Libraries and Learning Services

University of Auckland Research Repository, ResearchSpace

Copyright Statement

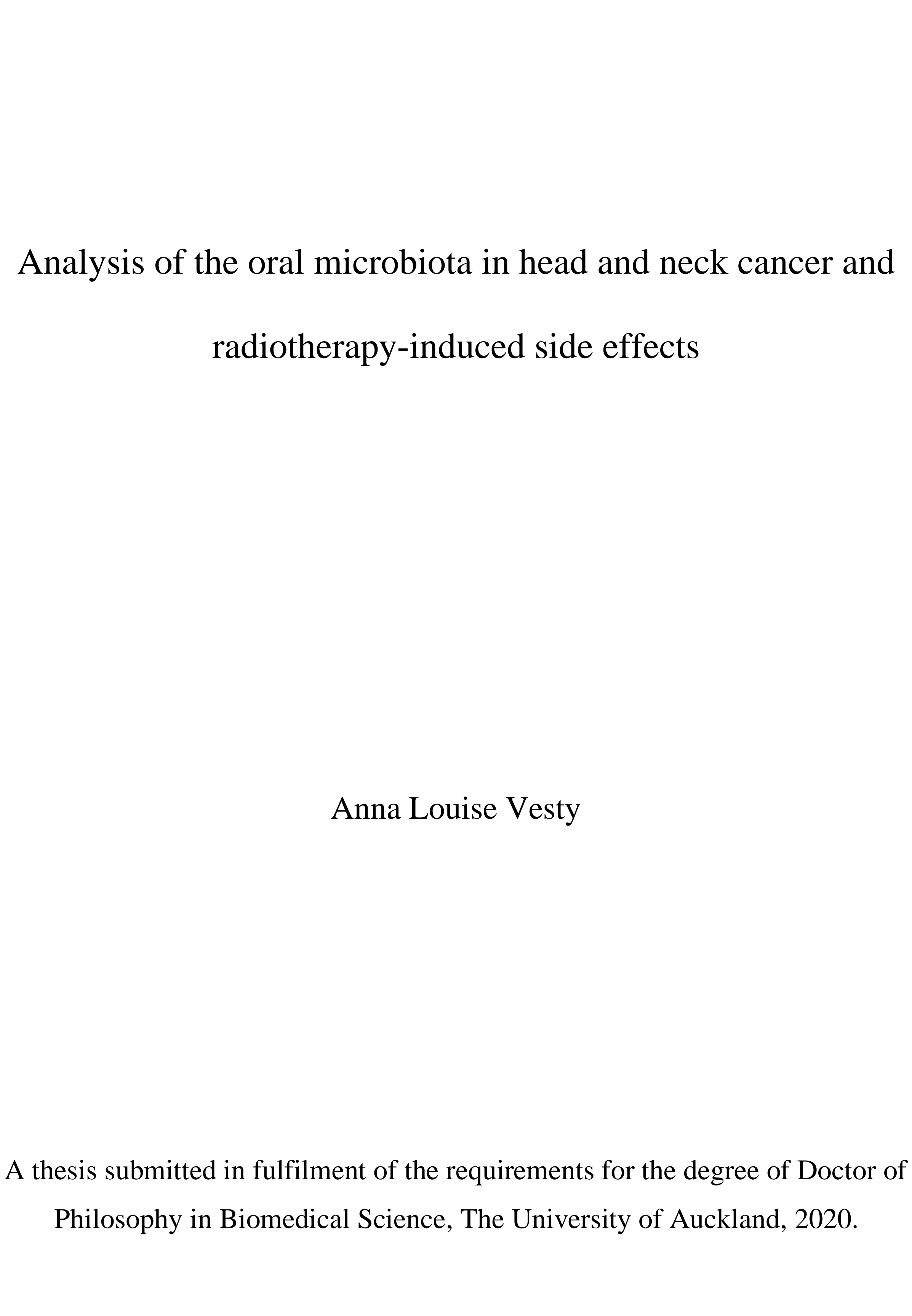
The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognize the author's right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from their thesis.

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the Library Thesis Consent Form and Deposit Licence.



Analysis of the oral microbiota in head and neck cancer and radiotherapy-induced side effects

Anna Louise Vesty

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Biomedical Science, The University of Auckland, 2020.

Abstract

Head and neck squamous cell carcinoma is an aggressive cancer that requires radiotherapy as part of its treatment approach. Carcinogenesis is traditionally linked to tobacco use and the human papilloma virus, though recent evidence associates variations in the oral microbiota with this group of cancers. Oral microbes also influence how well radiotherapy is tolerated and post-radiotherapy oral health. This thesis examines the oral microbiota as a biomarker for head and neck cancer and seeks to determine the role played by the microbiota in the development of acute and chronic oral health side effects of radiotherapy.

Comparison of genomic DNA extraction methods revealed the capability to simultaneously study the bacterial and fungal communities that exist in dental plaque and saliva, an approach vital to our understanding of the oral microbiota in head and neck cancer and its treatment. The optimal DNA extraction method was applied to saliva samples in order to evaluate microbial biomarkers of head and neck cancer. These data, along with inflammatory cytokine concentrations, revealed that the microbial and inflammatory profiles of head and neck cancer patients are more reflective of patients with compromised oral health than healthy controls. During radiotherapy, oral mucositis, an acute side effect characterised by ulceration and inflammation of the oral mucosa, was positively correlated with the abundance of anaerobic Gram-negative bacilli, including those implicated in periodontal disease. Post-radiotherapy, a four-week intervention with oral probiotic *Streptococcus salivarius* M18 had minimal impact on the composition and diversity of the oral microbiota and post-radiotherapy oral health.

By applying molecular-based approaches to study the oral microbiota pre-, during and post-radiotherapy, this research presents a theme that links periopathogenic oral microbes to

negative clinical outcomes, thereby establishing the presence of a microbial-based risk factor for head and neck cancer and its treatment.

Acknowledgements

First and foremost, thank you to all the patients who participated in the studies presented in this thesis. Without you, this research would not be possible.

Thank you Kristi, Mike T and Richard for your commitment, encouragement, guidance and support. I could not have asked for a more talented or nicer team of supervisors and I am incredibly lucky to have had the opportunity to work with you all. It was a tough road but we made it in the end.

Kim, I am so grateful to have had you on this journey with me – none of this would be possible without you. Thank you for sticking by me and for being so kind and supportive. Your commitment to the well-being of your patients will continue to inspire me.

Thank you Sharon Boutell for your involvement in Chapter 5. Your rapport with patients is so admirable and you have been such a wonderful and positive person to work with.

A huge thank you to the amazing team of scientists I have been fortunate enough to work with or alongside over the years at the School of Biological Sciences in the Taylor and Lear lab groups, especially Anna Lau, Brett, Carmen, Danielle, Elahe, Giselle, Jieyun, Mike H and Syrie. We've had some good times together!

At the Grafton campus, I'm lucky to have worked with Fiona and Sharon Waldvogel-Thurlow and alongside Christian (literally), plus many other students in the Douglas research group who have been and gone over the years.

A special thank you to Brett and Syrie – coffee/wine/gin club has been the best chapter of my thesis by far and one that will not be finished any time soon! Thank you for believing in me and for being constant sources of inspiration.

Lisa, you never cease to amaze me and I am so grateful to have you as a friend, particularly one who understands the PhD journey so well (too well, perhaps). Thank you to any friend who has heard more about my PhD than necessary, especially Liz and Renee.

To my colleagues at LabPlus, particularly the night duty and molecular teams – thank you for understanding my ongoing commitment and for your interest and support.

Mum, Dad, Michael, Anna, Clare and Bede – thank you for supporting my decision to pursue this goal and for your encouragement. George, Hazel and Florence, thank you for bringing so much happiness into my life – news of your adventures brightened the many weekends spent working on this thesis.

Thank you all for your support over the years, I appreciated it so much.

Table of Contents

Abstract......II

Acknowledgements IV

Chapter 1: General Introduction 1

1.1 The oral microbiota......2

1.1.1 Investigating the oral microbiota: culture and molecular approaches 2

1.1.2 Defining healthy oral microbiota......3

1.2 Periodontal disease......4

1.2.1 Periodontal disease and microbial dysbiosis......4

1.2.2 Saliva as a proxy for oral health 5

1.2.3 Bacterial complexes in plaque biofilms......6

1.2.4 Pathogenesis of microbial-induced periodontal inflammation 9

1.3 The oral mycobiota 10

1.3.1 Defining the oral mycobiota 11

1.3.2 The oral mycobiota and oral disease......11

1.4 Head and neck cancer 13

1.4.1 Risk factors 13

1.4.2 Side effects of radiotherapy treatment 14

1.5 Radiotherapy-induced oral mucositis 16

1.5.1 Risk factors 16

1.5.2 Pathogenesis of oral mucositis 17

1.5.3 Oral mucositis assessment scale 18

1.6 Sequelae of radiotherapy-induced xerostomia 20

1.6.1 Long-term effects of xerostomia 20

1.6.2 Management and treatment of xerostomia 21

1.6.3 Use of oral probiotics to manipulate oral microbiota 22

1.7 Thesis objectives 23

Chapter 2: Evaluating the impact of DNA extraction method on the representation of human oral bacterial and fungal communities 25

2.1 Introduction 26

2.2 Materials and methods 28

2.2.1 Sample collection 28

2.2.2 DNA extraction 29

2.2.3 Amplification of target genes and sequencing preparation 32

2.2.4 Bioinformatic analyses 35

2.2.5 Statistical analyses 36

2.3 Results 37

2.3.1 Influence of extraction method on DNA quantity and yield 37

2.3.2 Effect of DNA extraction on microbial communities in plaque 40

2.3.3 Effect of DNA extraction method on microbial communities in saliva 46

2.3.4 Quality and yield of DNA extracted from control fungal strains 48

2.4 Discussion 49

Chapter 3: Microbial and inflammatory-based salivary biomarkers of head and neck squamous cell carcinoma 52

3.1 Introduction 53

3.2 Materials and methods 55

3.2.1 Participant populations and sample collection 55

3.2.2 DNA extraction, amplification of target genes and sequencing preparation 56

3.2.3 Bioinformatic analyses 58

3.2.4 Cytometric bead array 59

3.2.5 Statistical analyses 60

3.3 Results 62

3.3.1 Sequencing data 62

3.3.2 Bacterial community profiles 62

3.3.3 Bacterial diversity 63

3.3.4 Fungal community profiles 64

3.3.5 Fungal diversity 64

3.3.6 Comparison of inflammatory cytokine concentrations 68

3.3.7 Correlation of microbial relative abundance data to IL-1β and IL-8 concentrations 69

3.4 Discussion 72

Chapter 4: Oral microbial influences on oral mucositis during radiotherapy treatment of head and neck cancer 75

4.1 Introduction....76

4.2 Materials and methods....78

4.2.1 Patient recruitment....78

4.2.2 Sample collection....78

4.2.3 Oral mucositis assessment....79

4.2.4 DNA extraction, amplification of target genes and sequencing preparation....79

4.2.5 Bioinformatic analyses....81

4.2.6 Analysis of the salivary bacterial microbiota during radiotherapy....81

4.2.7 Analysis of bacterial community data from swabs....82

4.2.8 Quantification of bacterial load in swabs....83

4.2.9 Predicted function of the bacterial community at sites of ulcerative mucositis....84

4.2.10 Analysis of fungal community data from swabs....84

4.3 Results....85

4.3.1 Oral mucositis prevalence....85

4.3.2 Salivary microbiota during radiotherapy....85

4.3.3 Core salivary bacteria during radiotherapy....89

4.3.4 Salivary microbiota and the development of oral mucositis....90

4.3.5 Bacterial communities in buccal mucosa and lateral tongue swabs....90

4.3.6 Bacterial genera correlated with ulcerative mucositis....94

4.3.7 Predictive modelling or oral mucositis outcomes....96

4.3.8 Estimation of bacterial load on the buccal mucosa....97

4.3.9 Functional prediction at sites of ulcerative mucositis 98

4.3.10 Fungal communities in buccal mucosa and lateral tongue swabs 98

4.4 Discussion 101

Chapter 5: Randomised, double-blind, placebo-controlled clinical trial of the oral probiotic *Streptococcus salivarius* M18 on head and neck cancer patients post-radiotherapy: a pilot study 104

5.1 Introduction 105

5.2 Methods 108

5.2.1 Patient enrolment and randomisation 108

5.2.2 Study design and sample collection 109

5.2.3 Oral health assessments 110

5.2.4 Assessment of probiotic viability 110

5.2.5 DNA extraction and sequencing preparation 111

5.2.6 Bioinformatic analyses 112

5.2.7 Network analyses 113

5.2.8 Statistical analyses 114

5.3 Results 115

5.3.1 Study cohort 115

5.3.2 Comparison of pre- and post-intervention clinical measures of oral health 118

5.3.3 Probiotic viability 119

5.3.4 Bacterial community profiles 120

5.3.5 Comparison of *Streptococcus* ZOTUs pre- and post-intervention 122

5.3.6 Bacterial community diversity pre- and post-intervention 127

5.3.7 Inference of bacterial community networks in plaque and saliva.........................132

5.4 Discussion 135

Chapter 6: General Discussion 138

6.1 Oncogenic potential of the oral microbiota 139

6.2 Oral microbiota and the pathogenesis of oral mucositis 141

6.3 Probiotic-induced modulation of post-radiotherapy oral microbiota...........................142

6.4 Future directions 144

6.5 Concluding remarks 145

APPENDIX A 146

APPENDIX B 148

APPENDIX C 152

APPENDIX D 159

Bibliography 196

School of Graduate Studies
AskAuckland Central
Alfred Nathan House
The University of Auckland
Tel: +64 9 373 7599 ext 81321
Email: postgradinfo@auckland.ac.nz

Co-Authorship Form

This form is to accompany the submission of any PhD that contains published or unpublished co-authored work. **Please include one copy of this form for each co-authored work**. Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements. Co-authored works may be included in a thesis if the candidate has written all or the majority of the text and had their contribution confirmed by all co-authors as not less than 65%.

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

Chapter 2: Evaluating the impact of DNA extraction on the representation of human oral bacterial and fungal communities

Vesty et al. 2017 doi:10.1371/journal.pone.0169877

Nature of contribution by PhD candidate	Experimental design, ethics approval, sample collection, laboratory work, analyses, manuscript draft
Extent of contribution by PhD candidate (%)	90

CO-AUTHORS

Name	Nature of Contribution
Kristi Biswas	Experimental design, ethics approval, manuscript review and editing
Michael W. Taylor	Experimental design, manuscript review and editing
Kim Gear	Sample collection, funding acquisition
Richard G. Douglas	Manuscript review and editing

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Kristi Biswas	[signature]	16/10/19
Michael W. Taylor	[signature]	16/10/19
Kim Gear	[signature]	16/10/19
Richard G. Douglas	[signature]	15/10/19.

School of Graduate Studies
AskAuckland Central
Alfred Nathan House
The University of Auckland
Tel: +64 9 373 7599 ext 81321
Email: postgradinfo@auckland.ac.nz

Co-Authorship Form

This form is to accompany the submission of any PhD that contains published or unpublished co-authored work. **Please include one copy of this form for each co-authored work**. Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements. Co-authored works may be included in a thesis if the candidate has written all or the majority of the text and had their contribution confirmed by all co-authors as not less than 65%.

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

Chapter 3: Microbial and inflammatory-based biomarkers of head and neck squamous cell carcinoma

Vesty et al. 2018 doi:10.1002/cre2.139

Nature of contribution by PhD candidate	Experimental design, ethics approval, patient consent, sample collection, clinical data acquisition, laboratory work, analyses, manuscript draft
Extent of contribution by PhD candidate (%)	90

CO-AUTHORS

Name	Nature of Contribution
Kim Gear	Sample collection, clinical data interpretation, funding acquisition
Kristi Biswas	Experimental design, ethics approval, manuscript review and editing
Fiona J. Radcliff	Assisted with cytometric bead array, manuscript review and editing
Michael W. Taylor	Experimental design, manuscript review and editing
Richard G. Douglas	Experimental design, ethics approval, manuscript review and editing

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Kim Gear	[signature]	16/10/19
Kristi Biswas	[signature]	16/10/19
Fiona J. Radcliff	[signature]	21/10/19
Michael W. Taylor	[signature]	16/10/19
Richard G. Douglas	[signature]	15/10/19

School of Graduate Studies
AskAuckland Central
Alfred Nathan House
The University of Auckland
Tel: +64 9 373 7599 ext 81321
Email: postgradinfo@auckland.ac.nz

Co-Authorship Form

This form is to accompany the submission of any PhD that contains published or unpublished co-authored work. **Please include one copy of this form for each co-authored work.** Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements. Co-authored works may be included in a thesis if the candidate has written all or the majority of the text and had their contribution confirmed by all co-authors as not less than 65%.

