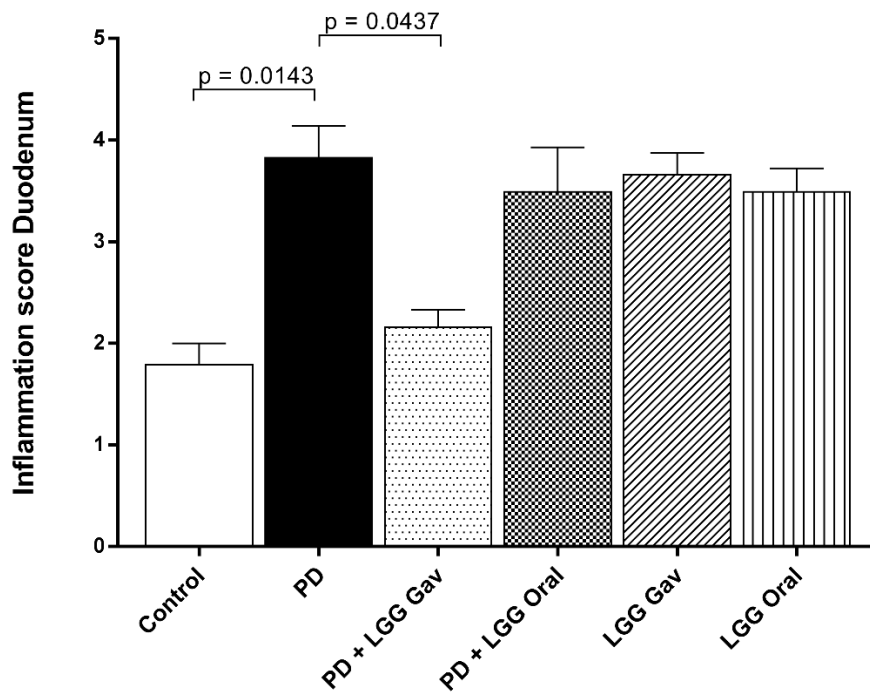


Figure 4: Disease mice (PD) presented significant inflammatory changes in the duodenum when compared with Control ($p = 0.0143$) or gavage treated group ($p = 0.0437$). Bars represent mean inflammatory score \pm SEM. PD = Periodontitis, Gav = Gavage

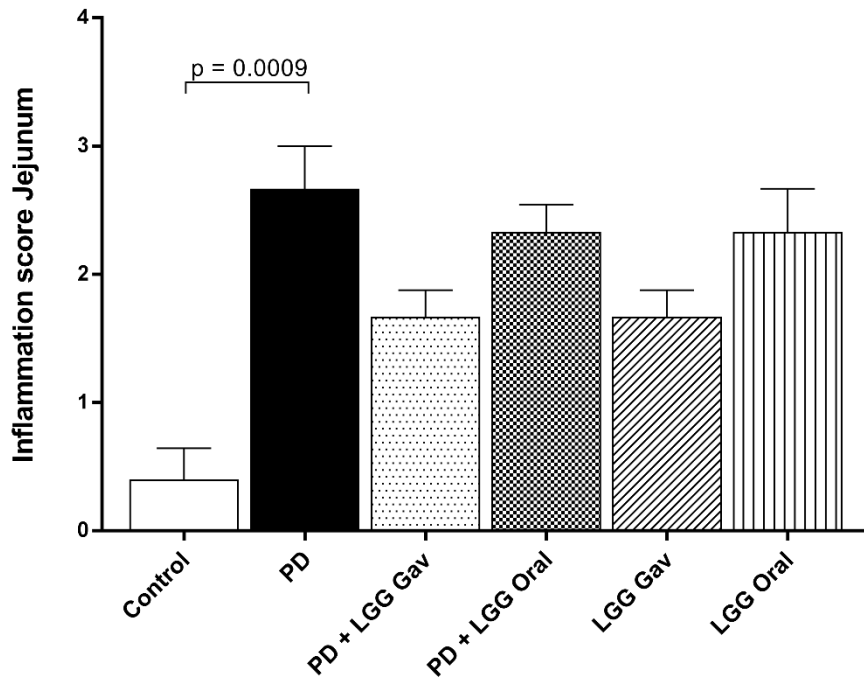


Figure 5: Disease mice (PD) presented significant inflammatory changes in the jejunum when compared with Control ($p = 0.0009$). Bars represent mean inflammatory score \pm SEM. PD Periodontitis, Gav = Gavage

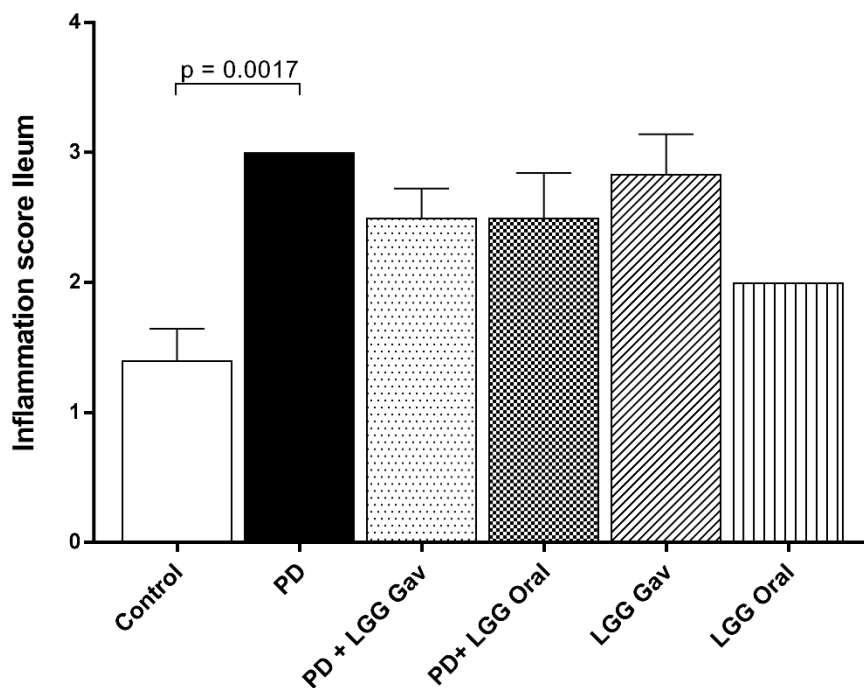


Figure 6: Disease mice (PD) presented significant inflammatory changes in the ileum when compared with Control ($p = 0.0017$). Bars represent mean inflammatory score \pm SEM. Abbreviations: PD = Periodontitis, Gav = Gavage