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

Chapter 4: Oral microbial influences on oral mucositis during radiotherapy treatment of head and neck cancer

Vesty et al., 2019 doi:10.1007/s00520-019-05084-6

Nature of contribution by PhD candidate	Experimental design, ethics approval, sample collection, clinical data acquisition, laboratory work, analyses, manuscript draft
Extent of contribution by PhD candidate (%)	85

CO-AUTHORS

Name	Nature of Contribution
Kim Gear	Experimental design, ethics approval, patient recruitment, sample collection, clinical data interpretation, funding acquisition
Kristi Biswas	Experimental design, ethics approval, manuscript review and editing
Brett Wagner Mackenzie	Assisted with ddPCR, manuscript review and editing
Michael W. Taylor	Experimental design, manuscript review and editing
Richard G. Douglas	Experimental design, ethics approval, manuscript review and editing

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Kim Gear	[signature]	16/10/19
Kristi Biswas	[signature]	16/10/19
Brett Wagner Mackenzie	[signature]	21/10/19
Michael W. Taylor	[signature]	16/10/19
Richard G. Douglas	[signature]	15/10/19.

Last updated: 28 November 2017

School of Graduate Studies
AskAuckland Central
Alfred Nathan House
The University of Auckland
Tel: +64 9 373 7599 ext 81321
Email: postgradinfo@auckland.ac.nz

Co-Authorship Form

This form is to accompany the submission of any PhD that contains published or unpublished co-authored work. **Please include one copy of this form for each co-authored work**. Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements. Co-authored works may be included in a thesis if the candidate has written all or the majority of the text and had their contribution confirmed by all co-authors as not less than 65%.

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.	
Chapter 5: Randomised, double-blind, placebo-controlled clinical trial of the oral probiotic Streptococcus salivarius M18 on head and neck cancer patients post-radiotherapy: a pilot study	
Nature of contribution by PhD candidate	Experimental design, ethics approval, patient consent, sample collection, clinical data acquisition, laboratory work, analyses, manuscript draft
Extent of contribution by PhD candidate (%)	75

CO-AUTHORS

Name	Nature of Contribution
Kim Gear	Experimental design, ethics approval, patient recruitment and liaison, clinical data interpretation
Sharon Boutell	Oral health assessments, sample collection, patient liaison
Michael W. Taylor	Experimental design, review and editing of chapter
Richard G. Douglas	Experimental concept and design, ethics approval
Kristi Biswas	Experimental design, ethics approval, review and editing of chapter, funding acquisition

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Kim Gear	[signature]	16/10/2019
Sharon Boutell	[signature]	14th October 19
Michael W. Taylor	[signature]	16/10/19
Richard G. Douglas	[signature]	15/10/19
Kristi Biswas	[signature]	16/10/19

Chapter 1

General Introduction

1.1 The oral microbiota

The human mouth is home to the oral microbiota, one of the most diverse microbial communities in the human body (Human Microbiome Project Consortium, 2012). To date, much focus has been on the bacterial constituents of this community, which have been thoroughly investigated by cultivation. However, culture-based techniques are limited to only cultivable organisms, which are estimated to capture less than half of the true bacterial diversity present in the mouth (Chen et al., 2010). More recently, the oral microbiota has been extensively characterised using DNA-based molecular techniques, primarily 16S ribosomal RNA (16S rRNA) gene sequencing-based identification, which indicates thousands of bacterial species comprise the oral microbiota across a wide range of health and disease states (Dewhirst et al., 2010; Human Microbiome Project Consortium, 2012; Keijser et al., 2008).

1.1.1 Investigating the oral microbiota: culture and molecular approaches

The use of next-generation sequencing (NGS) as a tool for analysing complex bacterial communities is continually gaining momentum. NGS platforms, including Illumina MiSeq, allow for the rapid sequencing of PCR-amplified microbial targets, an approach that yields thousands of sequences per sample, enabling both abundant and rare members of oral microbial communities to be identified. Amplicon sequencing of conserved genetic regions has the advantage over culture-based methods in that it captures a snapshot of the entire microbial community present in a sample, without *a priori* knowledge of culture requirements, including those of fastidious organisms that may be difficult to isolate. Molecular-based approaches are

still limited by inherent biases, for example, the choice of DNA extraction protocol, therefore optimising methodology to yield a true representation of the oral microbiota is vital. When phenotypic testing is required, cultivation is the better approach, therefore, methodology should be guided by the research objective.

1.1.2 Defining healthy oral microbiota

Within the oral microbiota, distinct bacterial niches occur including those found in plaque, saliva and on oral mucosal surfaces (Aas, Paster, Stokes, Olsen, & Dewhirst, 2005; Mager, Ximenez-Fyvie, Haffajee, & Socransky, 2003; Segata et al., 2012). Several studies have attempted to define the bacterial microbiota of the oral cavity. Aas et al. (2005) utilised 16S rRNA gene amplification, cloning and sequencing of samples taken from five subjects with no signs of oral disease at nine different oral sites to investigate the oral microbiota in health. Several dominant species belonging to the genera *Gemella*, *Granulicatella*, *Streptococcus* and *Veillonella* were common to all sites (Aas et al., 2005). A more recent study utilised the increased throughput and improved sensitivity of NGS to investigate oral bacterial communities of 200 normal adults enrolled in the Human Microbiome Project that were sampled at nine different oral sites: buccal mucosa, keratinised gingiva, hard palate, saliva, tongue, tonsils, throat and sub- and supra-gingival plaque (Segata et al., 2012). Members of the phylum *Firmicutes* dominated all of the oral sites with relatively high abundances of *Streptococcus*, *Gemella* and *Veillonella* (Segata et al., 2012).

1.2 Periodontal disease

Periodontal disease is a highly prevalent, inflammatory disease whereby a bacterial biofilm (dental plaque) builds up on teeth and within periodontal pockets around the teeth, initiating a destructive, host-driven inflammatory response in surrounding periodontal tissue (Darveau, 2010; Tatakis & Kumar, 2005). There is evidence to suggest ancient populations suffered from alveolar bone loss, the hallmark of periodontitis that leads to periodontal pocketing, and it continues to be a major cause of tooth loss globally in the 21st century (Petersen & Ogawa, 2012). Dental plaque is the primary reservoir for periopathogenic bacteria and the use of DNA sequencing methods to elucidate the bacterial composition of dental plaque in both healthy and diseased states has greatly improved our understanding of periodontal disease (Dewhirst et al., 2010).

1.2.1 Periodontal disease and microbial dysbiosis

Commensal microbes help maintain homeostasis of the mutualistic host-microbial relationship (Hooper, Littman, & Macpherson, 2012). When the equilibrium of the oral bacterial community is disrupted, the host becomes prone to the dysbiosis-associated periodontal disease (Marsh, 1994). The polymicrobial aetiology of periodontal disease is well characterised as a microbial-shift disease, owing to the shift (or dysbiosis) from predominantly Gram-positive to predominantly Gram-negative bacteria that occurs during the transition from oral health to periodontal disease (Darveau, 2010; Nishihara & Koseki, 2004). The Gram-negative organisms identified in this transition are primarily obligate and facultative anaerobic Gram-

negative bacilli (GNB) (Marsh, 1994) and are included in the group of bacteria, or periopathogens, that are associated with periodontal disease: *Aggregatibacter actinomycetemcomitans*, *Campylobacter* spp., *Capnocytophaga gingivalis*, *Eikenella corrodens*, *Eubacterium nodatum*, *Fusobacterium nucleatum*, *Parvimonas micra*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, *Tannerella forsythia* and *Treponema denticola* (Socransky, Haffajee, Cugini, Smith, & Kent, 1998).

1.2.2 Saliva as a proxy for oral health

Saliva contains microbes shed from intraoral sites, including tooth surfaces, therefore it acts as a simple proxy to study the oral microbiota and related oral health (Belstrøm et al., 2013; Kageyama et al., 2017; Sakamoto, Umeda, Ishikawa, & Benno, 2000). Benefiting from its non-invasive ease of collection, saliva is a valuable tool for detecting and estimating the relative abundance of plaque-derived periopathogenic bacteria, with data suggesting the presence of, and changes in, the relative abundances of specific plaque microbiota are accurately reflected in saliva (Belstrøm et al., 2017; Kageyama et al., 2017). Many studies have investigated the salivary microbiota for this reason and due to its temporal stability (Belstrøm et al., 2016). Typically, across several recent studies, the healthy salivary microbiota is dominated by the following genera: *Granulicatella*, *Haemophilus*, *Neisseria*, *Prevotella*, *Rothia*, *Streptococcus* and *Veillonella* (Belstrøm et al., 2016; Human Microbiome Project Consortium, 2012; Takeshita et al., 2016). In health, the bacterial diversity of saliva is lower than in patients with dental decay and periodontal disease (Takeshita et al., 2016); an increase in phylogenetic

diversity coincides with tooth decay, periodontal pocketing, high plaque indices and other manifestations of poor oral health (Takeshita et al., 2016).

1.2.3 Bacterial complexes in plaque biofilms

1.2.3.1 The 'red complex'

Within the polymicrobial communities of plaque biofilms, bacterial complexes exist. Cluster analysis and community ordination techniques have been used to define microbial communities in plaque samples taken from subjects with varying degrees of periodontal disease (Socransky et al., 1998). Five major bacterial complexes were identified and given a colour designation, summarised in Figure 1.1. The 'red complex' is a group of three specific bacterial species implicated in periodontal disease: the three anaerobic GNB of this periopathogenic complex are *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*. The detection of each of the three species is associated with detection of the other two and very strongly correlated with the manifestation of periodontal disease (Dewhirst et al., 2010; Rôças, Siqueira, Santos, & Coelho, 2001; Socransky et al., 1998).

1.2.3.2 Porphyromonas gingivalis as a keystone pathogen

P. gingivalis is considered a keystone-pathogen in plaque biofilms, yet acts as only a weak activator of inflammation (Darveau, 2010; Darveau, Hajishengallis, & Curtis, 2012). The keystone-pathogen hypothesis arises from ecological studies where it is defined as a species that is present in low abundance, yet it has a major role in the remodelling of a microbial community (Hajishengallis, Darveau, & Curtis, 2012). The role of *P. gingivalis* in the

pathogenesis of periodontal disease relies on its ability to subvert the innate immune response, a mechanism that benefits the entire microbial community (Lamont, Koo, & Hajishengallis, 2018). Specifically, *P. gingivalis* inhibits Toll-like receptor (TLR)-4 and disables macrophage-mediated bacteria-killing mechanisms to evade host detection and clearing of itself, as well as other oral microbes (Darveau et al., 2012; Wang et al., 2010). This leads to an increase in oral microbial load and a shift from symbiotic to dysbiotic oral microbiota, which promotes inflammation and accelerates the disease process (Darveau et al., 2012).

1.2.3.3 The 'orange complex'

The 'orange complex' is a group of bacteria that are generally found together and have a strong association with periodontal disease: *Campylobacter* spp., *Eubacterium nodatum*, *Fusobacterium nucleatum*, *Parvimonas micra*, *Prevotella intermedia*, *Prevotella nigrescens* and *Streptococcus constellatus* (Socransky et al., 1998). A significant association between increasing periodontal pocket depth and the orange complex has been demonstrated (Socransky et al., 1998). The orange and red complexes are closely associated: red complex species are rarely found in the absence of orange complex members, yet orange complex species are often found in the absence of red complex members; such observations have led to speculation that colonisation by the orange complex precedes red complex colonisation (Socransky et al., 1998).

1.2.3.4 Other complexes

The other bacterial complexes in plaque described in Figure 1.1 are earlier colonisers of the biofilm and are associated with a lower risk of periodontal disease (Socransky et al., 1998).

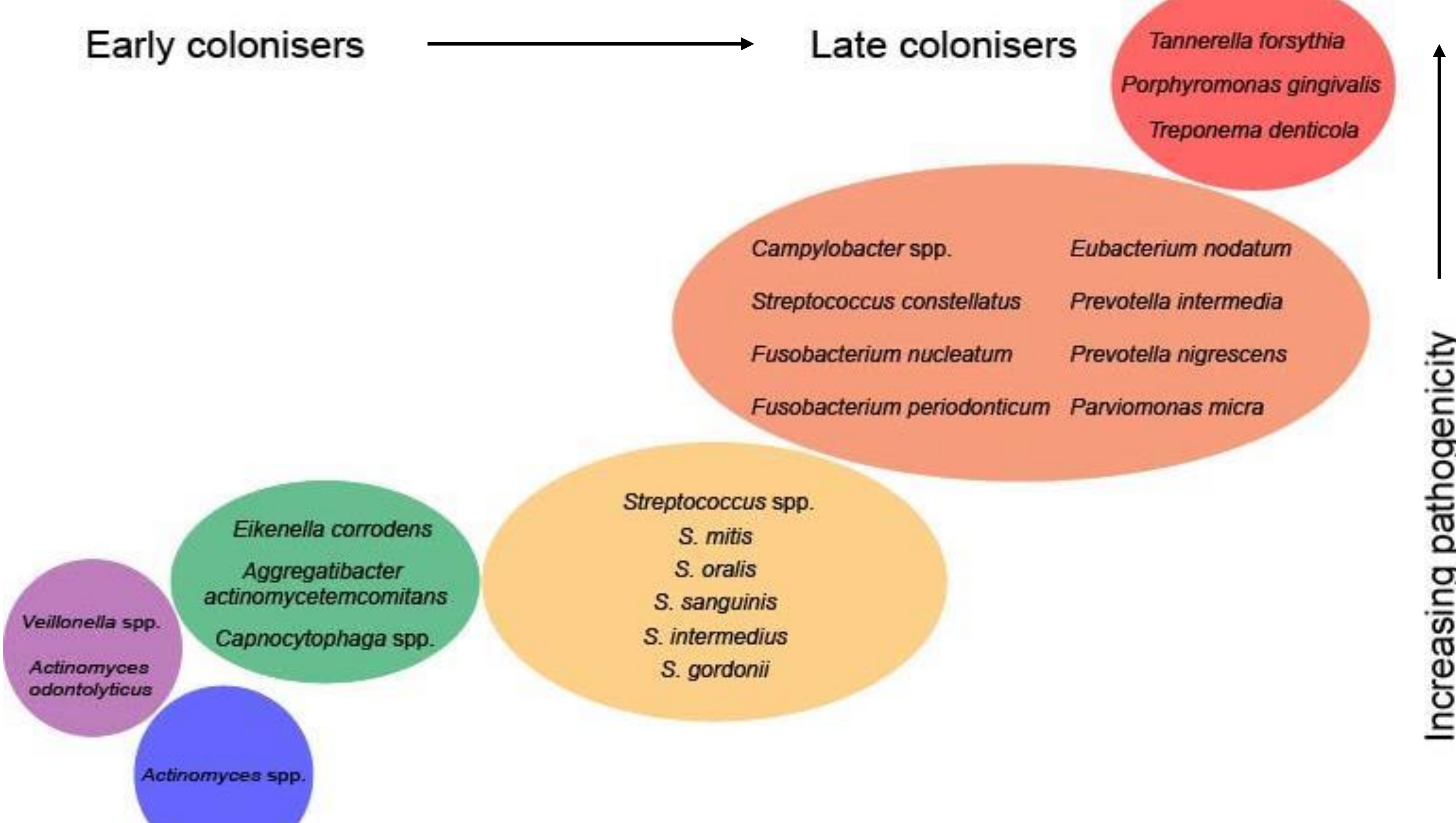


Figure 1.1. Schematic of the bacterial complexes in dental plaque. An adaptation based on multiple cluster and community ordination analyses by Socransky et al., 1998.

1.2.4 Pathogenesis of microbial-induced periodontal inflammation

The pathogenesis of periodontal disease is driven by the host inflammatory response to periopathogenic bacteria in the dental plaque biofilm. Dysregulation of the host inflammatory pathway leads to the chronic inflammation and tissue destruction associated with periodontal diseases. The oral epithelium has tight inter-cellular junctions to prevent entry of oral microbes and their metabolites. When exposed to lipopolysaccharide, the cell-wall component of Gram-negative bacteria, junctional epithelial cells are triggered to release cytokines and stimulate neurons to produce neuropeptides, resulting in vasodilation in the adjacent periodontium (Cekici, Kantarci, Hasturk, & Van Dyke, 2014). TLRs in periodontal tissue recognise pathogen-associated molecular patterns (PAMPs) (Cekici et al., 2014; Uehara & Takada, 2007). When PAMPs bind to TLRs, an intracellular cascade is initiated via the transcription factors NF-κB and interferons, ultimately leading to the production of cytokines, chemokines and antimicrobial peptides involved in the innate immunity response (Kagnoff & Eckmann, 1997). Immune cells present in the periodontal tissues (neutrophils, macrophages, monocytes and mast cells) migrate towards the site of inflammation and amplify the inflammatory response by releasing pro-inflammatory cytokines including tumour necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-8, chemotactic molecules, histamine and collagen-degrading matrix metalloproteinases (Cekici et al., 2014).

1.3 The oral mycobiota

Studies of the oral microbiota have largely focused on the bacterial communities that reside in the oral cavity, with far less attention given to their fungal counterparts. Fungi are abundant and diverse organisms that live on or in humans as commensals, yet many have the capacity to act as opportunistic pathogens. Therefore, their inclusion in our understanding of the resident human oral microbiota is necessary (Diaz, Strausbaugh, & Dongari-Bagtzoglou, 2014; Limon, Skalski, & Underhill, 2017). A lack of appreciation and the scarcity of data relating to the oral mycobiota stems from challenges in the recovery of adequate fungal DNA to different approaches in bioinformatic analysis. Fungi have thick cell walls that require harsher extraction techniques than the bacterial cells for which many extraction methods are optimised, therefore the representation of fungal communities is sensitive to DNA extraction method (Vesty, Biswas, Taylor, Gear, & Douglas, 2017). Variability in fungal targets, e.g. internal transcribed spacer (ITS) regions, the small subunit (18S rRNA gene) and the large subunit (28S rRNA), and subsequent analysis pipelines that produce taxonomic resolution equivalent to the bacterial 16S rRNA gene reduces inter-laboratory comparability of data (Diaz, Hong, Dupuy, & Strausbaugh, 2017; Halwachs et al., 2017; Hoggard et al., 2018). Difficulties with phylogenetic classification due to redundant fungal naming systems (Hibbett & Taylor, 2013) and the assignment of large portions of sequences as uncultivable or unidentified fungi may also complicate results (Limon et al., 2017). Furthermore, until the development of well-curated fungi databases such as UNITE (Nilsson et al., 2018), correct assignment of taxonomy using publically available repositories was hindered by erroneously annotated sequences and chimeras (Bandara, Panduwawala, & Samaranayake, 2018; Dupuy et al., 2014; Limon et al., 2017; Nilsson, Kristiansson, Ryberg, Hallenberg, & Larsson, 2008). Nonetheless, the few

reported studies of the oral mycobiota have endeavoured to shed light on this community's core members.

1.3.1 Defining the oral mycobiota

The seminal study of the healthy oral mycobiota by Ghannoum et al. (2010) utilised high throughput pyrosequencing of the ITS1 region and identified 85 fungal genera in saliva, with *Candida* the most frequently detected genus. *Malassezia* was later proposed as a prominent genus in saliva (Dupuy et al., 2014). These two studies identified several consensus members of the "basal salivary mycobiome": *Candida*, *Cladosporium*, *Alternaria*, *Aspergillus*, *Fusarium*, *Cryptococcus* and *Aureobasidium* (Dupuy et al., 2014; Ghannoum et al., 2010). These data are consistent with the results of a longitudinal, cultivation-based study of salivary mycobiota, which found *Candida* was the most commonly isolated fungal genus in the oral cavity and included three species: *C. albicans*, *C. parapsilosis* and *C. tropicalis* (Sampaio-Maia, 2014). This study also demonstrated inter-individual variability within the mycobiota, but intra-individual stability over time (Sampaio-Maia, 2014).

1.3.2 The oral mycobiota and oral disease

The role of the oral mycobiota in oral diseases such as periodontitis and dental decay is not well understood. The mycobiota of dental plaque is seldom investigated using NGS

approaches, but the few studies available have shown that *C. albicans* is consistently the most abundant fungal species in plaque for a range of disease states including healthy, dentally compromised and patients infected with human immunodeficiency virus (HIV) (Diaz et al., 2017; Fechney et al., 2019; Mukherjee et al., 2014). As part of the polymicrobial dental biofilm, *C. albicans* can synergistically interact with *P. gingivalis* to increase gingival epithelial cell invasion and promote biofilm formation (Sztukowska et al., 2018; Tamai, Sugamata, & Kiyoura, 2011). Based on ITS1 sequencing, the abundance of *Candida* is reportedly higher in the oral washes of patients with periodontal disease, compared to subjects with good oral health (Peters, Wu, Hayes, & Ahn, 2017). However, the presence of *C. albicans* in healthy subjects makes such data difficult to interpret and the role of the oral mycobiota in periodontal disease and dental decay is thus uncertain.

1.4 Head and neck cancer

Head and neck cancer describes a group of malignant tumours that present in the oral cavity (buccal mucosa, gums, front two-thirds of the tongue, hard palate and the floor of the mouth), nasal cavity, paranasal sinuses, salivary glands, nasopharynx, hypopharynx, larynx and the oropharynx (back third of the tongue, soft palate, tonsils and throat walls). The majority (> 90%) of these cancers are squamous cell carcinomas, an aggressive disease with a five year survival rate of less than 50% and a high risk of recurrence in survivors (Silverman 2001; Wang et al. 2013). Treatment involves surgery, radiotherapy and chemotherapy or a combination of all three and requires a multidisciplinary approach involving specialists in dentistry, dietetics, oncology, oral health, psychology, radiotherapy, surgery and speech and language therapy.

1.4.1 Risk factors

Traditionally, the major risk factors associated with head and neck cancer are tobacco smoking and excess alcohol consumption (Shaw & Beasley, 2016). A rise in the incidence of oropharyngeal cancers amongst a younger population has been linked to viral aetiologies, particularly exposure to the human papilloma virus (HPV) (Chaturvedi, Engels, Anderson, & Gillison, 2008). More recently, it is speculated that oral microbes may play a role in the pathogenesis of head and neck cancer (Chattopadhyay & Panda, 2019). Considerable attention reviewing potential associations between oral microbes and oral carcinogenesis has failed to associate specific bacteria with oral cancers; yet, the consistent theme is that an etiological link between the two cannot be excluded (Gholizadeh et al., 2016; Hooper, Wilson, & Crean, 2009;

Perera, Al-Hebshi, Speicher, Perera, & Johnson, 2016; Vyshenska, Lam, Shulzhenko, & Morgun, 2017). Recent saliva-based oral microbiota studies show that changes in the relative abundance of several bacterial genera are tentatively associated with head and neck cancer (Guerrero-Preston et al., 2016; Pushalkar et al., 2011; Wolf, Moissl-eichinger, Perras, Koskinen, & Peter, 2017).

1.4.2 Side effects of radiotherapy treatment

Significant morbidity is associated with radiation to the head and neck region. Due to their proximity to the radiation target area, salivary glands are often damaged during treatment, resulting in a decrease in saliva production (hyposalivation) and xerostomia (dry mouth) (Rogers, Ahad, & Murphy, 2007). Aside from a loss of taste, xerostomia is one of the most frequent complaints of head and neck cancer patients during and after radiotherapy treatment and can substantially reduce quality of life (Rogers et al., 2007). In addition to its discomfort for patients, who may experience associated difficulties with mastication and speaking, a reduction in salivary flow has detrimental effects on oral health (Marsh, Do, Beighton, & Devine, 2016). The antimicrobial properties of saliva, along with its buffering capacity, help protect the mucosal membranes it coats in the oral cavity; therefore, a reduction in saliva can lead to changes in the oral microbial community (Marsh et al., 2016). Acute side effects associated with changes in the oral microbial community include oral candidiasis, which is considered a secondary infection, and oral mucositis (Dreizen, Daly, Drane, & Brown, 1977). Long-term changes after radiotherapy involve a shift to acidogenic and cariogenic bacteria that leaves patients prone to gingivitis (gingival inflammation), periodontal disease and dental

decay (Brown, Dreizen, Handler, & Johnston, 1975; Hameed, Zafar, & Ghafoor, 2018; Porter, Fedele, & Habbab, 2010).

Table 1.1 Side effects associated with radiotherapy treatment of head and neck cancer.

Acute	**Chronic**
altered taste sensation	*dental decay
dysphagia	*gingivitis
*oral candidiasis	*periodontal disease
*oral mucositis	osteoradionecrosis
oral pain	xerostomia
weight loss	
xerostomia	

* *Influenced by oral microbes.* Adapted from Khaw et al., 2014

1.5 Radiotherapy-induced oral mucositis

Radiotherapy uses high-energy radiation that targets rapidly dividing tumour cells with the goal of destroying them. Since the oral mucosal epithelium is rapidly dividing to maintain its role in alimentation, it is also prone to the damaging effects of radiotherapy. All patients undergoing radiotherapy, but particularly head and neck cancer patients, are at risk of developing oral mucositis (Maria, Eliopoulos, & Muanza, 2017; Rodríguez-Caballero et al., 2012; Sonis, 2009). Radiotherapy-induced oral mucositis and its sequelae are significant complications of head and neck radiotherapy. The painful inflammation and ulceration associated with oral mucositis can be debilitating for patients, who often experience trouble eating, swallowing and speaking (Bressan et al., 2016). In severe cases, patients require the insertion of a feeding tube or percutaneous endoscopic gastrostomy (PEG) and hospitalisation (Maria et al., 2017). Additionally, patients may elect to reduce their treatment or stop radiotherapy altogether – both situations resulting in suboptimal cancer treatment (Maria et al., 2017; Sonis et al., 2004).

1.5.1 Risk factors

Factors that influence the incidence and severity of oral mucositis can be divided into two categories: treatment-related or patient-related. Treatment-related risk factors include concomitant chemotherapy, cumulative radiation dose and tumour location (Maria et al., 2017; Vera-Llonch, Oster, Hagiwara, & Sonis, 2006). Patient-related risk factors are less clearly defined. Age, body mass index, gender, periodontal disease, systemic inflammation, tobacco

use and tumour stage have been identified as possible patient-related risk factors for oral mucositis (Maria et al., 2017; Vera-Llonch et al., 2006).

Oral microbes are thought to influence the pathogenesis of oral mucositis, although their exact role remains unclear (Laheij & de Soet, 2014; Vanhoecke, De Ryck, Stringer, Van de Wiele, & Keefe, 2015). Periopathogens are potential microbial risk factors for oral mucositis. Recent studies suggest that an overabundance of anaerobic Gram-negative bacilli are associated with the onset and severity of oral mucositis (Hou et al., 2018; Zhu et al., 2017), with peak abundances of *Prevotella*, *Fusobacterium*, *Treponema* and *Porphyromonas* being positively associated with oral mucositis severity (Hou et al., 2018). A positive trend was reported between patients with periodontal disease and the development of oral mucositis (Khaw, Liberali, Logan, Keefe, & Bartold, 2014) and a 'two hit model' has been used to describe the hypothesis in which patients are primed by periodontal disease for an exaggerated inflammatory response to radiotherapy, that manifests as oral mucositis (Khaw, Logan, Keefe, & Bartold, 2014).

1.5.2 Pathogenesis of oral mucositis

Oral mucositis is now well accepted to be the consequence of a series of interacting biological events that occur in five stages (Sonis, 2004). During the initial stage, radiation directly injures cellular DNA, leading to cell death and activation of an inflammatory cascade. The nuclear factor kappa-B (NF-κB) pathway is one of the best studied in terms of oral mucositis (Sonis, 2002); it regulates the expression of several genes involved in the inflammatory process and its activation results in an increase in production of inflammatory cytokines, including IL-1β

and IL-6 (Sonis, 2009). During signalling and amplification (stages two and three), signalling molecules produced during the initial response to radiotherapy create a feedback loop (Sonis, 2004). For example, TNF-α, a pro-inflammatory cytokine, forms a positive feedback loop in which it further activates NF-κB, matrix metalloproteinases and mitogen-activated protein kinase signalling, thus amplifying the inflammatory response. The result is an amplification of the initial radiation injury, leading to tissue damage (Al-Dasooqi et al., 2013; Sonis, 2004, 2012). During the fourth stage, ulcers and a pseudomembrane form in response to a breach in the oral mucosa – this is the most significant phase for patients and clinicians, as it is associated with pain, risk of infection and hospitalisation (Sonis, 2009). The pseudomembrane is colonised by bacteria and, once the integrity of the mucosal layer is lost, microbes invade and further perpetuate the inflammatory response (Vasconcelos et al., 2016). The final healing stage generally occurs spontaneously (Sonis, 2004).

1.5.3 Oral mucositis assessment scale

The World Health Organisation Oral Mucositis Assessment Scale is among the mostly widely used scales to assess oral mucositis, summarised in Table 1.2 (Miller, Hoogstraten, Staquet, & Winkler, 1981). At the lowest grade, grade 0, no changes are present and mucositis is absent. Grade 1 is considered mild, erythema is present without ulceration. At grades 2, 3 and 4 ulcerations are present with increasing severity and pain (moderate, severe and life-threatening, respectively). A normal diet can generally be maintained at ≤ grade 2, with liquids only at grade 3; alimentation may only be achieved via PEG by grade 4.

Table 1.2 Summary of the World Health Organisation Oral Mucositis Assessment Scale.

Grade	Severity	Erythema	Ulceration	Diet	Example
0	nil	absent	absent	Normal	-
1	mild	present	absent	normal	
2	moderate	present	present	normal	
3	severe	present	present	liquid	
4	life-threatening	present	present	feeding tube or PEG	

Photographs from Maria et al. 2017 https://doi.org/10.3389/fonc.2017.00089

1.6 Sequelae of radiotherapy-induced xerostomia

Post-radiotherapy, quality of life for head and neck cancer patients is negatively affected by xerostomia, which is characterised by a dry mouth, and often results in difficulties with eating and speaking, loss of taste and oral pain that interferes with daily life (Dirix, Nuyts, & Van Den Bogaert, 2006; Epstein et al., 1999). Salivary gland damage during radiotherapy and the subsequent hyposalivation results in a loss of salivary buffering and antimicrobial capacities – ultimately increasing bacterial load and changing the oral microbiota to conditions that favour gingivitis, periodontitis and rampant dental decay (Brown et al., 1975; Eliasson, Carlén, Almståhl, Wikström, & Lingström, 2006; Hong et al., 2010; Joyston-Bechal, Hayes, Davenport, & Hardie, 1992; Kielbassa, Hinkelbein, Hellwig, & Meyer-Lückel, 2006; Porter et al., 2010).

1.6.1 Long-term effects of xerostomia

Radiotherapy inflames periodontal tissues and increases periodontal attachment loss and gingival recession (Epstein, Lunn, Le, & Stevenson-Moore, 1998; Markitziu, Zafiropoulos, Tsalikis, & Cohen, 2006). Hyposalivation exacerbates these effects on periodontal tissue as the flushing mechanism of saliva helps reduce the accumulation of plaque on teeth and periodontal tissues (Yadav & Prakash, 2017). In patients suffering from xerostomia, this capacity is reduced, and leads to a build-up of dental plaque, increasing the risk of gingivitis and periodontitis (Chambers, Garden, Kies, & Martin, 2004). Dental decay becomes a chronic consequence of radiotherapy-induced xerostomia and a significant physical and financial

burden for many patients (Jawad, Hodson, & Nixon, 2015). Increases in acidogenic and cariogenic bacteria (promoters of dental decay), including *Streptococcus mutans* and *Lactobacillus* spp., occur simultaneously with a decrease in non-cariogenic bacteria (Almståhl, Finizia, Carlén, Fagerberg-Mohlin, & Alstad, 2018; Brown et al., 1975; Joyston-Bechal et al., 1992). Based on Koch's postulate principles, *S. mutans*, a sugar-fermenting, acidogenic species is considered the primary etiological agent of dental decay (Hamada & Slade, 1980; Loesche, 1986), although molecular-based studies are improving our understanding of the polymicrobial nature of dental decay (Banas & Drake, 2018; Simón-Soro & Mira, 2015).

1.6.2 Management and treatment of xerostomia

Careful management and monitoring of oral health and effective dental hygiene is important before, during and after radiotherapy (Chambers et al., 2004; Porter et al., 2010). Post-radiotherapy management of xerostomia aims to increase salivary flow or relies on saliva substitutes (Mercadante, Al Hamad, Lodi, Porter, & Fedele, 2017; Porter et al., 2010). Minimising the risk of gingivitis, periodontitis and dental decay requires implementation of an effective oral hygiene routine that includes plaque removal (tooth brushing and flossing) and the use of fluoridated toothpaste or prescription fluorides (Chambers et al., 2004; Porter et al., 2010).

1.6.3 Use of oral probiotics to manipulate oral microbiota

S. mutans is the target of many therapeutic strategies aimed at reducing dental decay and is inhibited by antimicrobial peptides called bacteriocin-like inhibitory substances (BLIS), produced by *S. salivarius* (James & Tagg, 1991). Probiotic lozenges containing *S. salivarius* M18, a strain of *S. salivarius* that produces BLIS, have demonstrated the potential to reduce dental decay *in vivo* (Di Pierro, Zanvit, Nobili, Risso, & Fornaini, 2015). In a recent randomised placebo-controlled trial, clinical measures of gingivitis show improvement following a four-week intervention of an oral probiotic containing a mix of *Lactobacillus* spp. (Keller, Brandsborg, Holmstrøm, & Twetman, 2018). Additionally, short-term consumption of probiotic yoghurts increases the alpha diversity of salivary microbiota but does not affect the overall structure of the bacterial community (Dassi et al., 2018). The beneficial properties of oral probiotics are poorly understood compared to their gut probiotic counterparts. The ability of oral probiotics to modulate the post-radiotherapy oral microbiota in head and neck cancer patients and potentially improve long-term oral health outcomes has not been explored.

Oral probiotics have the potential to manipulate and optimise the oral microbiota post-radiotherapy; this hypothesis warrants investigation and may lead to discoveries that are translatable to our understanding of the role played by oral microbiota in the pathogenesis of head and neck cancer and radiotherapy-induced oral mucositis.

1.7 Thesis objectives

This thesis has four main objectives:

1. Evaluate approaches for the extraction of genomic DNA from dental plaque and saliva and compare the subsequent microbial community profiles generated by amplification and sequencing of bacterial and fungal DNA.

This aim is addressed in Chapter 2 by comparing the quality and yield of DNA extracted using four different approaches. The bacterial 16S rRNA gene and the fungal ITS1 region from extracted DNA were amplified and sequenced to compare resulting microbial community signatures.