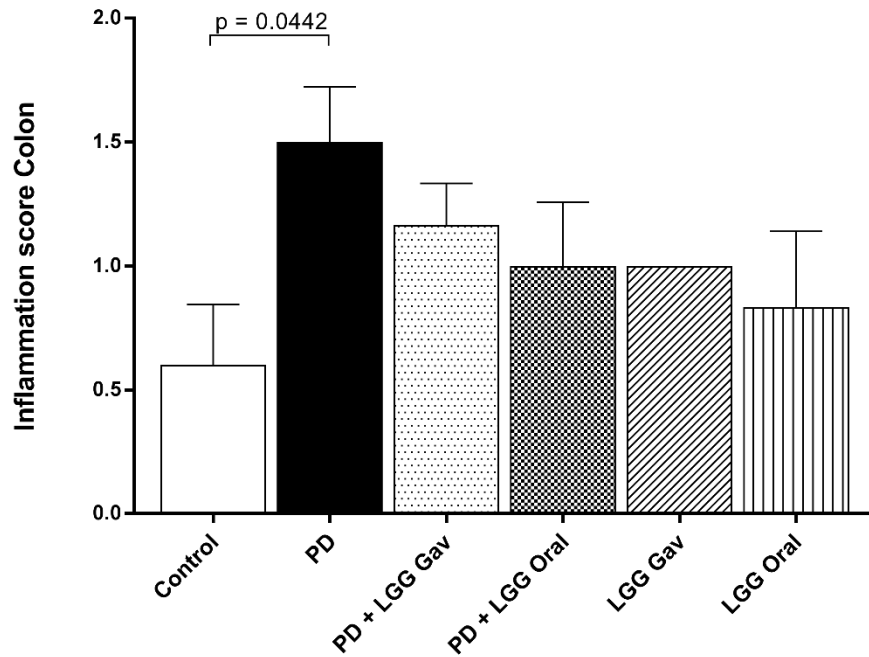


Figure 7: Disease mice (PD) presented significant inflammatory changes in the colon when compared with Control ($p = 0.0442$). Bars represent mean inflammatory score \pm SEM. Abbreviations: PD = Periodontitis, Gav = Gavage

5.4.1.2 *P. gingivalis* and *F. nucleatum* administration induced elevated IL-6 protein expression in the ileum

IL-6 expression was elevated in the ileum of the disease mice when compared with controls ($p = 0.052$) (Figures 12, 13).

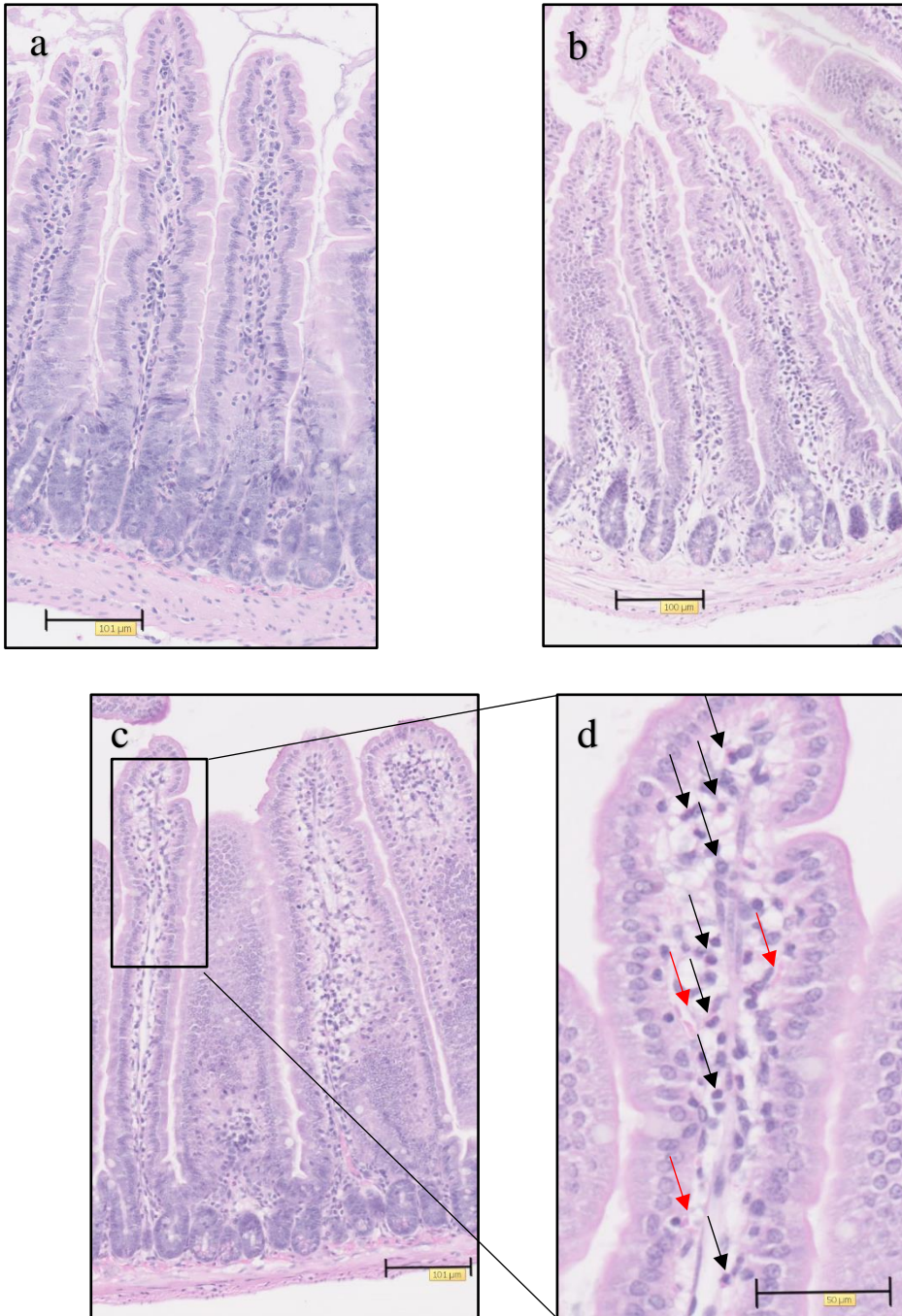


Figure 8: Representative images of standard haematoxylin and eosin staining of the duodenum in group: Control (a), PD (c, d) and PD + LGG Gav (b); PD group presented an increase in inflammatory infiltrate (polymorphonuclear cells and lymphocytes)(black arrows), and dilation of lymphatics and capillaries; (red arrows); scale bars represent 100 μm (a, b, c, d) and 50 μm (e); Abbreviations: PD = Periodontitis, Gav = Gavage