2. Identify microbial and inflammatory biomarkers in the saliva of head and neck cancer patients.

This aim is addressed in Chapter 3 and identifies microbial and inflammatory biomarkers in saliva collected from head and neck cancer patients and investigates their distinguishability from healthy patients and those with compromised dental health.

3. Describe changes in the oral microbiota during radiotherapy treatment of head and neck cancer and correlate with the onset and severity of oral mucositis.

This aim is addressed in Chapter 4 by prospectively collecting saliva and oral swabs from patients at regular intervals during radiotherapy. Microbial data were analysed and correlated with oral mucositis outcomes.

4. Determine the ability of *Streptococcus salivarius* M18 to modify the post-radiotherapy oral microbiota and its impact on clinical measures of oral health.

This aim is addressed in Chapter 5, a pilot study of *S. salivarius* M18 oral probiotic lozenges in a cohort of head and neck cancer patients post-radiotherapy.

Chapter 2

Evaluating the impact of DNA extraction method on the representation of human oral bacterial and fungal communities

This chapter is a modified version of: Vesty, A., Biswas, K., Taylor, M.W., Gear, K., Douglas, R.G. (2017). "Evaluating the impact of DNA extraction method on the representation of human oral bacterial and fungal communities". ***PLoS One*** *12(1): e0169877. doi: 10.1371/journal.pone.0169877*

2.1 Introduction

The oral cavity harbours one of the most diverse microbiomes in the human body (Human Microbiome Project Consortium, 2012). Within the oral cavity, several distinct niches occur, including those found in plaque and saliva (Aas et al., 2005; Xu et al., 2015), where dysbiosis and the presence of specific microbes can be associated with disease (Darveau, 2010; Socransky et al., 1998; Wade, 2013). Choice of DNA extraction protocol has the potential to influence our perception of microbiome structure. DNA extraction is achievable via different cell lysis procedures, including chemical, enzymatic, mechanical and heat. Recent studies demonstrate that the cell lysis method used during DNA extraction from oral samples can impact the recovery of specific bacterial phyla (Lazarevic, Gaïa, Girard, François, & Schrenzel, 2013). Mechanical lysis increases the number of different bacterial phyla recovered from saliva (Lazarevic et al., 2013), while the addition of lysozyme to mechanical lysis improves overall bacterial DNA yield from saliva (Sohrabi et al., 2016). Similarly, in plaque a mechanical lysis step and the addition of lysozyme maximises recovery at the species level (Abusleme, Hong, Dupuy, Strausbaugh, & Diaz, 2014). As all methods have inherent biases, an approach that provides a true representation of the oral microbiome is vital if we are to more fully understand its clinical manifestations.

Studies of DNA extraction bias have largely focused on the bacterial constituents of the oral microbiome, thus data pertaining to the fungal community (mycobiome) are lacking by comparison. Identical host (human) and fungal sequences at 18S rRNA gene primer binding sites render this gene an unsuitable target for many human samples, as sequences obtained may be predominantly human- rather than fungal-derived. Partly for this reason, the intergenic internal transcribed spacer (ITS) region has become an attractive alternative to 18S rRNA gene sequencing for fungal community analyses, due to its greater sequence variability which

differentiates it from the host DNA and also allows for greater taxonomic resolution (Nilsson et al., 2008). However, the advantages of ITS sequencing have not yet led to a greatly improved understanding of the oral mycobiome. To date, only a handful of studies have attempted to describe this community, with even less emphasis on how DNA extraction may affect its representation (Dupuy et al., 2014; Ghannoum et al., 2010; Weerasekera et al., 2013).

Physical and chemical interactions occur between bacteria and fungi in the oral environment, driving the structure and behaviour of the oral microbial community and potentially contributing to the pathogenesis of oral diseases (Krom, Kidwai, & Cate, 2014; Xu & Dongari-Bagtzoglou, 2015; Zijnge et al., 2010). Hence, it would be advantageous to simultaneously study DNA from both bacterial and fungal communities, in order to understand the clinical associations of both intra- and inter-domain relationships.

In this chapter, four genomic DNA (gDNA) extraction methods are systematically evaluated in order to compare bacterial and fungal community profiles, and determine which methods provide a suitable representation of microbial diversity in human dental plaque and saliva samples.

2.2 Materials and methods

2.2.1 Sample collection

Twelve patients were recruited from the Oral Health Unit, Green Lane Hospital, Auckland, New Zealand to participate in this study. All patients consented to providing sub- and supra-gingival dental plaque for this study, collected during a whole mouth scale. Plaque was removed by a registered dentist using sterile periodontal scalers and placed in a screw cap tube containing 1 mL of RNA*later*® (AMBION, Inc., Austin, TX, USA). Samples were stored at -20°C until further processing. Twelve healthy volunteers from The University of Auckland, New Zealand consented to providing a fresh saliva sample. At least 1 mL of saliva was collected from each participant via passive secretion into a sterile container and frozen neat at -20°C. Ethical approval for this study was granted by the Southern Health and Disability Ethics Committee (HDEC) (14/STH/121) and institutional approval was gained from the Auckland District Health Board, New Zealand. Written consent was obtained from all participants.

To create a homogenous sample of adequate volume for the comparison of multiple DNA extraction methods, 1 mL of each of the 12 plaque samples in RNA*later*® (AMBION, Inc., Austin, TX, USA) was pooled in a 50 mL CELLSTAR® Polypropylene Tube (Greiner Bio-One) and vortexed until a consistent solution was achieved. In a similar manner, 1 mL of each of the 12 saliva samples was pooled and mixed thoroughly. The plaque and saliva pooled homogenates (totalling approximately 12 mL each) were divided separately into 200 µL aliquots for subsequent testing.

2.2.2 DNA extraction

Four extraction techniques were performed in triplicate on the 200 µL aliquots of both the pooled plaque and pooled saliva homogenates (Table 2.1). Three commonly used commercial DNA extraction kits were used as per manufacturer's instructions, namely the MoBio PowerSoil® DNA Isolation Kit, Qiagen QIAamp® DNA Mini Kit and the Zymo Bacterial/Fungal DNA Mini Prep™ (Table 2.1). Additionally, a previously described phenol:chloroform-based method for DNA isolation from saliva was used (Polgárová, Behuliak, & Celec, 2010). Mechanical cell rupture was performed using a Qiagen TissueLyser II at 30 Hz for 2 x 50 s, as applicable. For each method, an extraction blank (PCR-grade water) was used to ascertain potential kit and/or reagent contamination.

Table 2.1 Summary of DNA extraction methods.

Extraction method	Lysis type	DNA isolation	Elution (µL)
MoBio PowerSoil® DNA Isolation Kit	Mechanical	Spin column	100
Qiagen QIAamp® DNA Mini Kit	Enzymatic, heat	Spin column	200
Zymo Bacterial/Fungal DNA Mini Prep ™	Mechanical	Spin column	100
Phenol:chloroform-based DNA isolation	Enzymatic, freeze-thaw	Phase separation	20

2.2.2.1 Efficiency of fungal DNA extraction

The four DNA extraction methods were employed in triplicate to extract fungal DNA separately from 200µL of ~7500 CFU/µL ATCC32045 *Cryptococcus neoformans* in RNA*later*® (AMBION, Inc., Austin, TX, USA) and 200µL of ~1150 CFU/µL ATCC10002 *Penicillium chrysogenum* in RNA*later*® (AMBION, Inc., Austin, TX, USA). DNA quality and yield were measured on the NanoPhotometer® N60 (IMPLEN, Inc., Westlake Village, CA, USA).

2.2.2.2 MoBio PowerSoil® DNA isolation kit

Aliquots were thawed on ice and pipetted into a supplied PowerBead Tube. The MoBio PowerSoil® DNA isolation kit employs a mechanical (bead beating) lysis step to rupture cells. Addition of the provided salt solution helped DNA bind to the silica spin column filter, and an ethanol-based solution washed the bound DNA. Finally, 100 µL of sterile elution buffer released the DNA from the spin column filter, yielding DNA for downstream applications.

2.2.2.3 Qiagen QIAamp® DNA mini kit

After thawing on ice, aliquots were pelleted at 5000 x *g* and resuspended in the supplied lysis buffer. Cell lysis and protein digestion were achieved using a Proteinase K incubation at 56°C for 1 h. The DNA was bound to a spin column filter, then washed with 96 – 100% ethanol, followed by two wash buffers (supplied). The bound DNA was eluted from the spin column filter with 200 µL of the supplied elution buffer.

2.2.2.4 Zymo Bacterial/Fungal DNA Mini PrepTM

Thawed plaque and saliva aliquots were centrifuged at 5000 x *g* and resuspended in 200 µL of sterile phosphate buffered saline (PBS) in a ZR BashingBead Lysis Tube. A lysis solution was added to help lyse cells during the mechanical lysis step. The supernatant of the lysed solution was filtered using a Zymo-Spin IV Spin Filter, then DNA was bound to a Zymo-Spin IIC column in the presence of DNA Binding Buffer, containing 0.5% (v/v) beta-mercaptoethanol. DNA was washed, then eluted with 100 µL of DNA Elution Buffer.

2.2.2.5 Phenol:chloroform-based DNA extraction

This phase separation method was adapted from a previously described method for gDNA extraction from saliva (Polgárová et al., 2010). Tris-EDTA (TE) buffer (400 µL) was added to thawed plaque and saliva aliquots and the mixture centrifuged at 8000 x *g* for 5 min. The pellet was resuspended in TE buffer containing lysozyme (5 mg/mL) and incubated for 1 h at 37°C. Proteinase K and 5% sodium dodecyl sulphate were added to final respective concentrations of 2 mg/mL and 1% (v/w), then tubes were incubated at 50°C for 2 h, with shaking at 300 rpm. Nucleic acids were released from the cells with three freeze-thaw cycles of -20°C (5 min) and 65°C (3 min). 200 µL of buffer-saturated phenol was added and tubes were vortexed, then centrifuged (13,000 x *g*, 4°C, 10 min). The aqueous phase was recovered, to which an equal volume of chloroform/isoamyl alcohol (24:1) was added. Tubes were vortexed and centrifuged again at 13,000 x *g*, 4°C for 10 min. The supernatant was transferred to a UV-sterilised Eppendorf tube and the nucleic acids were precipitated with 0.6 volume of isopropanol overnight at 4°C. The following day, DNA was pelleted (13,000 x *g*, 4°C, 10 min), washed with 70% ethanol and resuspended in 20 µL of sterile water.

2.2.2.6 Evaluation of DNA quality and yield

DNA yield was determined fluorometrically using the High Sensitivity dsDNA kit (Invitrogen Co., Carlsbad, CA, USA) on the Qubit® Fluorometer 1.0. Absorbance ratios were measured spectrophotometrically on the NanoDrop® ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA) to assess DNA purity: A260/280 nm for protein contamination and A260/230 nm for salt and phenol contamination. Since DNA absorbs light at 260 nm, ratios of 1.8 – 2.0 (for A260/280 nm) and > 1.8 (for A260/230 nm) indicated the sample was likely to be free from contamination by the respective substances. Genomic DNA (3 µL) was visualised on a 1% agarose gel (w/v) containing SYBR Safe DNA Gel Stain (Invitrogen Co., Carlsbad, CA, USA). DNA quantity and quality for each sample was further assessed using an Agilent DNA 1000 chip (Agilent Technologies, Waldbronn, Germany), which uses fragment size to assess DNA integrity and degradation.

2.2.3 Amplification of target genes and sequencing preparation

DNA extracts were diluted in UltraPure™ distilled water (Invitrogen Co., Carlsbad, CA, USA) to achieve equimolar concentrations. For each triplicate extraction and single extraction blank from the four different methods, gDNA was subjected to PCR amplification of both the bacterial 16S ribosomal RNA (16S rRNA) gene and fungal internal transcribed spacer 1 (ITS1) region.

2.2.3.1 16S rRNA gene amplification

Illumina-adapted primers S-D-Bact-0341-b-S-17 (5' TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG 3') and S-D-Bact-0785-a-A-21 (5' GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GAC TAC HVG GGT ATC TAA TCC 3') were used to amplify the V3 – V4 region of the 16S rRNA gene (template specific sequences are underlined; Klindworth et al., 2013). Each PCR reaction contained: 1× High Fidelity PCR Buffer, 2 mM magnesium sulphate, 0.5 mM dNTPs, 0.004× Platinum® Taq DNA Polymerase High Fidelity (Invitrogen Co., Carlsbad, CA, USA), 0.2 µM of each primer, 18.9 µL PCR-grade water and 1 µL of each normalised gDNA template. For each amplification run, 1 µL *Escherichia coli* gDNA was used as a positive control and 1 µL of PCR-grade water as a negative control. PCR was performed using the following thermocycling conditions: initial denaturation at 94°C for 3 min, followed by 32 cycles consisting of denaturation (94°C for 45 s), annealing (55°C for 45 s) and extension (72°C for 90 s), with a final extension step at 72°C for 10 min. Duplicate amplifications were performed for each reaction, then pooled to give a total volume of 50 µL. Two microlitres from each pooled PCR reaction were run on a 1% agarose gel (w/v) containing SYBR Safe DNA Gel Stain (Invitrogen Co., Carlsbad, CA, USA) and visualised under ultraviolet light.

2.2.3.2 ITS1 region amplification

Illumina-adapted primers ITS1F (5' TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CTT GGT CAT TTA GAG GAA GTA A 3') (Gardes & Bruns, 1993) and ITS2 (5' GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GCT GCG TTC TTC ATC GAT GC 3') (White, Bruns, Lee, & Taylor, 1990) were used to amplify the ITS1 region (template specific sequences are underlined). Each PCR reaction contained: 1X High Fidelity PCR Buffer, 2 mM magnesium sulphate, 0.5 mM dNTPs, 0.004X Platinum® Taq DNA Polymerase

High Fidelity (Invitrogen Co., Carlsbad, CA, USA), 0.2 µM ITS1 forward primer, 0.2 µM ITS2 reverse primer, 18.9 µL PCR-grade water and 1 µL of each DNA template. For each reaction, 1 µL *Candida albicans* gDNA was used as a positive control and 1 µL of PCR-grade water as a negative control. The following thermocycling conditions were used: initial denaturation at 95°C for 3 min, followed by 38 cycles consisting of denaturation (94°C for 45 s), annealing (50°C for 30 s) and extension (72°C for 90 s), with a final extension step at 72°C for 10 min. Duplicate amplifications were performed for each reaction, then pooled to give a total volume of 50 µL. Two microlitres from each pooled PCR amplification were run on a 1% agarose gel (w/v) containing SYBR Safe DNA Gel Stain (Invitrogen Co., Carlsbad, CA, USA).

2.2.3.3 Preparation of PCR amplicons for Illumina MiSeq

For each of the pooled 16S rRNA gene and ITS1 amplicons, 40 µL was purified using AMPure XP magnetic beads (Beckman Coulter Inc., Beverly, MA, USA) to a final volume of 20 µL in sterile water. DNA concentration was measured for all purified samples using the High Sensitivity dsDNA kit (Invitrogen Co., Carlsbad, CA, USA) on the Qubit® Fluorometer 1.0. Purified amplicons were subjected to further quality and quantity checks before sequencing on the Illumina MiSeq platform using paired-end sequencing with read lengths of 300 bp, performed by the Centre for Genomics, Proteomics and Metabolomics through New Zealand Genomics Ltd at The University of Auckland. Sequence data were uploaded to the NCBI Sequence Read Archive, under accession number SRP079075.

2.2.4 Bioinformatic analyses

2.2.4.1 Taxonomic assignment and bacterial diversity analyses

Forward and reverse bacterial 16S rRNA reads were merged with a minimum merge length of 200 bp and a maximum of five mismatches in the alignment using the fastq_mergepairs command, then simultaneously filtered to remove singletons and chimeras in USEARCH v9.2 (Edgar, 2010). Samples were rarefied to 6000 reads, and alpha diversity metrics were calculated using the core_diversity_analyses.py command (observed species, Chao1, Shannon, Simpson) in QIIME 1.9 (Caporaso et al., 2010). Operational taxonomic units (OTUs) were defined based on 97% sequence similarity using the UPARSE-OTU algorithm (Edgar, 2013), and taxonomy was assigned to individual OTUs through the Ribosomal Database Project (RDP) classifier (Wang, Garrity, Tiedje, & Cole, 2007) using the Human Oral Microbiome Database (HOMD) (Chen et al., 2010). Aligned sequences were used to build a phylogenetic tree with the make_phylogeny.py command through FastTree (Price, Dehal, & Arkin, 2009) in QIIME 1.9, which was used for subsequent phylogenetic-based beta diversity measures. Dissimilarity matrices (weighted UniFrac, unweighted UniFrac and Bray-Curtis) were generated in QIIME 1.9 and visualised through multidimensional scaling (MDS) plots built in PRIMER v6 (Anderson, Gorley, & Clarke, 2008).

2.2.4.2 Taxonomic assignment and fungal diversity analyses

Fungi sequences were processed using a similar pipeline: forward and reverse fungal ITS1 reads were merged using the USEARCH command fastq_mergepairs with a minimum merge length of 100 bp and the default number of misalignments. Sequences were filtered with an abundance threshold of more than four sequence counts, to exclude rare genera (Dupuy et al.,

2014). Samples were sub-sampled to 959 reads. Alpha diversity was estimated in QIIME 1.9 using the 'observed species', Chao1, Shannon and Simpson diversity metrics (Caporaso et al., 2010). Taxonomy was assigned using the Ribosomal Database Project (RDP) classifier (Wang et al., 2007) with UNITE OTUs (v12_11) (Kõljalg et al., 2013) as the database using a 97% sequence similarity threshold, based on 75% of fungal species containing ≤ 3% ITS1 intraspecific variation (Nilsson et al., 2008). *Candida* OTUs unresolved to species-level were further classified using BLASTn, with species-level taxonomic classification based on ≥ 98% sequence similarity over the length of the representative OTU.