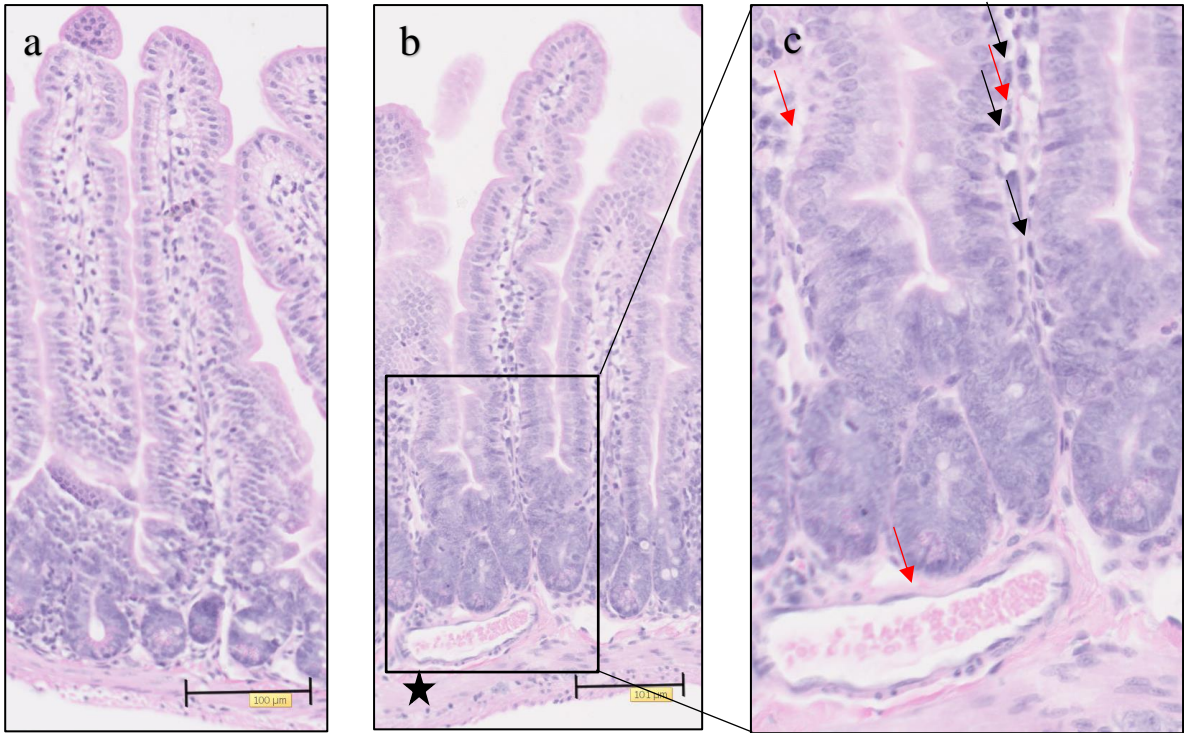


Figure 9: Representative images of standard haematoxylin and eosin staining of the jejunum in group: Control (a), PD (b,c); PD group presented an increase in inflammatory infiltrate of polymorphonuclear cells and lymphocytes (black arrows), dilation of lymphatics and capillaries (red arrows) and thickening of the mucosa (black star); scale bars represent 100 μm . Abbreviations: PD = Periodontitis, Gav = Gavage

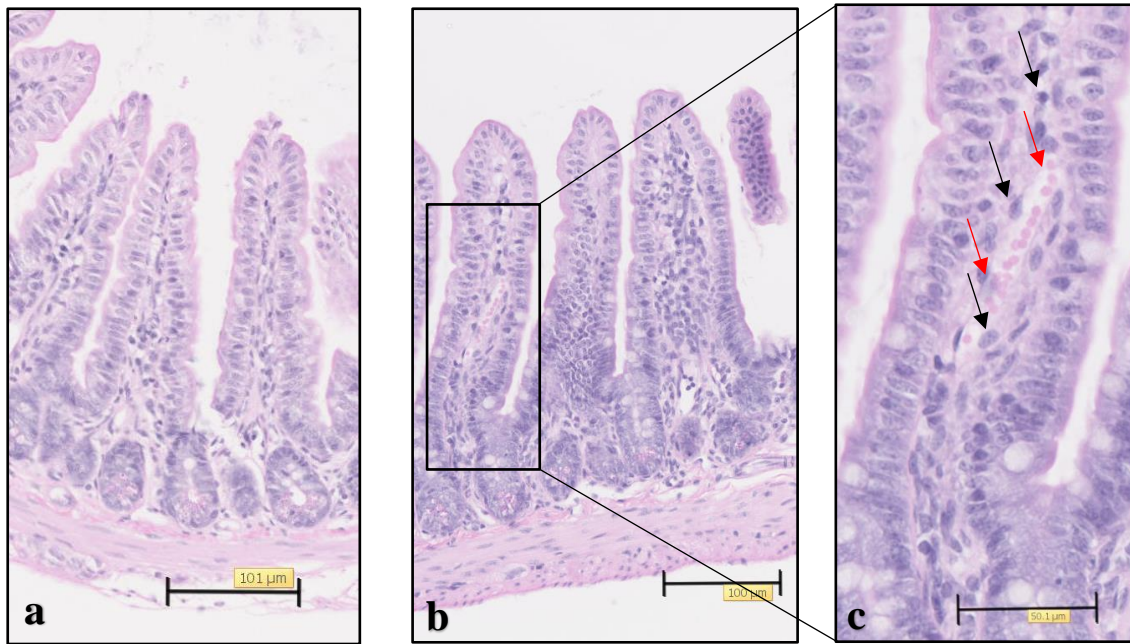


Figure 10: Representative images of standard haematoxylin and eosin staining of the ileum in group: Control (a), PD (b); PD group presented an increase in inflammatory infiltrate of polymorphonuclear cells and lymphocytes (black arrows) and dilation of lymphatics and capillaries (red arrows); scale bars represent 100 µm (a, b and 50 µm (c). Abbreviations: PD = Periodontitis, Gav = Gavage

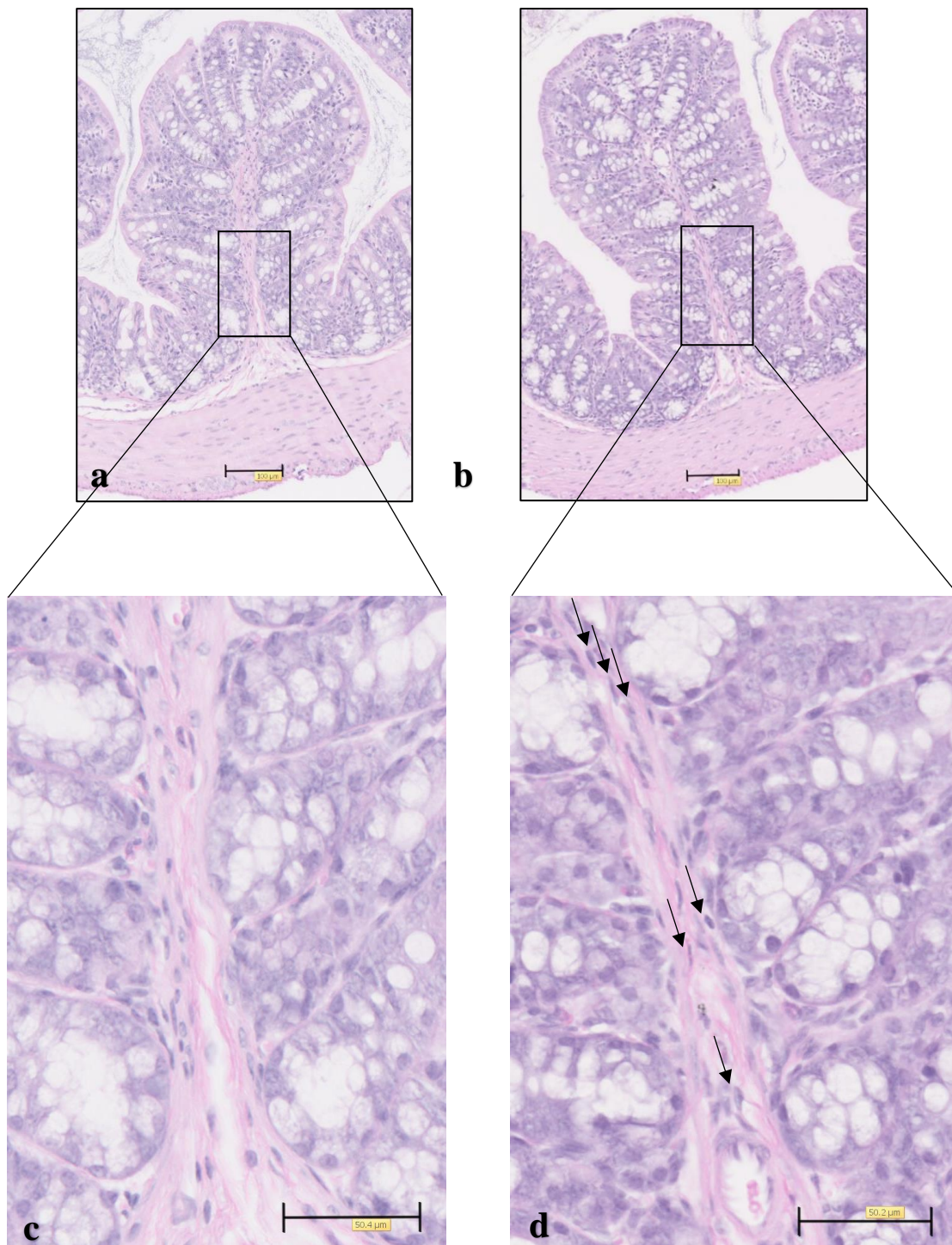


Figure 11: Representative images of standard haematoxylin and eosin staining of the colon in group: Control (a, c) and PD (b, d). PD group presented an increase in inflammatory infiltrate (polymorphonuclear cells and lymphocytes) (black arrows) (scale bars represent 100 µm (a, b) and 50 µm (c, d). Abbreviations: PD = Periodontitis, Gav = Gavage