2.2.5 Statistical analyses

To compare qualitative and quantitative data from DNA extractions across the four methods, a one-way analysis of variance (ANOVA) was used to compare means, with the Tukey-Kramer post-hoc test to account for multiple pairwise comparisons. To compare the reproducibility of each method, coefficients of variation were determined to describe the percentage of variability in DNA yield relative to the mean for each DNA extraction method. Statistical analyses were conducted in Prism v6 for Windows (GraphPad Software, La Jolla, CA, USA). Paired, two-tailed *t*-tests with Bonferroni adjustment for multiple pairwise comparisons were used to statistically assess differences in the relative abundance of taxon-assigned OTUs between DNA extraction methods. Permutational multivariate analysis of variance (PERMANOVA) was conducted in PRIMER v6 software (Anderson et al., 2008) and used to unbiasedly assess multivariate data. Values were obtained using type III (partial) sum of squares with 9999 permutations of residuals under a reduced model.

2.3 Results

2.3.1 Influence of extraction method on DNA quantity and yield

Agarose gel images revealed the presence of gDNA for all extraction methods from both plaque and saliva. The Qiagen QIAamp® DNA Mini Kit yielded the highest concentration of gDNA per 100 µL from both plaque and saliva, while phenol:chloroform-based extractions yielded the lowest in both. Based on normalised Qubit concentrations (mean ± SEM), a significant difference in DNA yield from both plaque and saliva was detected when Qiagen QIAamp® DNA Mini Kit extractions (4.87 ± 0.79 ng/µL) were compared separately to each of the three remaining DNA extraction methods (Fig 2.1). Coefficients of variation (CV) calculated for each method within sample type indicated that all methods were reproducible for DNA yield from both plaque (MoBio PowerSoil® DNA Isolation Kit = 40%, Qiagen QIAamp® DNA Mini Kit = 28%, Zymo Bacterial/Fungal DNA Mini PrepTM = 10%, phenol:chloroform = 59%) and saliva (MoBio PowerSoil® DNA Isolation Kit = 18%, Qiagen QIAamp® DNA Mini Kit = 28%, Zymo Bacterial/Fungal DNA Mini PrepTM = 15%, phenol:chloroform = 19%).

DNA purity was assessed across the four methods using the combined average plaque and saliva NanoDrop® values. Overall, the mean (± SEM) of the MoBio PowerSoil® DNA Isolation Kit extractions produced the highest A260/280 nm ratios (2.28 ± 0.27), significantly higher than all three other DNA extraction methods: Qiagen QIAamp® DNA Mini Kit (1.72 ± 0.04), Zymo Bacterial/Fungal DNA Mini PrepTM (1.57 ± 0.03) and phenol:chloroform (1.56 ± 0.06), however the MoBio PowerSoil® DNA Isolation Kit A260/280 nm ratio range was greater than 1.8 – 2.0. Average A260/230 nm ratios indicated the phenol:chloroform-based (1.88 ± 0.14) and Qiagen QIAamp® DNA Mini Kit (1.77 ± 0.14) methods both had

significantly less residual carryover than the MoBio PowerSoil® DNA Isolation Kit (0.92 ± 0.21) and Zymo Bacterial/Fungal DNA Mini PrepTM (0.72 ± 0.05) methods.

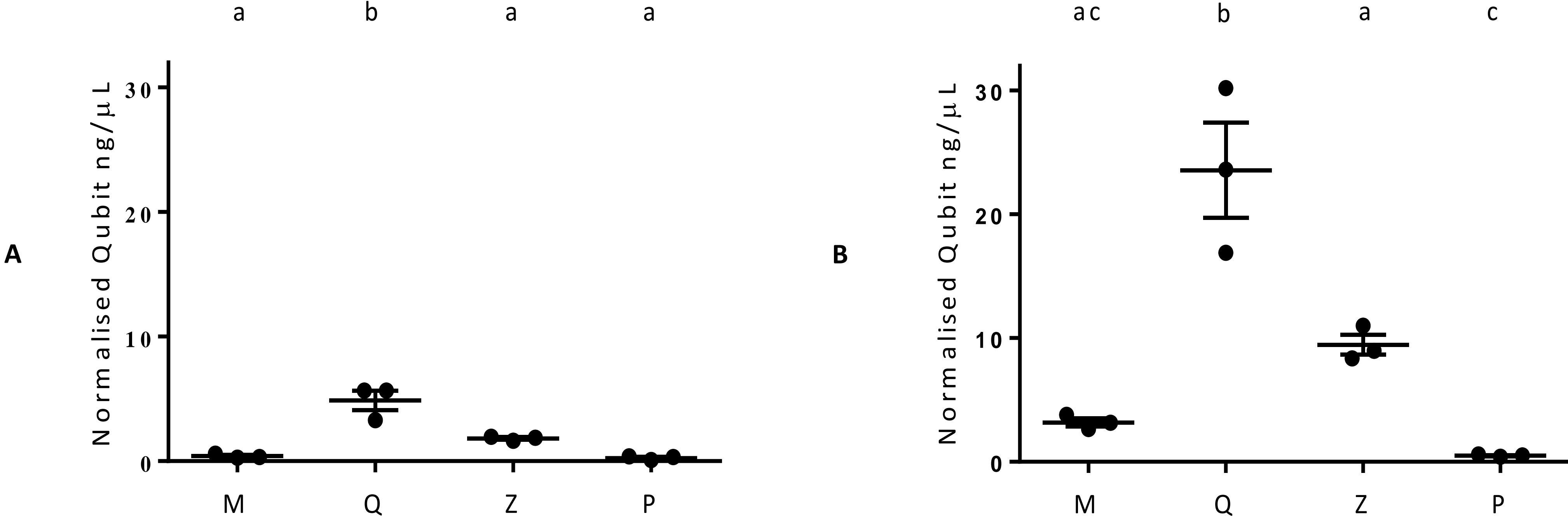


Figure 2.1. Normalised DNA yield (ng/µL) from (A) plaque and (B) saliva (mean ± SEM). M: MoBio PowerSoil® DNA Isolation Kit, Q: QIAamp® DNA Mini Kit, Z: Zymo Bacterial/Fungal DNA Mini Prep™, P: Phenol:chloroform-based DNA isolation. DNA extraction methods not linked by the same letter are significantly different to each other (Tukey-Kramer $p \leq 0.05$).

2.3.2 Effect of DNA extraction on microbial communities in plaque

The 12 pooled plaque samples (n = 4 methods × n = 3 replicates) returned a total of 238,945 unique 16S rRNA gene sequence reads (average length 456 bp). After removal of chimeras (0.9% of unique sequences), *de novo* OTU picking of the remaining sequences returned 325 unique OTUs. Sequencing of ITS1 amplicons from the same 12 plaque samples returned a total of 47,352 unique reads, with an average length of 256 bp. After removal of chimeras (5.4% of unique sequences), *de novo* OTU picking returned 22 unique OTUs.

Multiple pairwise comparisons between DNA extraction methods did not reveal any significant differences in bacterial and fungal species richness and evenness for plaque samples between methods (Table 2.2). Fungal diversity in plaque was much lower than bacterial diversity, with the mean number of observed OTUs ranging from 2.37 – 3.07 for fungi and 197 – 202 for bacteria, for all four methods (Table 2.2). Furthermore, PERMANOVA analysis of the plaque bacterial community profiles rarefied to 6,000 sequences per sample for unweighted UniFrac indicated that there was no evidence that DNA extraction method drove differences in bacterial community composition (Figure 2.2A).

The relative abundance of individual bacterial and fungal taxon-assigned OTUs identified within plaque did not significantly differ across any of the four DNA extraction methods. Sequences assigned to the bacterial genera *Capnocytophaga*, *Fusobacterium*, *Leptotrichia*, *Prevotella*, *Selenomonas* and *Streptococcus* represented the greatest relative abundances in plaque for each method and these genera were recovered at ≥ 5% of the plaque bacterial community in all replicates of each method (Figure 2.3A). Additionally, *Corynebacterium* comprised > 5% of the plaque community from MoBio PowerSoil® DNA Isolation Kit and Zymo Bacterial/Fungal DNA Mini Prep™ replicates, while *Veillonella* was found at > 6% in

the Qiagen QIAamp® DNA Mini Kit and phenol:chloroform-based methods. Genera present at ≥ 1% were consistently recovered from plaque by all four DNA extraction methods, including the periodontal disease-associated genera *Porphyromonas*, *Tannerella* and *Treponema*.

Fungal community profiles from plaque were closely related across the four DNA extraction methods, thus indicating comparable ability of the four approaches to extract fungal DNA from plaque. The plaque mycobiome was almost entirely dominated by *Candida* species, with > 99% of sequences for all methods assigned to this genus (Figure 2.3B). The closest match (100% sequence identity) through NCBI BLASTn indicated that *C. albicans* was the dominant species in this genus, across all four methods. Other species also identified within this genus included *C. dubliniensis* and *C. tropicalis*, although these were less abundant.

Table 2.2. Alpha diversity measurements (mean ± SEM) for triplicate data from each of the four DNA extraction methods, amplified with 16S rRNA gene primers and ITS1 primers.

		Chao1	**Observed OTUs**	**Shannon**	**Simpson**
16S rRNA, plaque	MoBio PowerSoil® DNA Isolation Kit	213 ± 11.33	197 ± 2.29	5.98 ± 0.01	0.97 ± 0.001
	Qiagen QIAamp® DNA Mini Kit	221 ± 5.44	201 ± 3.43	6.26 ± 0.02	0.98 ± 0.001
	Zymo Bacterial/ Fungal MiniPrep™	220 ± 5.43	202 ± 2.79	6.18 ± 0.03	0.97 ± 0.001
	Phenol:chloroform-based isolation	225 ± 2.18	202 ± 1.92	6.32 ± 0.11	0.98 ± 0.003
16S rRNA, saliva	MoBio PowerSoil® DNA Isolation Kit	153 ± 5.34	120 ± 0.73	4.61 ± 0.02	0.92 ± 0.002
	Qiagen QIAamp® DNA Mini Kit	143 ± 6.51	121 ±2.50	4.73 ± 0.06	0.93 ± 0.003
	Zymo Bacterial/ Fungal MiniPrep™	162 ± 4.70	122 ± 1.40	4.37 ± 0.05	0.90 ± 0.006
	Phenol:chloroform-based isolation	145 ± 5.14	112 ± 2.10	4.53 ± 0.07	0.92 ± 0.005
ITS1, plaque	MoBio PowerSoil® DNA Isolation Kit	3.22 ± 0.35	3.07 ± 0.39	0.67 ± 0.16	0.28 ± 0.09
	Qiagen QIAamp® DNA Mini Kit	2.37 ± 0.09	2.37 ± 0.09	0.56 ± 0.05	0.23 ± 0.03
	Zymo Bacterial/ Fungal MiniPrep™	3.02 ± 0.06	2.90 ± 0.08	0.37 ± 0.03	0.13 ± 0.02
	Phenol:chloroform-based isolation	3.27 ± 1.09	3.00 ± 0.71	0.57 ± 0.15	0.23 ± 0.08

		Chao1	Observed OTUs	Shannon	Simpson
ITS1, salvia	MoBio PowerSoil® DNA Isolation Kit	16.16 ± N/A	14.5 ± N/A	1.28 ± N/A	0.40 ± N/A
	Qiagen QIAamp® DNA Mini Kit	N/A	N/A	N/A	N/A
	Zymo Bacterial/ Fungal MiniPrep™	11.90 ± 0.90	11.90 ± 0.90	2.58 ± 0.24	0.77 ± 0.06
	Phenol:chloroform-based isolation	26.98 ± 4.28	23.2 ± 5.20	2.66 ± 0.15	0.70 ± 0.01

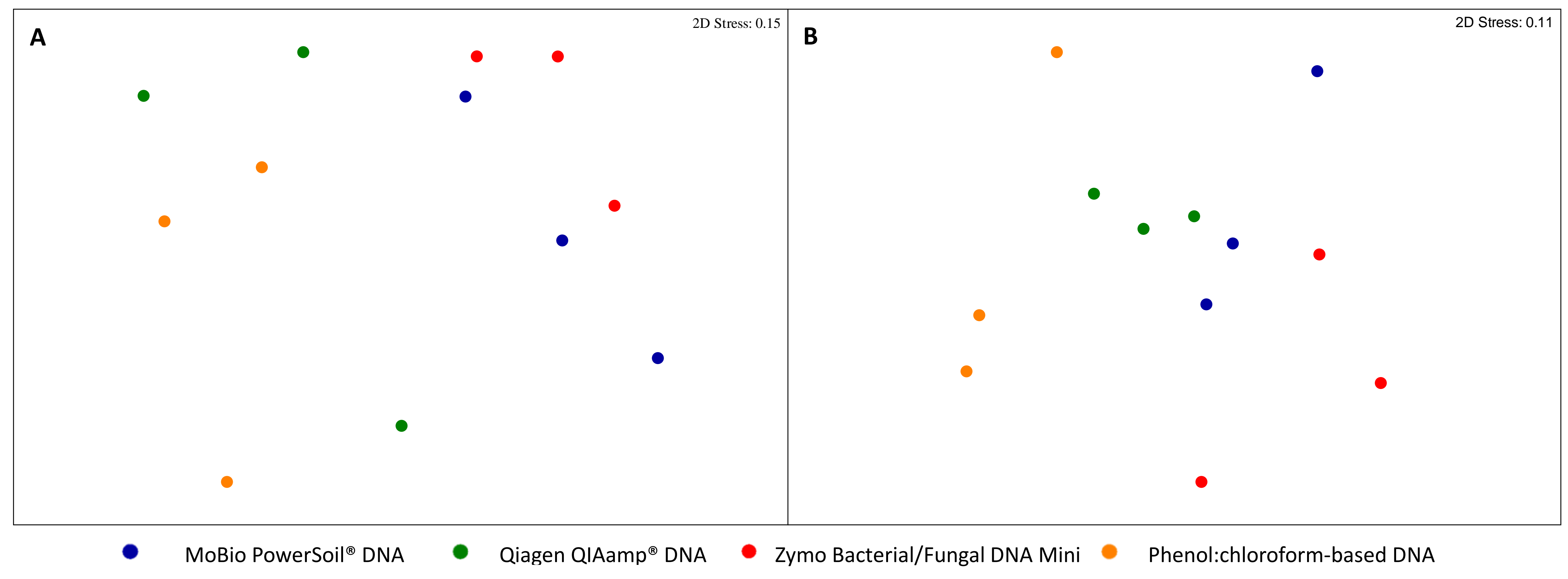


Figure 2.2. Non-metric multidimensional scaling (nMDS) plots comparing relative unweighted UniFrac phylogenetic distances of bacterial communities in (A) plaque and (B) saliva across the four DNA extraction methods.

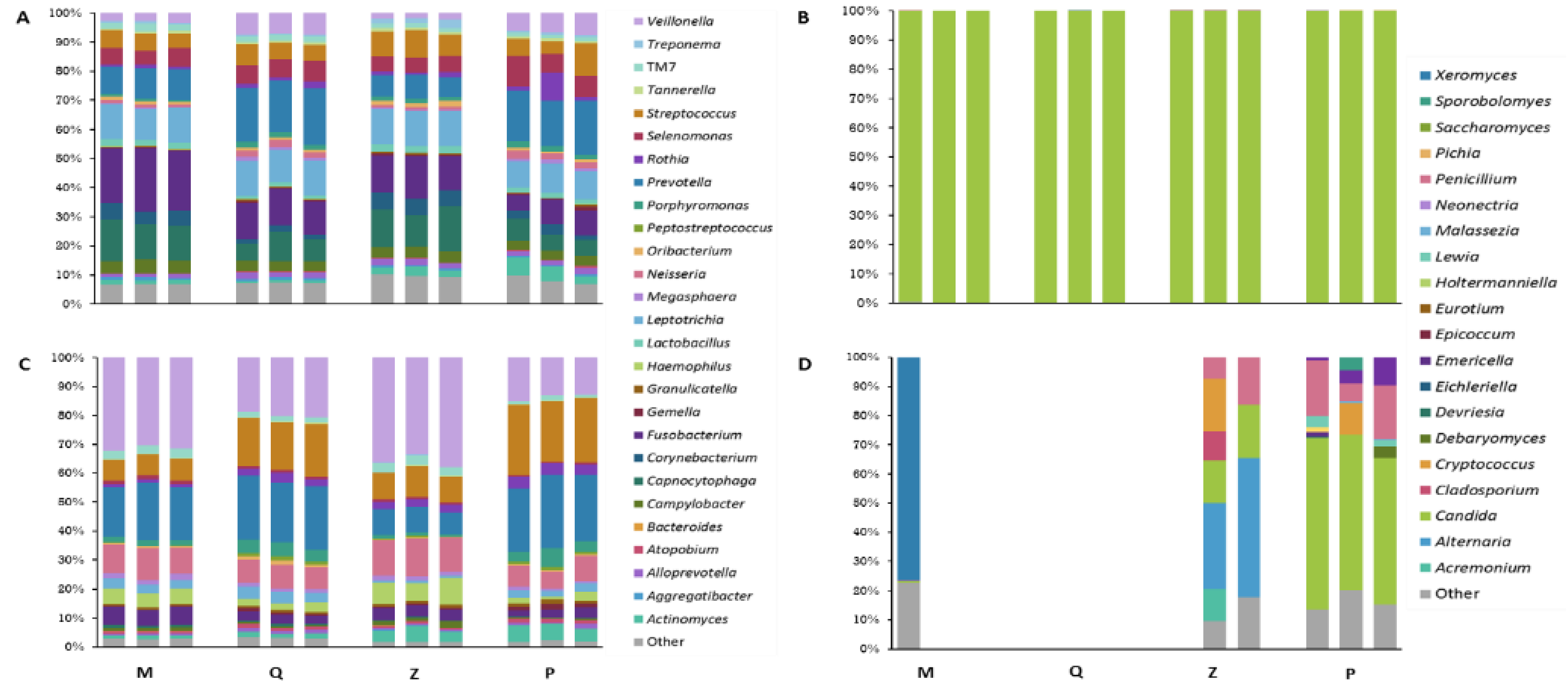


Figure 2.3. Taxa plots summarising the relative abundance of taxon-assigned OTUs identified in pooled homogenates for (A) bacterial genera in plaque; (B) fungal genera in plaque; (C) bacterial genera in saliva and (D) fungal genera in saliva. Each bar represents sequencing from three replicates, rarefied to 6000 sequences per sample for bacterial genera and 959 for fungal genera, with replicates that did not meet these criteria excluded. M: MoBio PowerSoil® DNA Isolation Kit, Q: QIAamp® DNA Mini Kit, Z: Zymo Bacterial/Fungal DNA Mini Prep™, P: Phenol:chloroform-based DNA isolation.