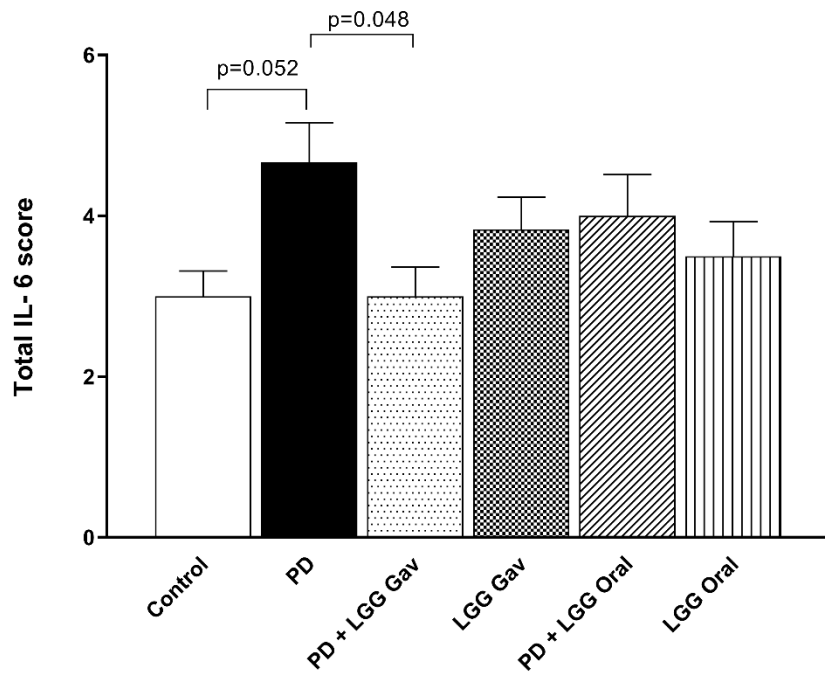


Figure 12: Total IL-6 immunohistochemistry ileum score; there was a significant difference LGG Gav + PD group when compared with PD ($p = 0.048$). Abbreviations: PD = Periodontitis, Gav = Gavage, IHC – Immunohistochemistry

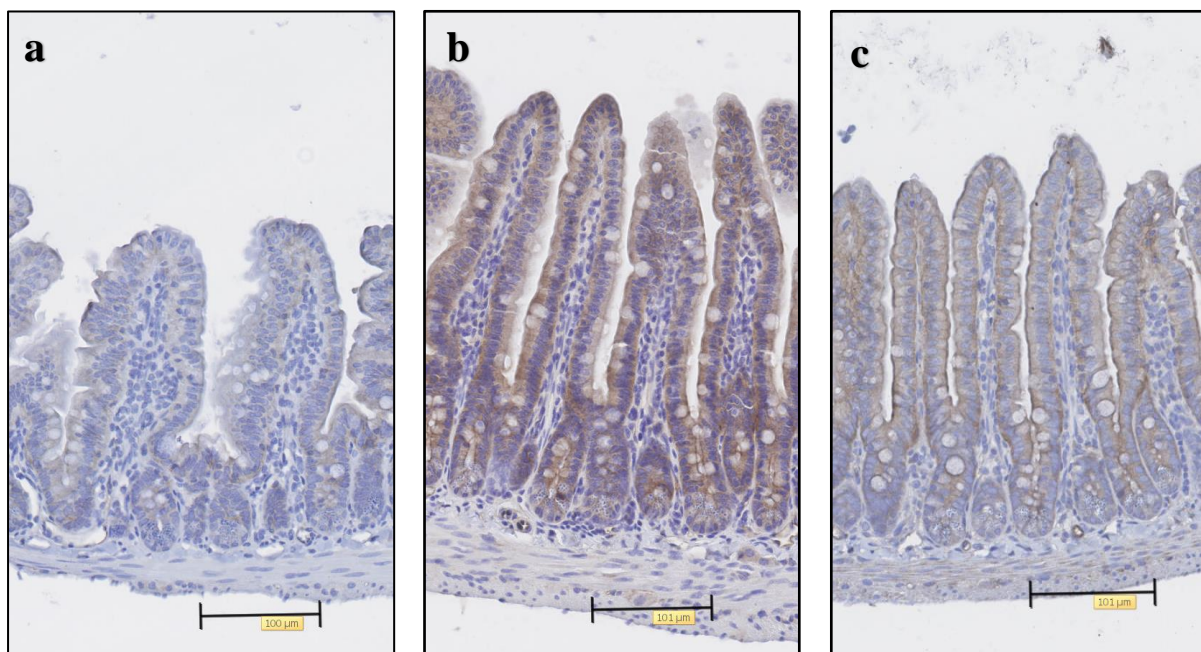


Figure 13: Representative images of IL-6 immunohistochemistry staining of the ileum. There was significant difference for Control (a) and PD + LGG Gav (c) groups when compared with PD (b). Scale bars represent 100 µm. Abbreviations: PD = Periodontitis, Gav = Gavage

5.4.1.3 *P. gingivalis* and *F. nucleatum* administration altered the gut microbiome

Mice inoculated with *P. gingivalis* and *F. nucleatum* presented with increased abundance and evenness of the caecal microbiome ($p = 0.068$, Figure 14) and a significant change in the microbial composition of the caecal microbiome ($p = 0.01$, Figure 15) when compared with controls. ANCOM analysis identified unclassified Clostridiales taxa belonging to the phylum Firmicutes present in the Control group and absent in disease mice.

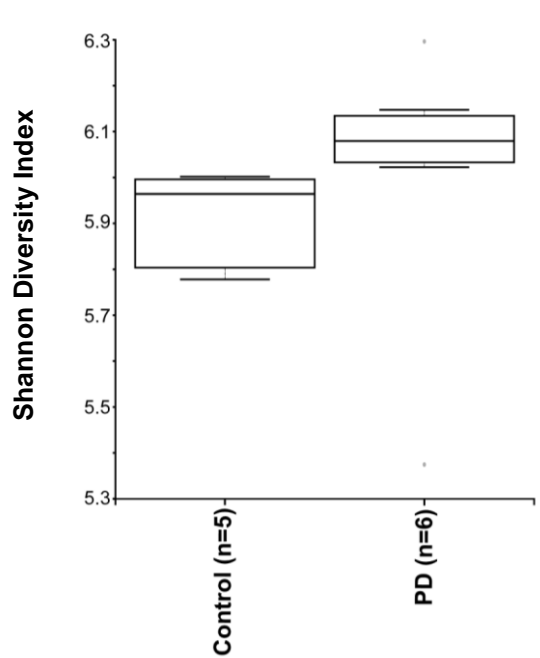


Figure 14: Shannon's diversity index (H) for Control and PD mice (9,500 sequences per sample for rarefaction, $H = 3.33$; $p = 0.068$). Abbreviations: PD = Periodontitis

5.4.1.4 *P. gingivalis* DNA was not detected in caecum and faecal samples

PCR testing was performed on a random selection of both caecal (n = 19) and faecal (n = 9) samples. *P. gingivalis* DNA was not detected in any of the samples (Figure 16). Some samples showed the presence of primer dimers. Metagenomics analysis of 16S DNA sequences of caecum samples and *P. gingivalis* culture identified *Porphyromonas* sp. present in the culture and absent in all caecum samples (Supplementary Figure 1, pg. 163). Sequencing for *F. nucleatum* provided a similar result with *Fusobacterium* sp. present in the *F. nucleatum* culture and absent in all caecum samples (Supplementary Figure 2, pg. 164).