2.3.3 Effect of DNA extraction method on microbial communities in saliva

Purified reactions of the 12 pooled saliva samples amplified with 16S rRNA gene-targeting primers (n = 4 methods × n = 3 replicates) returned a total of 360,337 sequences (221,370 unique), with an average length of 459 bp. Chimeras (1.1% of unique reads) were removed and *de novo* OTU picking of the remaining sequences returned 201 unique OTUs. Sequencing of the ITS1 amplicons from the same 12 saliva samples returned a total of 143,832 sequences (19,694 unique reads, average length 287 bp), ranging from 11 to 86,096 sequences per sample. Chimera removal (0.1% of the unique reads) and *de novo* OTU picking returned 92 unique OTUs.

Similarly to plaque, multiple pairwise comparisons of the four DNA extraction methods returned no significant differences in the numbers of observed OTUs in bacterial communities from saliva samples. The Zymo Bacterial/Fungal DNA Mini PrepTM yielded the highest number of observed bacterial OTUs (122 ± 1.40), followed by the QIAamp® DNA Mini Kit (121 ± 2.50), MoBio PowerSoil® DNA Isolation Kit (120 ± 0.73) and finally phenol:chloroform (112 ± 2.10). There were no significant differences in Chao1, Shannon or Simpson diversity indices (Table 2). PERMANOVA analysis of the saliva bacterial community profiles rarefied to 6,000 sequences per sample for unweighted UniFrac did not exhibit any significant differences in bacterial community profiles across the four DNA extraction methods (Figure 2.2B).

The rarefaction threshold excluded several saliva samples from analyses: 3 × Qiagen QIAamp® DNA Mini Kit, 2 × MoBio PowerSoil® DNA Isolation Kit and 1 × Zymo Bacterial/Fungal DNA Mini PrepTM; the phenol:chloroform-based method was the only technique to yield adequate numbers of sequences from all three replicates for fungal community diversity analyses. Accordingly, the three phenol:chloroform replicates obtained

the highest number of observed fungal OTUs from saliva (23.2 ± 5.20), followed by MoBio PowerSoil® DNA Isolation Kit (14.5, n = 1) and Zymo Bacterial/Fungal DNA Mini PrepTM (11.9 ± 0.90, n = 2). Pairwise comparisons between DNA extraction methods for the average observed fungal OTUs, Chao1, Shannon and Simpson diversity indices did not return any significant differences (Table 2.2).

Neisseria, *Prevotella*, *Streptococcus* and *Veillonella* dominated the bacterial community present in the pooled saliva across all four DNA extraction methods, with the average relative abundance of OTUs assigned to these genera within a method between 7 – 36% (Figure 2.3C). Recovery of genera that comprised ≥ 1% of the saliva bacterial community in this study was consistent across all DNA extraction methods.

My findings using the phenol:chloroform-based approach indicate that the fungal community in the 12 pooled saliva samples was dominated by members of the genus *Candida*, found at >50% in each replicate. The relative abundance of *Penicillium* was 6 – 19%, while 14 – 20% of sequences could not be identified to genus level. *Saccharomyces* and *Malassezia* were only found in phenol:chloroform replicates at <10% and < 1% relative abundance, respectively (Figure 2.3D). Additionally, this method was not only the sole DNA extraction method to yield adequate sequencing reads for fungal diversity analyses, it was the only method in which *Malassezia* was detected (present in all saliva replicates). *Emericella*, *Lewia*, *Malassezia* and *Saccharomyces* were found in at least two of the three replicates, while *Cryptococcus* was identified from a single replicate and *Epicoccum* was identified in low numbers from two of the three phenol:chloroform plaque replicates (Figure 2.3D).

From the Zymo Bacterial/Fungal DNA Mini PrepTM extractions, fungal sequences assigned to the genus *Alternaria*, a ubiquitous plant pathogen, represented 29% and 48% of the relative abundance of the fungal community in two replicates (Figure 3D). *Candida* was the next most

abundant genus, at 15% and 18%. *Cladosporium*, a common indoor and outdoor mould, comprised 10% of sequences in a single replicate but was not found in any other sample that satisfied the rarefication criterion across the four DNA extraction methods. Unassigned sequences made up 9% and 18% of the relative abundance in the two Zymo Bacterial/Fungal DNA Mini PrepTM replicates. The single replicate from MoBio PowerSoil® DNA Isolation Kit to meet our rarefication threshold was largely dominated by *Xeromyces* (77%), a food spoilage mould, which was not present in replicates from the other methods. Additionally, *Candida* and *Penicillium* made up only 0.7% and 0.1% of the respective relative abundances, and sequences to which genus-level identification was not assigned made up 23% (Figure 2.3D).

2.3.4 Quality and yield of DNA extracted from control fungal strains

No significant differences were detected in DNA yield or A260/280 and A260/230 ratios across the four methods when attempting to extract fungal DNA from ATCC32045 *C. neoformans* and ATCC10002 *P. chrysogenum* (Appendix A, Table A1).

2.4 Discussion

Our understanding of the oral microbiome in health and disease is dependent on obtaining an accurate description of the oral microbial community. The many variations in DNA extraction methodology and sequence curation steps that occur between laboratories diminishes consistency and comparability between studies, and may confound results. In this chapter, I examined how different DNA extraction approaches influence our assessment of oral microbial communities by comparing bacterial and fungal diversity and composition using different DNA extraction protocols.

My findings suggest that the overall quality and yield of gDNA is influenced by DNA extraction approach. The enzymatic approach employed by the Qiagen QIAamp® DNA Mini Kit produced good quality gDNA with significantly greater yield compared to the other three DNA extraction methods. An enzymatic approach was previously demonstrated to enhance extraction of gDNA from saliva, when compared with mechanical lysis (Sohrabi et al., 2016); this is consistent with the highest gDNA yield obtained using the Qiagen QIAamp® DNA Mini Kit in this study, but does not hold true for the phenol:chloroform-based approach. Therefore, these quantitative results may factor into method selection when gDNA is required for multiple downstream applications.

The diversity of bacterial communities in plaque and saliva was largely unaffected by DNA extraction method, with no significant differences in the relative abundance of taxon-assigned OTUs across all four DNA extraction methods. However, comparison of relative abundances at genus-level would provide additional information, while increasing the number of replicates used for each method would improve the power of this study. The four DNA extraction methods tested, with different cell lysis approaches, did not significantly impact on microbial

diversity in plaque and agreed with previous findings that DNA extraction method is not an influencing factor on plaque community composition (Abusleme et al., 2014). The results presented in this chapter are consistent with previous studies that reported *Capnocytophaga*, *Fusobacterium*, *Leptotrichia*, *Prevotella*, *Selenomonas* and *Streptococcus* as dominant members of the bacterial dental plaque community (Xiao, Ran, Huang, & Liang, 2016; Xu et al., 2015). The bacterial communities in saliva were consistent between the four DNA extraction methods. *Neisseria*, *Prevotella*, *Streptococcus* and *Veillonella* were the most abundant genera detected, in agreement with previous studies that identified these genera as dominant members of the salivary bacterial microbiome (Segata et al., 2012).

Diversity assessment of the pooled plaque mycobiome also failed to identify discrepancies between DNA extraction methods, however as this community was > 99% *Candida* species, the diversity of our homogenate may have been inadequate for a rigorous comparison of methods. Given the current lack of available data on the plaque mycobiome, there are no other ITS1 sequencing-based studies against which these results can be validated. However, an 18S rRNA gene-based study identified *C. albicans* as the dominant species in the subgingival plaque of HIV patients with low viral loads and high CD4 levels, which may support the current findings (Aas et al., 2007).

The performance of the three commercial DNA extraction kits fell short of the phenol:chloroform-based isolation method when analysing the salivary mycobiome. Various factors could account for this observation. Firstly, given that yeasts and other fungi often have a cell wall which is harder to lyse than bacterial cell walls, the kits utilised in this study may not be optimised for fungal DNA extractions (although the name of at least one of the kits implies otherwise). As suggested by Dupuy and colleagues (2014), cell lysis methodology is likely to have a significant effect on identifying *Malassezia* as a fungal community member in saliva. The unique presence of *Malassezia* in the phenol:chloroform extractions suggests that

this approach was the only one of the four studied to reliably detect this genus in saliva. Data obtained using the phenol:chloroform-based method confirmed most genera of the fungal community common to the only two previous studies of the human salivary mycobiome (Dupuy et al., 2014; Ghannoum et al., 2010): *Candida*, *Emericella*, *Lewia*, *Malassezia* and *Saccharomyces*. In contrast to the previous studies, *Cladosporium/Davidiella*, *Fusarium/Gibberella*, *Aureobasidium* and *Epicoccum* were absent in all replicates. Given the variation in salivary mycobiome data between methods, the ability of the four DNA extraction approaches employed here to extract fungal DNA warranted investigation. Data suggested that certain extraction approaches are more efficient than others when attempting to extract gDNA from fungal cells.

Our understanding of the oral mycobiome is further hindered by the inherent obstacles faced when studying fungal communities. Several factors contribute to a general lack of human oral mycobiome studies, including: the need for well-curated databases comparable to those used for bacterial 16S rRNA gene-based studies, the varying use of primers targeting different regions of the ITS operon between studies, and the unreliable alignment of these targeted ITS sequences.

In this study, oral bacterial communities were largely unaffected by DNA extraction approach, however the salivary mycobiome was affected by extraction method. The phenol:chloroform-based DNA isolation method tested was the only one of the four assessed DNA extraction methods to yield sufficient fungal sequences for analysis from all three saliva replicates. Such discrepancies reinforce the importance of selecting an appropriate DNA extraction method to study oral microbial communities, which should be guided by its ability to produce sufficient and accurate data that address the research question.

Chapter 3

Microbial and inflammatory-based salivary biomarkers of head and neck squamous cell carcinoma

This chapter is a modified version of: Vesty, A., Gear, K., Biswas, K., Radcliff, F.J., Taylor, M.W., Douglas, R.G. (2018). "Microbial and inflammatory-based salivary biomarkers of head and neck squamous cell carcinoma". ***Clinical and Experimental Dental Research*** *4(6): 255-262. doi: 10.1002/cre2.139*

3.1 Introduction

Traditionally, the major risk factors associated with head and neck cancer are tobacco use and heavy alcohol consumption. More recently, viral infections – particularly infection with human papilloma virus subtype 16 – have been implicated in the increasing incidence of this group of cancers, most notably in younger adults (Chaturvedi et al., 2008). Emerging hypotheses suggest that oral microbial imbalances and variations to microbial community structure may modulate viral infections by regulating host susceptibility to oncogenic viruses (Vyshenska et al., 2017).

Salivary microbes are gaining attention as potential diagnostic markers for oral cancers. Recent studies suggest that increases in the relative abundances of several bacterial genera (including *Dialister*, *Selenomonas*, *Streptococcus* and *Treponema*) occur in the saliva of oral cancer patients, when compared to healthy controls (Guerrero-Preston et al., 2016; Pushalkar et al., 2011; Wolf et al., 2017). While *Streptococcus anginosus* has been implicated in head and neck squamous cell carcinoma (HNSCC) (Morita et al., 2003; Tateda et al., 2000), a majority of the focus has been on *Porphyromonas gingivalis*, which displays oncogenic properties directly linked to oral carcinogenesis (Ha et al., 2015). Detection of *P. gingivalis* and other oral pathobionts in healthy subjects makes it difficult to attribute carcinogenic potential to specific bacteria (Katz, Onate, Pauley, Bhattacharyya, & Cha, 2011). *Candida albicans* is implicated in carcinogenesis because of its capacity to produce carcinogenic levels of acetaldehyde and induce host immune responses (Ramirez-Garcia et al., 2016). Candidiasis may play a role in the malignant transformation of oral lesions (Bakri, Hussaini, Holmes, Cannon, & Rich, 2010). Furthermore, *C. albicans* is overrepresented (average relative abundance of 61.2%) in the mycobiome of oral squamous cell carcinoma (OSCC) biopsies (Perera et al., 2017).

Oral pathobionts elicit a host inflammatory response characterised by an increase in cytokines, chemokines and growth factors that promote cell survival and proliferation, actions which may contribute to carcinogenesis (Cekici et al., 2014). A review of several studies reports higher concentrations of inflammatory cytokines in the saliva of oral cancer patients, particularly interleukin (IL)-1β, IL-6 and IL-8, relative to healthy controls (Cheng, Rees, & Wright, 2014). It is unclear what induces these inflammatory-based biases but oral microbes may play a role.

Investigating oral and oropharyngeal carcinogenesis by studying oral microbiology or immunology is potentially confounded by oral health, given that HNSCC patients often present with poor oral health (Galvão-Moreira & Da Cruz, 2016; Tezal et al., 2009). Poor oral health may be the factor that leads to differences in the microbiology and immune response seen between patients with HNSCC and healthy controls.

In order to unmask oral health-dependent bias and help decipher the link between HNSCC, oral microbes and inflammation, this chapter investigates oral bacterial and fungal communities in conjunction with salivary inflammatory markers in three disease groups: HNSCC patients, dentally compromised patients, and healthy controls.

3.2 Materials and methods

3.2.1 Participant populations and sample collection

3.2.1.1 Head and neck cancer patients

Fourteen patients (11 male and 3 female, aged 49 – 81 years) diagnosed with HNSCC were recruited for this study during routine pre-radiotherapy dental assessments at the Oral Health Unit, Green Lane Hospital, Auckland, New Zealand. This group of patients included two current smokers, six ex-smokers, five non-smokers and one patient for whom this information was not obtained. Dental and oral health information was extracted from clinical notes and orthopantomograms (Appendix B, Table B1).

3.2.1.2 Dentally compromised patients

Nine patients (seven male and two female, aged 28 – 68 years) who attended Relief of Pain Clinics at the Oral Health Unit, Green Lane Hospital, Auckland, New Zealand were recruited to participate in this study. Two patients were current smokers, three ex-smokers and four non-smokers. Based on the World Health Organisation definition of oral health (World Health Organisation, 2012), this group of patients was deemed dentally compromised as the result of periodontal disease and/or tooth decay that caused significant pain and necessitated tooth extraction at this clinic. Samples were collected prior to clinical intervention.

3.2.1.3 Healthy controls

Seven volunteers from The University of Auckland, New Zealand participated in this study representing healthy controls. To maintain anonymity, participant demographics including age and sex were not assigned to samples, however participants were non-smokers and aged approximately 20 – 35 years at the time of sample collection.

3.2.1.4 Sample collection

Approximately 1 mL of unstimulated whole saliva was collected in a sterile container from each participant and divided into 200 µL aliquots. All samples were frozen at -20°C until further processing. Ethical approval for this study was granted by the Southern Health and Disability Ethics Committee (14/STH/121). Written informed consent was obtained from all participants.

3.2.2 DNA extraction, amplification of target genes and sequencing preparation

3.2.2.1 DNA extraction

Genomic DNA (gDNA) was extracted using the phenol:chloroform-based DNA isolation method described in 2.2.2.5. In brief, this phase separation DNA isolation method utilised an enzymatic (lysozyme and Proteinase K) and freeze-thaw approach to release nucleic acids from the cells, which were separated out with buffer-saturated phenol and chloroform:isoamyl alcohol. Nucleic acids were precipitated with isopropanol, pelleted and washed with ethanol before re-suspension in 20 µL of sterile water. PCR-grade water was included as a negative control for the DNA extraction procedure. The quality and quantity of gDNA were assessed

spectrophotometrically using the NanoDrop® ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA).