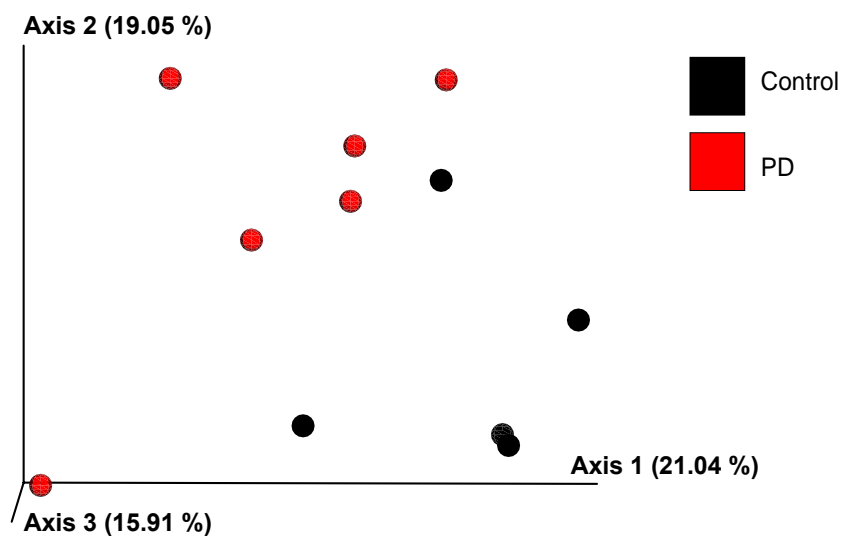


Figure 15: PCoA plot of beta diversity index showing statistically significant differences between Control and PD group (PERMANOVA pseudo F test statistic: 2.08592; $p = 0.01$). Abbreviations: PD = Periodontitis, Gav = Gavage

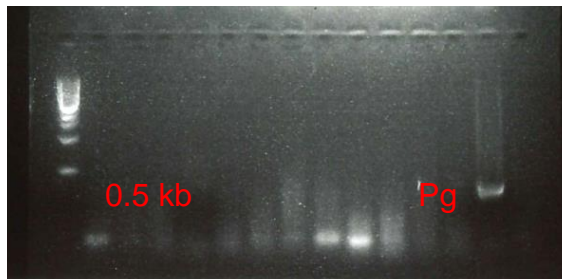
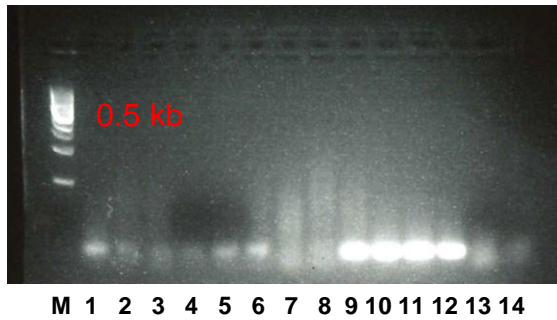


Figure 16: *P. gingivalis* DNA was not detected by PCR in any faeces or caecum samples. Lane M: molecular weight marker (100bp); lane 1-8: negative faecal samples, 9-27: negative cecum samples; lane 28: positive control of *P. gingivalis*; lane 29: negative control (water); Pg – *P. gingivalis*

5.4.2 The effects of LGG administration in mice

5.4.2.1 The method of LGG administration had no effect on intestinal inflammation

Mice treated exclusively with LGG, either via oral inoculation (LGG Oral) or via oral gavage (LGG Gav), presented no differences in inflammatory scores for the duodenum, jejunum, ileum or colon when compared with controls ($p > 0.05$) (Figures 4, 5, 6, 7). No change was seen in IL-6 expression in the ileum of treated animals when compared with controls. (Figure 12).

5.4.2.2 LGG administration altered the gut microbiome

Abundance of the caecal microbiome was increased in mice orally gavaged with LGG ($p = 0.068$) and significantly increased in mice orally inoculated with LGG ($p = 0.028$) when compared with control mice (Table 5, Figure 17). There was no significant difference in abundance and evenness between the two different delivery methods, oral inoculation and oral gavage (Alpha diversity Kruskal Wallis pairwise; $H = 0.0256$; $p = 0.8272$, Figure 17).

Administering LGG using either the gavage or oral inoculation method significantly changed the bacterial composition of the caecal microbiome when compared with controls (Control and LGG Oral, $p = 0.012$, Control and LGG gavage, $p = 0.009$, Figure 18). ANCOM analysis identified Lachnospiraceae family, belonging to the order Clostridiales, present in control mice and absent in the LGG Oral group. For the LGG Gav group, ANCOM identified Bacteroidales and unclassified bacteria belonging to the order Clostridiales, present in the treated mice and absent in controls.

Table 5: Shannon diversity index (H) between control and all other groups (Kruskal-Wallis pair-wise comparisons). Abbreviations: PD = Periodontitis, Gav = Gavage

Group	H value	p value
LGG Oral (n=6)	4.80	0.028
LGG Gav (n=6)	3.333	0.068
PD (n=6)	3.333	0.068
PD + LGG Oral	0.30	0.584
PD + LGG Gav	1.20	0.273

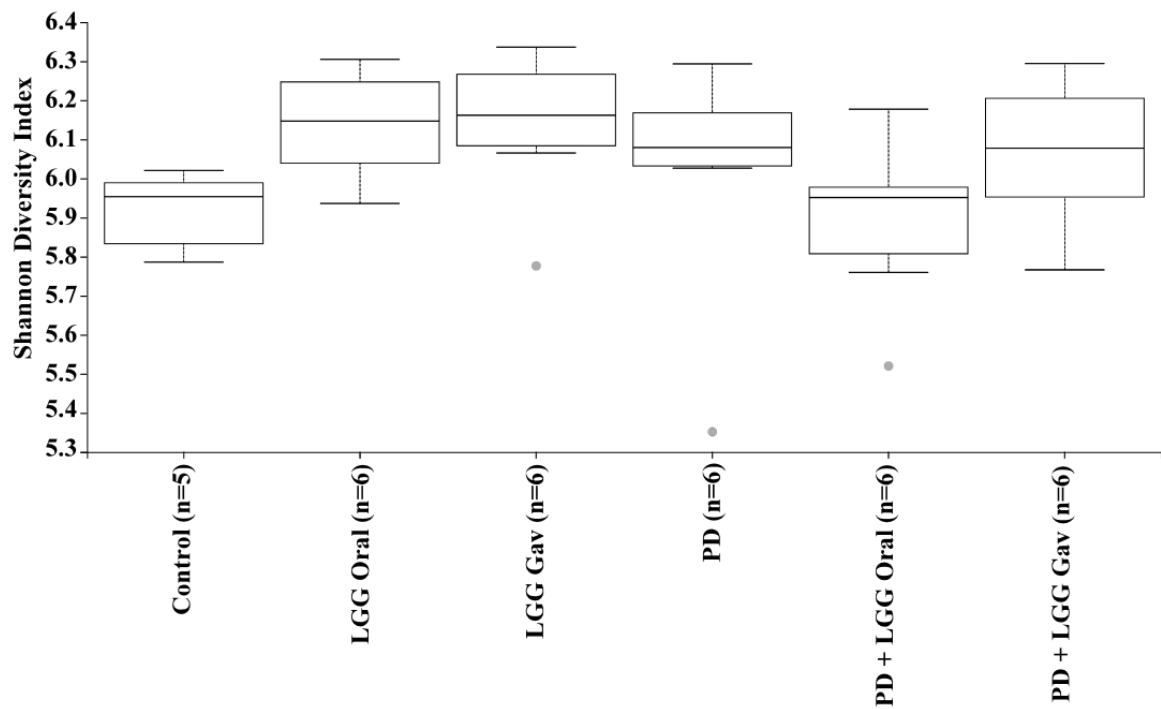


Figure 17: Shannon's diversity index for all groups (9,500 sequences per sample for rarefaction). Abbreviations: PD = Periodontitis, Gav = Gavage

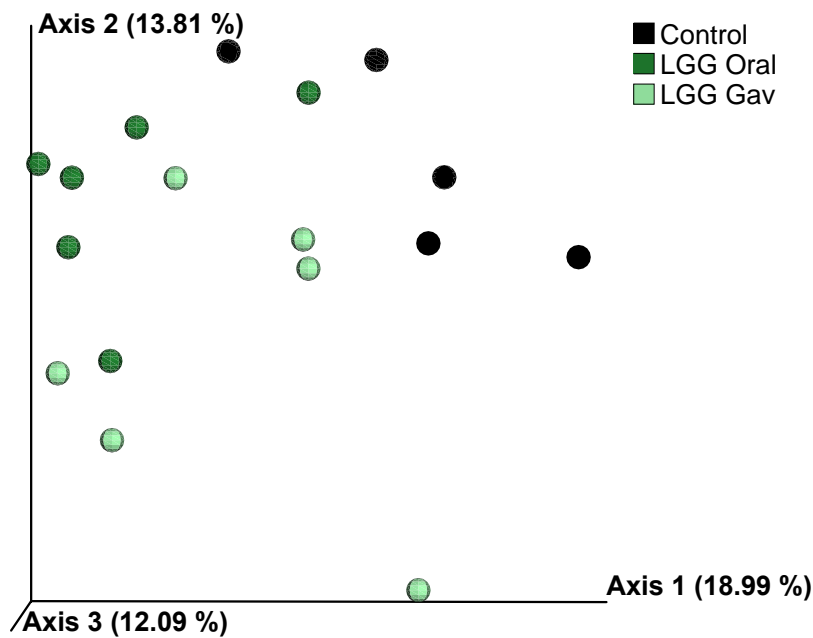


Figure 18: PCoA plot of beta diversity index shows statistically significant differences between Control and LGG Oral (PERMANOVA pseudo-F; test stat 1.8719; $p = 0.012$) and Control and LGG Gav (PERMANOVA pseudo-F; test stat 2.4596; $p = 0.009$) groups. Abbreviations: PD = Periodontitis, Gav = Gavage

5.4.3 The effects of LGG treatment prior to *P. gingivalis* and *F. nucleatum* administration

5.4.3.1 Prior use of LGG prevents intestinal inflammatory changes induced by *P. gingivalis* and *F. nucleatum*

Mice treated with LGG via oral gavage (PD + LGG Gav) presented significantly lower inflammatory scores in the duodenum than disease mice (PD) ($p = 0.0437$) (Figures 4 and 8c). No significant inflammatory differences in the jejunum, ileum or colon were detected for any of the treatment groups PD + LGG Gav and PD + LGG Oral when compared with disease (Figures 5, 6 and 7). Expression of IL-6 in the ileum was significantly decreased in animals treated with LGG via gavage (PD + LGG Gav) when compared with disease ($p < 0.05$) (Figures 12 and 13c).

5.4.3.2 Treatment with LGG prevents gut microbiota changes associated with *P. gingivalis* and *F. nucleatum* inoculation

Metagenomics analysis found no significant differences in the abundance of the caecal microbiome between treatment groups PD + LGG Oral and PD + Gav LGG and Control ($p > 0.05$, Table 5, Figure 17). The use of LGG prior to *P. gingivalis* and *F. nucleatum* inoculation did not change the abundance and evenness of the caecal microbiome of treated mice when compared with disease (PD and PD + LGG Oral, $H = 1.64$; $p = 0.22$; PD and PD + LGG Gav, $H = 1.0$; $p = 1$, Figure 17). Bacterial composition of mice caecal microbiota was significantly altered in LGG treated mice

when compared with disease, with orally administered LGG having a greater influence than the gavage treatment (PD and PD + LGG Oral, $p = 0.015$ (PERMNOVA; pseudo $F = 2.08$), PD and PD + LGG Gav $p = 0.024$ (PERMNOVA; pseudo $F = 1.82$, Figure 19)). ANCOM analysis identified Lachnospiraceae family, belonging to the order Clostridiales, present in PD + LGG Gav mice and absent in PD. When comparing PD + LGG Oral with PD, ANCOM analysis identified Cyanobacteria present in PD and absent in PD + LGG Oral, and presence and absence of separate sequences of Clostridiales taxa between the two groups.

5.4.4 Firmicutes DNA detected in caecal and faecal samples

PCR testing detected presence of Gram positive bacterial species (Firmicutes) in caecal and faecal samples (Figure 20). All faecal and caecum samples were subsequently micro-sequenced.

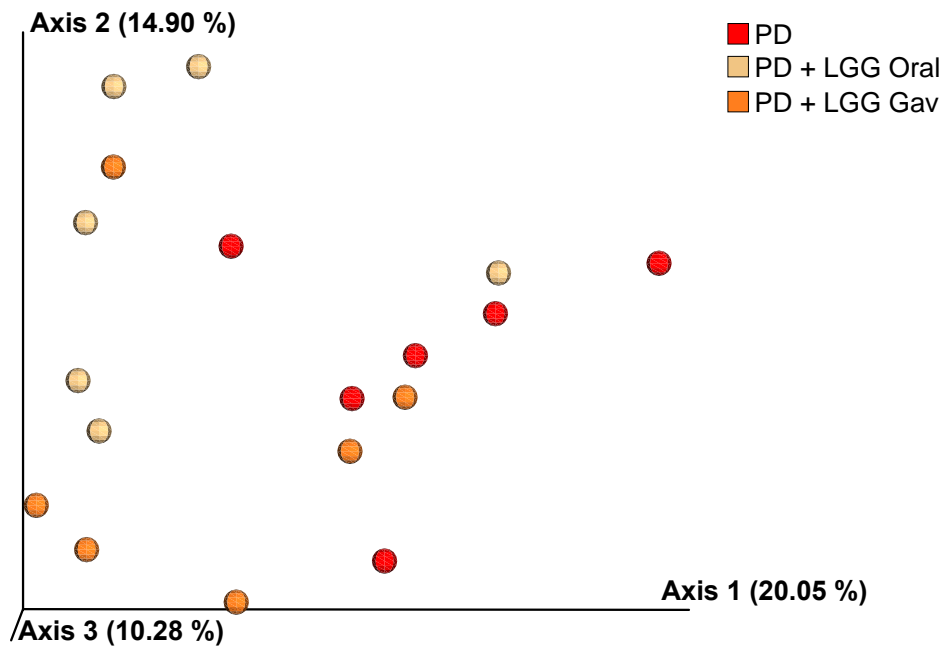


Figure 19: PCoA plot of beta diversity index mice shows statistically significant differences between PD and PD + LGG Oral (PERMANOVA pseudo-F; test stat 2.08; $p = 0.015$) and PD and PD + LGG Gav (PERMANOVA pseudo-F; test stat 1.82; $p = 0.024$) groups.