3.2.2.2 16S rRNA gene amplification

The V3 – V4 region of the bacterial 16S rRNA gene was amplified using the primers described in 2.2.3.1, namely S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 (Klindworth et al., 2013). Each PCR reaction contained: HotStar PCR Buffer (1×), 2 mM $MgCl_2$, 0.5 mM of each dNTP, 0.5U HotStar DNA Polymerase (Qiagen, Hilden, NRW, Germany), 0.2 μM of each primer, 1 μL of gDNA template and PCR-grade water to a final volume of 25 μL. Positive and negative PCR controls were included (*Escherichia coli* and water, respectively). PCR was performed using the following conditions: initial denaturation at 95˚C for 15 min, followed by 32 cycles of denaturation (95˚C for 30 s), annealing (55˚C for 30 s) and extension (70˚C for 40 s), with a final extension step at 70˚C for 3 min.

3.2.2.3 ITS1 region amplification

Illumina-compatible primers ITS1F and ITS2 (described in 2.2.3.2) were substituted in place of the 16S rRNA primers in the reaction mix to amplify the internal ITS1 region of fungal DNA. *C. albicans* was used as a positive control and water as a negative control. PCR was performed using the following conditions: initial denaturation at 95˚C for 15 min, followed by 38 cycles of denaturation (95˚C for 30 s), annealing (50˚C for 30 s) and extension (70˚C for 40 s), with a final extension step at 70˚C for 3 min.

3.2.2.4 Preparation of PCR amplicons for Illumina MiSeq

Duplicate PCR reactions were prepared for sequencing as described in 2.2.3.3. Samples were sequenced using the Illumina MiSeq platform, through New Zealand Genomics Ltd. Sequencing data analysed are available from the NCBI SRA, uploaded under accession number SRP126472.

3.2.3 Bioinformatic analyses

3.2.3.1 Processing of bacterial 16S rRNA gene sequence data

Using a USEARCH (v9.2) pipeline similar to that described in 2.2.4.1, sequences were merged and filtered to remove poor quality sequences, singletons and sequences < 350 bp (Edgar, 2010). Operational taxonomic units (OTUs) were defined based on 97% 16S rRNA gene sequence similarity and simultaneously checked for chimeras as described in 2.2.4.1. A second chimera check was performed against the HOMD (v13.2) (Chen et al., 2010). Taxonomic assignment was performed in QIIME (v1.9) through the RDP classifier using the HOMD (v13.2) (Caporaso et al., 2010; Wang et al., 2007). Sequences were rarefied to 1996 reads per sample for subsequent analyses.

3.2.3.2 Processing of fungal ITS1 sequence data

Fungal sequences were processed using a pipeline to that described in 2.2.4.2: data were filtered to remove sequences < 100 bp and rare fungal sequences were filtered out using an abundance threshold of four sequences (Dupuy et al., 2014). OTUs were defined based on 97% sequence similarity and simultaneously checked for chimeras, followed by a second chimera check using

UNITE (v7.0) as a reference. Taxonomic assignment was performed in QIIME (v1.9) using the BLAST method against UNITE (v7.0). Non-fungal derived sequences were removed before subsampling to 229 reads per sample.

3.2.3.3 Estimation of diversity metrics

Alpha diversity metrics and Bray-Curtis dissimilarity were estimated independently for bacterial and fungal communities in QIIME (v1.9) using the core_diversity_analyses.py command (Caporaso et al., 2010). Beta diversity was visualised in R (v3.4.2) using the ggplot2 and vegan packages; specifically, Bray-Curtis dissimilarity was used to generate non-metric multidimensional scaling (nMDS) plots using the metaMDS function with a maximum of 999 restarts (R Core Team, 2017).

3.2.4 Cytometric bead array

Thawed saliva was diluted 1:1 with phosphate buffered saline and prepared for flow cytometry (according to manufacturer's instructions) using the BDTM Cytometric Bead Array Human Inflammatory Cytokine Kit (BD Biosciences, NJ, USA) to quantitatively measure IL-8, IL-1β, IL-6, IL-10, IL-12p70 and tumour necrosis factor (TNF). Samples were acquired on an LSR II using FACSDivaTM Software (v6.1.1) (BD Biosciences, NJ, USA) and sample concentrations interpolated from standard curves for each cytokine using the radioimmunoassay analysis option in GraphPad Prism (v7.03).

3.2.5 Statistical analyses

3.2.5.1 Bacterial and fungal community data

Bacterial and fungal community data were compared independently for each group. The relative abundance of individual taxon-assigned OTUs was statistically assessed using paired, two-tailed *t*-tests with Bonferroni adjustment for multiple pairwise comparisons, with a significance value of 0.05. Alpha diversity metrics were assessed using a non-parametric *t*-test. Bray-Curtis distance matrices were used to statistically assess beta diversity with pairwise, PERMANOVA in PRIMER v6 software, using type III (partial) sum of squares with unrestricted permutation of raw data and 999 permutations. Homogeneity of dispersions was calculated using PERMDISP in PRIMER v6 based on pairwise comparisons of deviations from the median with 999 permutations. Contribution of disease group to partitioning of Bray-Curtis distance matrices was assessed using the *Adonis* function in the Vegan package in R, with 999 permutations.

3.2.5.2 Linear discriminant analysis effect size

Linear Discriminant Analysis (LDA) Effect Size (LEfSe) was used to identify bacterial genera that differentiated the groups, based on rarefied relative abundance data (Segata et al., 2011). The non-parametric factorial Kruskal-Wallis sum-rank test ($\alpha \leq 0.05$) with a subsequent (unpaired) Wilcoxon rank-sum test ($\alpha \leq 0.05$) and an all-against-one multi-class analysis were used; only genera reaching an LDA score threshold of ≥ 3 were reported.

3.2.5.3 Inflammatory cytokine concentrations

IL-1β and IL-8 concentrations were $\log_{10}$ transformed to produce normally distributed data and excluded if the relevant cytokine concentration was below the limits of detection. IL-1β and IL-8 were compared between the three disease groups using a two-way ANOVA and Tukey's post-hoc test for multiple comparisons of means, with a 95% family-wise confidence interval. Spearman's rank correlation coefficients (ρ) and corresponding p values were calculated with a null hypothesis in R to determine the statistical dependence between IL-1β and IL-8 concentrations and the relative abundance of the most abundant 30 bacterial and five fungal genera. *Treponema* was also included in this analysis due to its potential clinical relevance and *Candida* was included at OTU level. Significant correlations ($p < 0.01$) were visualised in R using corrplot, with hierarchical clustering (Wei & Simko, 2016).

3.3 Results

3.3.1 Sequencing data

After post-processing and filtering, 533,317 bacterial 16S rRNA gene sequences were obtained, yielding 173 OTUs, classified into 68 genera. Fungal data returned 1,036,947 sequences of which 8,435 (0.8%) were non-fungal derived and therefore removed. Of the 1,028,512 fungal sequences that were retained, 44 OTUs from 34 genera were detected. Rarefaction curves based on the 'observed species' metric indicated that sequencing depth was sufficient to capture the vast majority of bacterial and fungal diversity (Appendix B, Figure B1).

3.3.2 Bacterial community profiles

Bacterial communities were largely dominated by *Streptococcus*, which accounted for an average of 47% of sequences across the three groups. *Prevotella*, *Neisseria*, *Rothia* and *Veillonella* were also dominant contributors to the bacterial communities in all groups, representing an average sequence abundance of 12%, 8%, 5% and 4% respectively (Figure 3.1A). Pairwise comparisons of the relative abundance of individual taxon-assigned OTUs identified no significant differences between the three disease groups. LEfSe analyses indicated that the genus *Treponema* was associated with the dentally compromised patients,

while *Actinomyces* and *Fusobacterium* were associated with the healthy controls (Appendix B, Figure B2). No genus was significantly associated with HNSCC patients.

3.3.3 Bacterial diversity

Pairwise comparisons of bacterial alpha diversity metrics failed to identify any significant differences between groups, although the number of 'observed species' in the dentally compromised group was slightly higher at 63.7 ± 14.6, compared to the HNSCC (56.8 ± 14.4) and healthy control (54.4 ± 20.9) groups (mean ± SD). Based on Bray-Curtis dissimilarity, disease group accounted for approximately 11% (R^2 = 11%) of the variation in bacterial data. PERMANOVA indicated that a significant proportion of bacterial community variation was attributable to differences between the HNSCC patients and the healthy control group (p = 0.03, Figure 3.2A). No other significant differences were identified in remaining pairwise PERMANOVA comparisons. Multivariate dispersions were compared based on the distance of each sample to the median of their disease group. The dentally compromised patients (0.37 ± 0.04) had the greatest average deviations from the median (mean ± SE). However, pairwise comparisons between disease groups revealed no significant differences, with deviations of 0.34 ± 0.03 for HNSCC patients and 0.32 ± 0.03 for healthy controls.

3.3.4 Fungal community profiles

Five of the seven healthy controls were excluded from fungal community analyses due to insufficient sequence reads, which probably reflects an absence or comparatively lower abundance of fungi in this group. There was no consistency between the two healthy control samples included in the fungal analysis, with one dominated by *C. albicans* and the other by *Saccharomyces* (Figure 3.1B). The remaining samples from all disease groups were largely dominated by *Candida* (Figure 3.1B). NCBI BLAST checks of OTUs belonging to the *Candida* genus revealed *C. albicans* (100% sequence identity) comprised on average about 80% of obtained sequence reads across all disease groups. However, fungal communities in HNSCC patients were almost entirely comprised of *C. albicans* (individual relative abundances of 96 – 100%). There was one exception where the mycobiome comprised 99.6% *C. tropicalis* (NCBI BLAST 100% sequence identity), however no obvious characteristic differentiated this HNSCC patient from the rest.

3.3.5 Fungal diversity

Dentally compromised patients exhibited the most diverse fungal communities, which included sequences belonging to the fungal genera *Candida*, *Fusarium*, *Mycosphaerella*, *Saccharomyces* and *Wickerhamomyces* (Figure 3.1B). This finding was reflected in alpha diversity metrics, with significantly more fungal 'observed species' found in the dentally compromised patients (5.3 ± 3.8) than in HNSCC patients (1.7 ± 0.5) ($p = 0.003$). Only two

samples from the healthy control group, which reached the rarefication threshold, were included in this analysis (1.7 ± 0.7).

Disease group also significantly contributed to variance in fungal communities (p = 0.03), explaining approximately 19% of observed mycobiota variation. PERMANOVA revealed that disease group was a significant driver of fungal community structure, with a significant difference identified between the HNSCC and dentally compromised patients (p = 0.04, Figure 3.2B). This result was supported by a pairwise comparison of dispersions based on deviations from the median for each disease group, with a difference trending towards significance between the HNSCC and dentally compromised patients (p = 0.06).

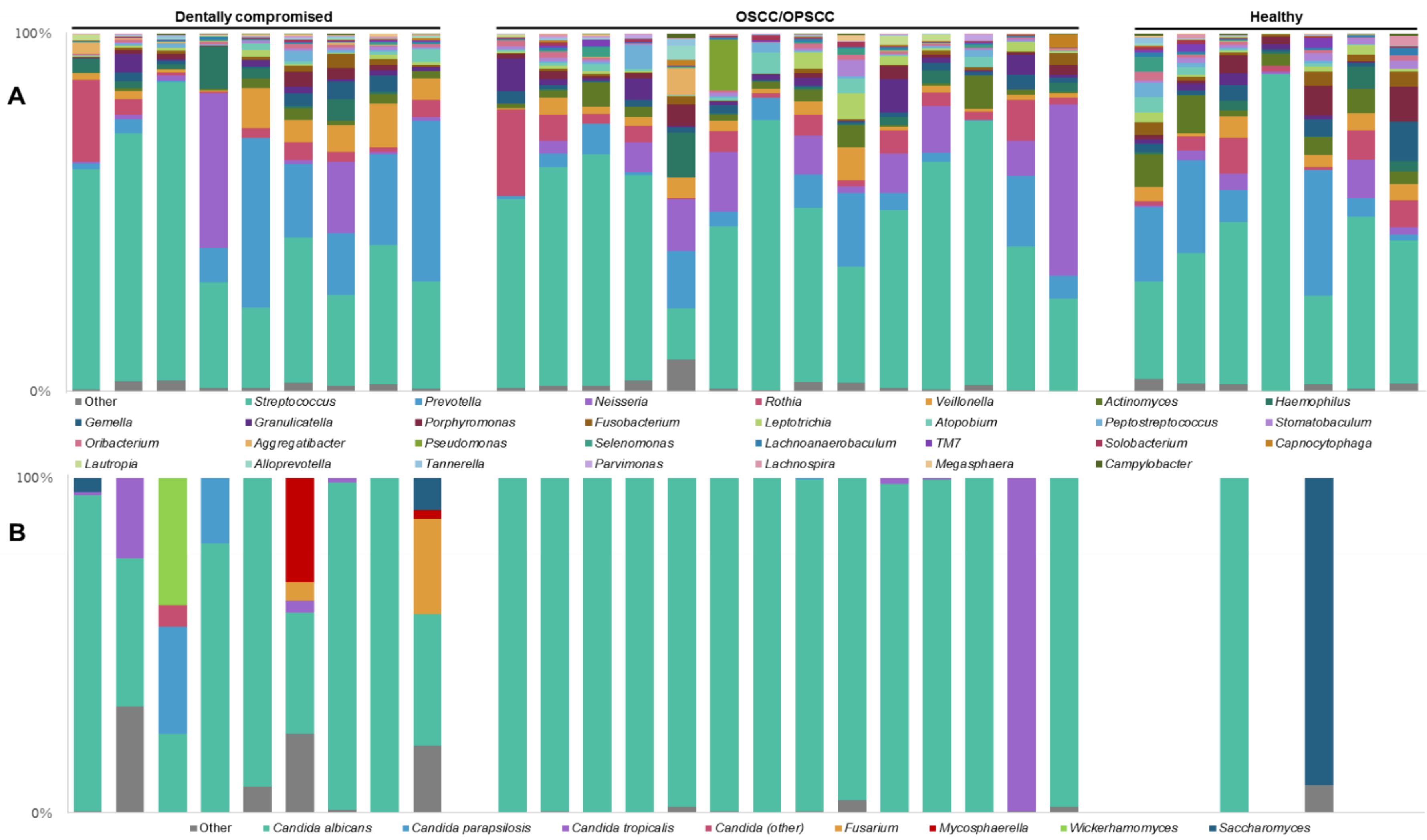


Figure 3.1. Genus-level summary of microbial communities in saliva by disease group. Each bar in (A) reflects the bacterial community in one sample and is aligned with its corresponding fungal community in (B). (A) 30 most abundant bacterial genera (on average); (B) five most abundant on average) fungal genera, excluding samples that did not meet the fungal rarefication criterion.

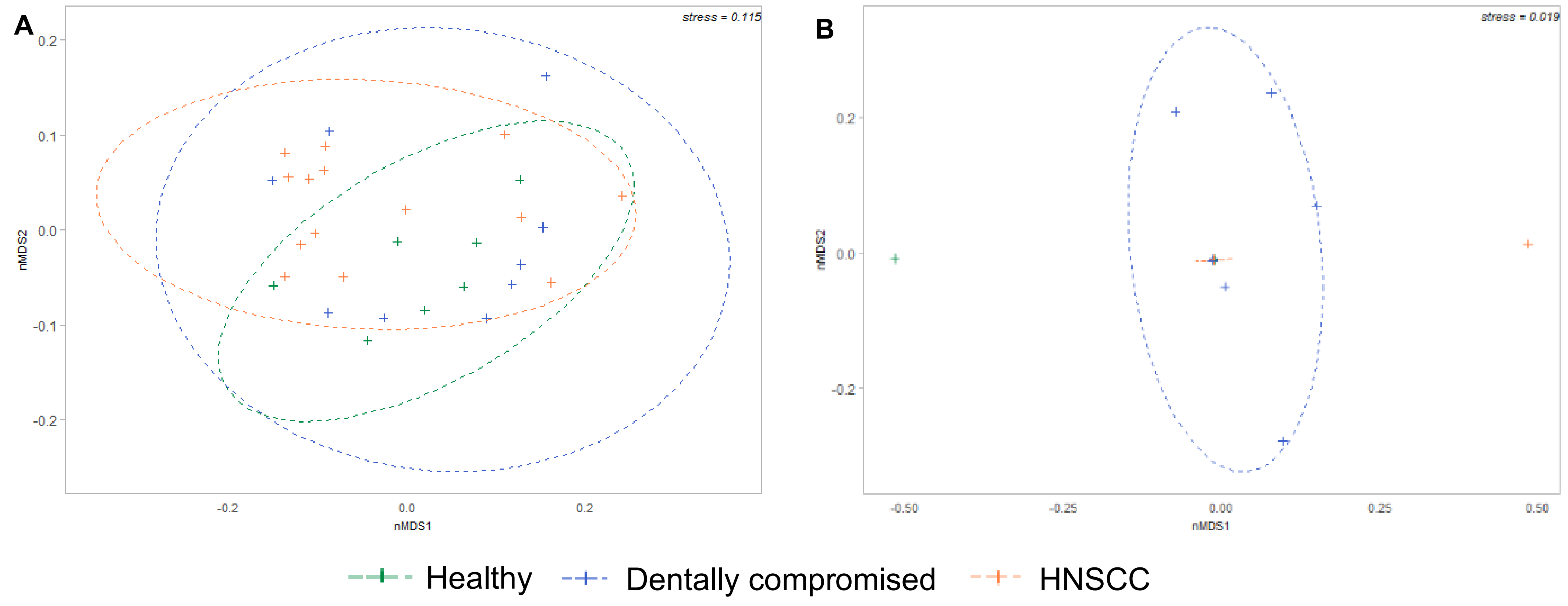


Figure 3.2. Bray-Curtis dissimilarity nMDS plots by disease group depicting: (A) bacterial beta diversity and (B) fungal beta diversity. Ellipses represent a 95% confidence interval for each disease group. Data that failed subsampling thresholds were excluded, therefore no ellipse was calculated for healthy controls (n = 2) in (B).