Abbreviations: PD = Periodontitis, Gav = Gavage



Figure 20: Gram positive bacterial (Firmicutes) DNA was detected by PCR in a random selection of intestinal, caecum and faecal samples. Lane M: molecular weight marker (100bp); lane 1, 2, 5-7 negative intestinal samples, lane 10 positive control of Firmicutes; lane 3-4 positive faecal samples, 8, 9, 11-14 positive caecal samples, lane 15 negative control (water)

5.4.5 Effects of LGG treatment on mouse C-reactive protein concentration in serum

No significant changes were seen in mouse C-reactive protein levels in mouse serum between groups (Figure 21).

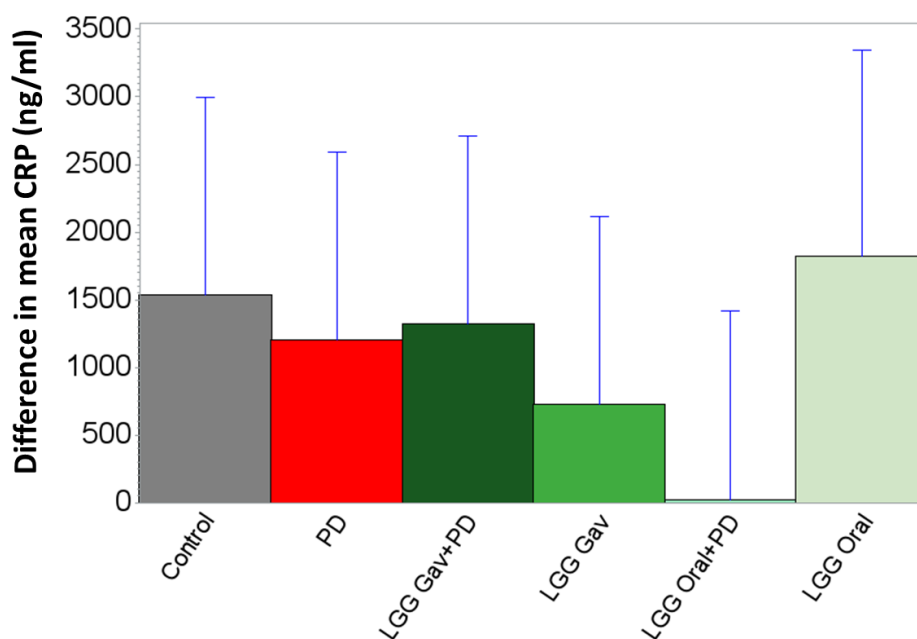


Figure 21: Difference in mean CRP; there was no significant difference in mean CRP for any groups. Abbreviations: PD = Periodontitis, Gav = Gavage, CRP – C-reactive protein

5.5 Discussion

The gut microbiome plays an important role in health and disease (20). An alteration of the composition of the gut microbiome has been associated with gastrointestinal

conditions such as inflammatory bowel disease and irritable bowel syndrome (21) and also with systemic conditions including type 2 diabetes (22) and obesity (23). Current research suggests the gut microbiome may also play an important role in regulating bone health. However, the mechanisms of the interactions between gut inflammation and bone loss are yet to be determined (24).

An important finding of this study is the systemic changes induced in mice by *P. gingivalis* and *F. nucleatum* inoculation. We have previously reported significant mean alveolar bone loss, increased presence of osteoclastic (TRAP) cells and inflammatory infiltrates in the PD group for this model (13, 25). In the current study, PD mice had significant inflammatory changes in the gastrointestinal tract, represented by increased numbers of inflammatory cells in the jejunum, ileum, duodenum and colon. Messori *et al.* (2013) first reported changes in the gastrointestinal structure of animals with induced bone loss in a 44 days study (26). Eight rats with ligature-induced periodontitis presented significant alteration of the intestinal morphology with significantly different mean values in the jejunum villous height and crypt depth (26). The authors suggested these changes were due to an increase in pathogenic bacterial counts in the gastrointestinal tract contributing to increased inflammation (26). In addition, in the present study, mice inoculated with *P. gingivalis* and *F. nucleatum* had a significant increase in the expression of the pro-inflammatory cytokine IL-6 in the ileum when compared with controls. This is in agreement with a previous study that found significantly elevated expression of IL6 in the small intestine in mice 48 hours after a single *P. gingivalis* oral administration (7). IL-6 is an important mediator secreted by T cells and macrophages (27). Although identified in the acute phase response of the inflammatory process or infection, persistent IL-6 production can lead to the development of immune-mediated

diseases including diabetes (28) and rheumatoid arthritis (29). IL-6 regulates T cell differentiation, activation and resistance against apoptosis with roles in maintaining chronic intestinal inflammation in inflammatory bowel diseases such as Crohn's disease and ulcerative colitis thus being considered a 'master regulator of intestinal disease' (30). Studies using *in vitro* and *in vivo* models identified IL-6 as a potential new target for the therapy of gastrointestinal inflammation (31). In periodontitis, IL-6 is a mediator of bone resorption, stimulating osteoclasts formation and thus being associated with the pathogenesis of this condition (32).

Furthermore, in the current study, oral inoculation with *P. gingivalis* and *F. nucleatum* led to a significant change in the bacterial composition of the caecum microbiome. Unclassified Clostridiales taxa belonging to the phylum Firmicutes were present in the caecum of control mice but were absent in mice administered with *P. gingivalis* and *F. nucleatum*. There has been growing evidence in recent years regarding the ability of these two bacteria in altering the microbial balance towards dysbiosis and systemic inflammation. Oral administration of *P. gingivalis* (10^{10} CFU/ml) twice a week for five weeks in mice resulted in altered ratio between Bacteroidetes and Firmicutes in the ileal microbiome with significant increase in the order Bacteroidales (6). These changes coincided with increases in IL-6 serum levels and insulin resistance, which further lead to inflammatory changes in adipose tissue and liver (6). Findings from the current study related to the absence of Clostridiales bacteria from the caecum of disease mice are consistent with a previous mouse study demonstrating that a single oral administration of 10^9 CFU/ml of *P. gingivalis* (strain W83) significantly decreased the proportion of Clostridiales in the gut of *P. gingivalis* infected mice when compared with controls (7). Reduction in the abundance of

intestinal Clostridiales bacteria has been previously associated with development of *Clostridium difficile* infections (33) and increased risk of colorectal cancer (34, 35).

Nakajima *et al.* (2015) also demonstrated that mice infected with *P. gingivalis* presented with higher quantities of bacterial DNA in their liver (7). Additionally, intestinal gene expression of proteins involved in intestinal permeability, such as *Tjp1* and *Ocln*, were down-regulated in the small intestine of infected mice (7). Gut microbiota changes, induced by oral administration of *P. gingivalis* and represented by an increased proportion of the phylum Bacteroidetes and decreases proportion of the phylum Firmicutes, preceded systemic inflammatory changes and could provide a mechanistic link in the associations between periodontitis and systemic disease (7). In the current study, bacterial DNA from *P. gingivalis* or *F. nucleatum* was not detected in caecum or faecal samples, suggesting these bacteria did not colonise the gastrointestinal tract. This finding suggests that changes induced by inoculation with these bacteria may be attributed to the ability of *P. gingivalis* and *F. nucleatum* to alter the composition and structure of the gut microbiome which may have significant impact on systemic inflammation (36).

Dysbiosis of the gut microbiome has been previously associated with changes in epithelial barrier function with increases in intestinal permeability regulated by tight junction proteins such as occludin and claudins (37). Arimatsu *et al.* (2014) reported downregulated expression of tight junction proteins in the ileum of mice orally inoculated with *P. gingivalis* (10^9 CFU/ml) twice per week for five weeks (6). The results of the current investigation seem to be based on mechanisms involving two factors. The first factor involves loosening of tight-junctions of the epithelial barrier by inflammatory cytokine IL-6 produced by T cells which may allow more antigens to cross the barrier and result in inflammation in the gut. The second and a more critical

role is played by the disruption of the gut microbiota that can influence the systemic production of pro-inflammatory cytokines and may be the cause for a disrupted epithelial barrier function.

Mice treated exclusively with LGG presented no significant differences in gut inflammation when compared with controls. This is in agreement with previous studies which have shown probiotics are able to maintain a healthy lining of the gastrointestinal tract (38). A key finding of the current study refers to mice pre-treated with LGG and subsequently inoculated with *P. gingivalis* and *F. nucleatum*. We have previously demonstrated that pre-treatment with LGG in a mouse model of experimental periodontitis significantly reduced bone loss ($p < 0.0001$) and gingival inflammation ($p < 0.0001$) for the treated groups when compared with the disease group (13). These results were seen irrespective of the mode of administration (oral gavage or oral inoculation) suggesting LGG colonisation in the mouth was not a prerequisite for the inhibition of bone loss. In the current study, treatment with LGG administered via oral gavage prior to and during inoculation with *P. gingivalis* and *F. nucleatum* demonstrated a significant protective effect on the ileum, preventing inflammatory changes induced by the two pathogenic bacteria such as increased inflammatory infiltrate in the lamina propria. Previous animal studies have shown that oral administration of *Bacillus subtilis* (1.5×10^8 CFU/ml) to Wistar rats significantly protected the small intestine from changes induced by ligature-induced periodontitis, probiotic treated rats presenting lesser or no defects of the villi, basal lamina degeneration and infiltration of neutrophils in the jejunum when compared with disease (39). In the current study, the underlying mechanism for these changes may be attributed to the ability of LGG in altering the composition of the gut microbiome. It has been previously shown that LGG improves markers of intestinal barrier function

occludin and claudin-1, and promotes beneficial bacteria Bacteroidetes and Firmicutes in the intestine of C57BL/J6 mice (40). It is clear that the immune mechanisms of probiotic action leading to prevention of gut inflammation and dysbiosis are complex (41) and future investigations are needed to elucidate the effects of probiotics in this model.

A limitation of the current study was the choice of mouse CRP as a marker of systemic inflammation. Previous human cross-sectional studies reported evidence of elevated plasma CRP levels in periodontitis when compared with controls (Paraskevas *et al.*, 2008). In contrast, a clinical study of 246 patients investigating inflammatory markers for acute pancreatitis found serum amyloid A to be a better predictor of inflammation severity than CRP (Mayer *et al.*, 2002). We have previously showed that, in the mouse model used in the present study, mice in the PD group presented with significantly higher systemic inflammation, represented by elevated serum levels of LIX, a mouse homologue of IL-8, when compared with controls (13). Future studies are needed to compare prognostic accuracy of mouse CRP with that of serum amyloid A as inflammatory markers in experimentally induced periodontitis.

In conclusion, my findings indicate that oral administration of *P. gingivalis* and *F. nucleatum* in this mouse model induced intestinal inflammation together with changes in the structure and composition of the gut microbiome. Pre-treatment with LGG via oral gavage prior to and during *P. gingivalis* and *F. nucleatum* inoculations significantly reduced intestinal inflammation for the probiotic treated groups compared with disease. Additionally, pre-treatment with LGG restored the composition of the caecal microbiome, irrespective of the mode of administration. Further studies are required to provide more insights into the mechanisms driving these observed changes.

5.6 References

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CHAPTER 6

General conclusion, clinical
significance and future directions

Chapter 6 General conclusion, clinical significance and future directions

The primary focus of the work described in this thesis was to gain an understanding of the clinical and microbiological effects of probiotics in experimentally induced periodontitis.

An initial review of the literature exploring the use of probiotics in prevention or treatment of periodontitis supported the notion that there was a place for probiotics in the treatment of periodontitis and that future independent studies were needed to investigate specific probiotic strains, delivery methods, treatment schedules and mechanisms of action. An *in vitro* experiment investigated the antimicrobial ability of *Lactobacillus rhamnosus* GG (LGG) against *Porphyromonas gingivalis* (*P. gingivalis*) and *Fusobacterium nucleatum* (*F. nucleatum*) and the results suggested that there was potential for the use of LGG in experimental periodontitis studies. Subsequently, the effect of LGG on bone loss and local and systemic inflammation was investigated in an *in vivo* mouse model of experimental periodontitis. Additionally, the impact of *P. gingivalis* and *F. nucleatum* inoculation on intestinal inflammation and the structure and diversity of the intestinal microbiome was investigated in this model together with the role of the probiotic LGG in altering these changes.

6.1 General conclusion

Repeated oral administration of *P. gingivalis* and *F. nucleatum* in the mouse model of experimental periodontitis induced significant mean alveolar bone loss and increased

presence of bone resorbing cells and inflammatory infiltrates in the jaw, results that are consistent with previous studies.

New findings arising from this project are administration of *P gingivalis* and *F nucleatum* also induced significant changes in intestinal and systemic inflammation and significant changes in the phylogenetic structure and composition of the gut microbiome. A possible explanation for this is that a disruption of the gut microbiota can influence the production of pro-inflammatory cytokines, which may be the cause for a disrupted epithelial barrier function that allows antigens to cross the barrier, and result in inflammation in the gut. More importantly, the results from the studies described in this thesis show for the first time that therapy with the probiotic LGG either administered via oral inoculation or via oral gavage effectively suppressed alveolar bone loss and local inflammation for all probiotic treated groups when compared with disease. Additionally, pre-treatment with LGG exerted a protective effect against intestinal and systemic inflammation and had a significant influence on the composition of the gut microbiome, promoting beneficial bacteria in the intestines of treated mice. The underlying mechanisms for these changes may be attributed to the ability of LGG in altering the composition of the gut microbiome. The mechanisms of probiotic action leading to prevention of gut inflammation and dysbiosis are complex and future investigations are needed to elucidate the effects of probiotics in this model.

6.2 Clinical significance and future directions

The results from the animal studies included in this thesis support the notion that there is a place for probiotics in the treatment of periodontitis. The approach

presented in this thesis was a preventive approach. Further investigations will look at treating the disease after it has already occurred and look into the specific mechanisms of actions driving the changes.

In the future, LGG may prove to be a good candidate for human probiotic-periodontitis studies and may offer a low risk, inexpensive, easy to use prevention or treatment option for adjunctive use in the management of periodontitis.

CHAPTER 7

Appendices

Supplementary Figure 1: 16S DNA sequence identified a single specific *Porphyromonas* species in *P. gingivalis* (strain W50) culture and not in caecal samples