3.3.6 Comparison of inflammatory cytokine concentrations

Three of the six tested inflammatory cytokines were detectable in saliva samples: IL-1β, IL-6 and IL-8. Across all groups, IL-8 was the most frequently detected, found in 28 of the 30 subjects (93%), followed by IL-1β in 26 subjects (87%); IL-6 was only detected in seven of the 30 subjects (23%), six of whom were HNSCC patients (Table 3.1). Comparisons for each inflammatory cytokine revealed the concentration of IL-1β was significantly higher in the HNSCC and dentally compromised patients, when these two disease groups were individually compared to the healthy controls (Table 3.2). The concentration of IL-1β was 5.1 times higher in the HNSCC patients when compared to healthy controls, yet no significant differences were found when the HNSCC patients were compared to the dentally compromised group. Comparison of IL-8 concentrations produced similar results: IL-8 was significantly higher (6.7 times) in the HNSCC patients compared to healthy controls and 6.5 times higher in the dentally compromised patients compared to healthy controls (Table 2). No significant difference in IL-8 concentration was detected between the HNSCC and the dentally compromised patients (Table 3.2).

Table 3.1 Summary of detectable inflammatory cytokines.

	HNSCC	**Dentally compromised**	**Healthy**
IL-1β[a]	11/14 (79%)	9/9 (100%)	6/7 (86%)
Concentration IL-1β[b]	5.1 ± 0.9	5.2 ± 1.5	3.5 ± 0.9
IL-6[a]	6/14 (43%)	1/9 (11%)	0/7 (0%)
Concentration IL-6[b]	-0.8 ± 1.2	-0.1 ± N/A	N/A
IL-8[a]	13/14 (93%)	9/9 (100%)	6/7 (86%)
Concentration IL-8[b]	7.2 ± 1.4	7.1 ± 1.2	5.3 ± 2.0

[a]*Number of patients cytokine detected in/total patients in disease group*

[b]*Cytokine concentration (log pg/mL) (mean ± SD)*

Table 3.2 Two-way ANOVA summary of IL-1β and IL-8 concentration comparisons.

	HNSCC vs. healthy	**Dentally compromised vs. healthy**	**HNSCC vs. dentally compromised**
Difference IL-1β[a]	5.1	5.4	0.93
p value[b]	**0.03**	**0.02**	0.99
Difference IL-8[a]	6.7	6.5	1.0
p value[b]	**0.04**	**0.05**	1.00

[a]*Fold difference in cytokine concentration A to B (A vs. B)*

[b]*Significant p values expressed in bold (α = 0.05). Tukey's post-hoc test, 95% confidence interval*

3.3.7 Correlation of microbial relative abundance data to IL-1β and IL-8 concentrations

Calculation of Spearman's rank correlation coefficients (ρ) comparing IL-1β and IL-8 with the 30 most abundant bacterial genera, plus *Treponema* and the five most abundant fungal genera (*Candida*-assigned sequences at OTU level) returned ρ values ranging from -0.56 to 0.37. The strongest positive correlation occurred between IL-8 and *C. albicans* ($\rho = 0.37$, $p = < 0.001$); IL-1β also showed significant positive correlation with the relative abundance of *C. albicans* ($\rho = 0.30$, $p = < 0.001$). Positive correlations between bacterial genera and IL-1β and IL-8 concentrations returned ρ values of ≤ 0.30, suggesting only weak correlations, as summarised in Figure 3.3. The relative abundance of the periopathogenic genera *Porphyromonas*, *Tannerella* and *Treponema* showed no significant positive correlations with cytokine concentrations. However, the relative abundances of these genera were significantly positively correlated with each other (Figure 3.3).

Several bacterial genera were negatively correlated with IL-1β and IL-8, with the strongest negative correlations occurring between IL-1β and *Lachnoanaerobaculum* ($\rho = -0.56$, $p = <$

0.001) and *Actinomyces* and IL-8 (ρ = -0.42, $p < 0.001$). Eight bacterial genera were significantly negatively correlated with both IL-1β and IL-8 ($p < 0.01$): *Actinomyces*, *Alloprevotella*, *Lachnoanaerobaculum*, *Megasphaera*, *Prevotella*, *Selenomonas*, *Stomatobaculum* and a genus within the candidate phylum Saccharibacteria (formerly TM7). Multiple significant correlations occurred between the relative abundances of individual microbial genera (summarised in Figure 3.3), and notably included a strong negative correlation between *Streptococcus* and *Prevotella*, two genera that dominated the salivary bacterial communities (ρ = -0.80, p = < 0.001).

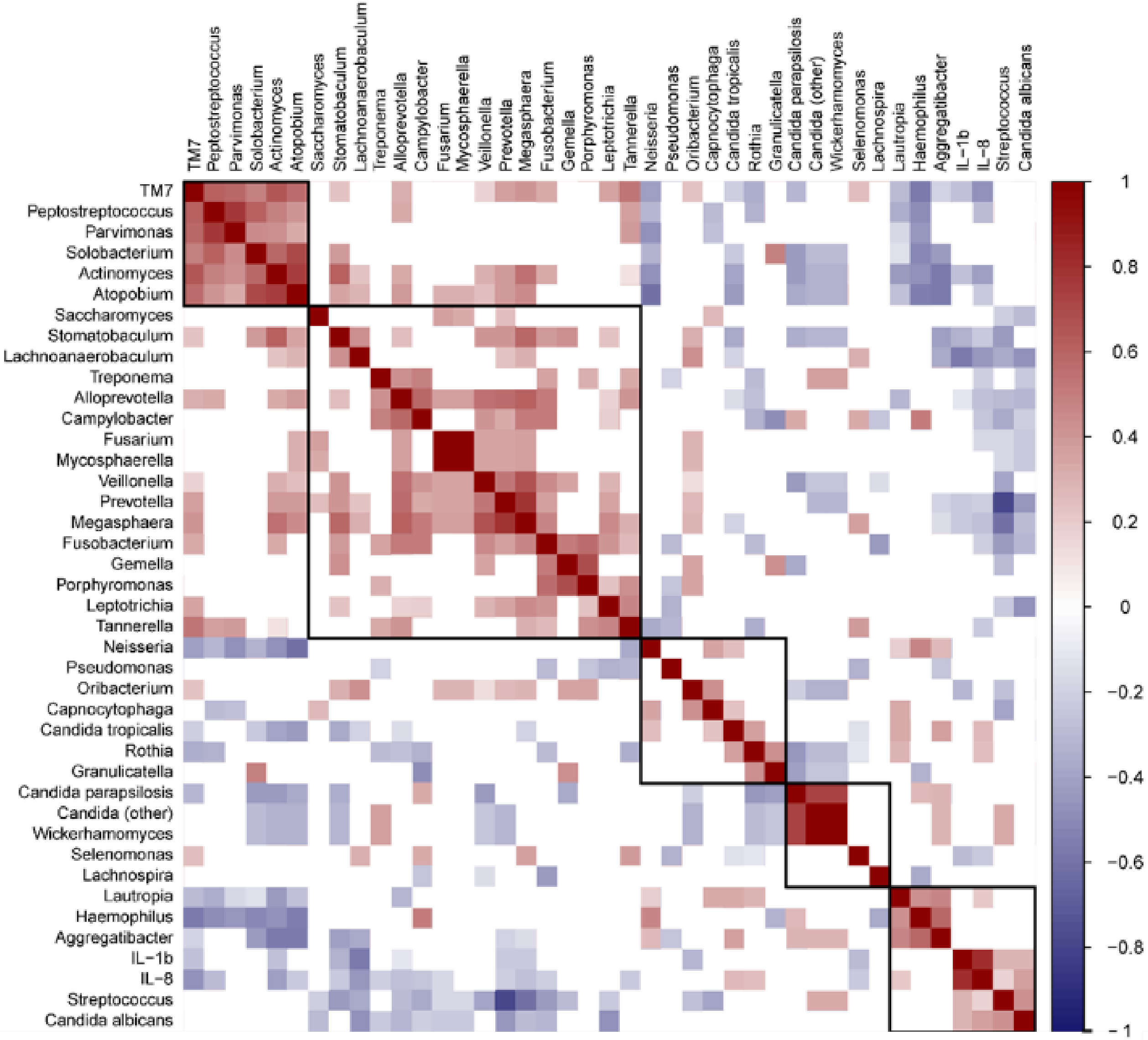


Figure 3.3. Correlation matrix visualising significant correlations ($p < 0.01$) between microbial relative abundance data and the concentrations of IL-1β and IL-8. Positive correlations are visualised in shades of red and negative correlations in shades of blue; correlations where the p value was ≥ 0.01 are left blank. Correlations are ordered by hierarchical clustering, with clusters outlined.

3.4 Discussion

The role of oral microbes in the pathogenesis of HNSCC is not well understood. Generally, it is considered that oral microbes contribute to carcinogenesis, potentially accounting for differences observed in the oral microbial communities of head and neck cancer patients. However, it could also be considered that oral microbial communities might be modified as a *consequence* of the cancer. Saliva-based microbiome studies have not yielded a consensus but do suggest that overall bacterial community composition, rather than the presence of a single pathogen, may be important in head and neck cancer. However, such conclusions can be confounded by the choice of control group, and may simply reflect oral health-related differences rather than differences related to cancer *per se*. To clarify this issue, a comparison to a group of dentally compromised patients in addition to healthy controls was included in this chapter.

PERMANOVA analysis of beta diversity metrics generated in this study indicated that there was a significant difference in salivary bacterial community structure between healthy controls and HNSCC patients ($p = 0.03$). However, no significant variations were detected in the bacterial beta diversity profiles of the HNSCC and dentally compromised patients, suggesting similarity in the bacterial communities of these two patient groups. Based on genus-level LEfSe analysis, HNSCC patients had no distinguishing bacterial characteristics.

Although several studies have focused on deciphering the bacterial features of oral cancers, far less attention has been devoted to the oral mycobiome. I found that *C. albicans* comprised 96 – 100% of fungal sequences in the saliva of most HNSCC patients, compared with an average of 80% of the mycobiome of all subjects reaching the rarefication threshold. The significantly more diverse mycobiome in dentally compromised patients highlights the overrepresentation

of *C. albicans* in HNSCC patients. *C. albicans* has been recognised as an etiological factor in oral carcinogenesis, due to its ability to induce host inflammatory responses (Ramirez-Garcia et al., 2016). This observation was supported by my finding that the relative abundance of *C. albicans* was positively correlated with the concentrations of IL-1β and IL-8 in saliva. However, the correlations between *C. albicans* and IL-1β and IL-8 were not particularly strong, potentially reflecting the presence of this OTU in most subjects.

Detection of IL-6 was most frequently observed in the HNSCC group and this cytokine has previously been implicated as a potential salivary biomarker of OSCC (Cheng et al., 2014). *In vitro*, IL-6-induced inflammation promotes tumorigenesis in oral cancer cells via aberrant DNA methylation (Gasche, Hoffmann, Boland, & Goel, 2011). IL-1β and IL-8 inflammatory profiles of HNSCC patients suggest the presence of comparable levels of inflammation to dentally compromised patients, with concentrations significantly higher in both of these patient groups when compared independently to healthy controls. Smoking has been linked to lower concentrations of IL-1β, IL-6 and IL-8 in saliva (Rathnayake et al., 2013), and although the healthy control group in this study was comprised only of non-smokers, this group still had the lowest average salivary concentrations of these cytokines. The presence of smokers/ex-smokers in the HNSCC and dentally compromised groups (and absence in the healthy controls) may reflect the epidemiology of head and neck cancer and periodontal disease, and that tobacco use is a strong risk factor for both (Petersen & Ogawa, 2012; Sturgis, Wei, & Spitz, 2004). Periodontal disease is linked with higher salivary concentrations of IL-1β and IL-8 (Jaedicke, Preshaw, & Taylor, 2016), yet in this study it was unclear whether inflammatory profiles were driven by differences in the respective microbial communities, with only weak associations detected between microbial relative abundances and cytokine concentrations.

The results presented in this chapter suggest that the use of salivary bacterial communities as a biomarker of HNSCC is limited due to its reduced distinguishability from dentally

compromised patients. Similarly, differentiation of HNSCC and dentally compromised patients based on inflammatory cytokines is limited, with only healthy controls significantly differentiated. However, application of IL-6 as a biomarker for HNSCC is potentially relevant due to its increased detection in HNSCC patients, although this finding requires confirmation with a larger cohort. The elevated relative abundance of *C. albicans* as a constituent of the salivary mycobiome is confounded by its presence in both healthy controls and dentally compromised patients. Therefore, future studies should consider examining the absolute abundance of *C. albicans* in the salivary mycobiome with a culture-based approach (e.g. using a selective and/or differential agar) or an alternative molecular approach (e.g. using species-specific quantitative PCR). Finally, this study highlights the importance of considering oral health when attempting to discern microbial and inflammatory biomarkers of HNSCC in saliva.

Chapter 4

Oral microbial influences on oral mucositis during radiotherapy treatment of head and neck cancer

This chapter is a modified version of: Vesty, A., Gear, K., Biswas, K., Wagner Mackenzie, B., Taylor, M.W., Douglas, R.G. (2019). "Oral microbial influences on oral mucositis during radiotherapy treatment of head and neck cancer". ***Supportive Care in Cancer*** *28(6): 2683-2691. doi: 10.1007/s00520-019-05084-6*

4.1 Introduction

Head and neck cancer (HNC) treatment often involves a course of radiotherapy, usually in conjunction with surgery and/or chemotherapy. Despite the use of intensity modulated radiotherapy to reduce the exposure of surrounding organs to radiation (de Sanctis et al., 2019), damage to the salivary glands often occurs during head and neck radiotherapy due to their close proximity to the radiation target region, resulting in hyposalivation and xerostomia (Bressan et al., 2016). The changes in saliva production and viscosity that occur during radiotherapy may alter the oral microbial community. Dynamic changes in microbial community composition likely influence the pathogenesis of oral mucositis, an acute side effect that has a particularly high prevalence in HNC patients, affecting the majority of those undergoing radiotherapy (Rodríguez-Caballero et al., 2012; Vera-Llonch et al., 2006). However, the exact role of oral microbes in the pathogenesis of oral mucositis remains unclear.

The painful inflammation and ulceration associated with oral mucositis can be debilitating for patients who may experience trouble eating, swallowing and speaking (Bressan et al., 2016). The well-characterised pathophysiology of oral mucositis occurs in five stages: initiation, primary response, signal amplification, ulceration – the most symptomatic stage for patients, during which a pseudomembrane forms and is colonised by microbes – and finally the healing stage (Sonis, 2009). Recently, and pertinent to this study, oral microbes have been suggested as patient-associated risk factors for oral mucositis (Stringer & Logan, 2015; Vanhoecke et al., 2015).

Data pertaining to changes in site-specific oral microbial communities of HNC patients during radiotherapy are variable with studies reporting a reduction or an increase in various genera (Almståhl et al., 2018; Hou et al., 2018; Zhu et al., 2017). Overall, microbial community

diversity is not always significantly altered by increasing radiation doses (Hou et al., 2018). Nonetheless, an emerging theme suggests that Gram-negative anaerobes, including periopathogenic genera *Porphyromonas* and *Treponema*, are associated with the onset and severity of oral mucositis in HNC patients (Hou et al., 2018; Zhu et al., 2017).

Such observations support the hypothesis that poor oral health may leave patients more susceptible to developing oral mucositis (Khaw, Logan, et al., 2014). As a group, HNC patients have microbial and inflammatory profiles more reflective of dentally compromised patients than healthy individuals (Robledo-Sierra et al., 2019; Vesty et al., 2018), which probably contributes to the high incidence of mucositis in this group of patients. Mucositis severity is influenced by microbial colonisation of ulcerations (Sonis, 2012), and the inflammatory process is potentially exacerbated to varying degrees depending on colonising microbes and their abundance. Candidiasis occurs as secondary infection, with several *Candida* species detected in oral mucositis ulcerations (Laheij et al., 2012).

In this chapter, changes in the oral microbiota of HNC patients during radiotherapy are described and correlated with the incidence and severity of oral mucositis.

4.2 Materials and methods

4.2.1 Patient recruitment

Patients were identified at the pre-radiotherapy dental clinic at Green Lane Hospital and at the oral mucositis clinic at Auckland City Hospital, both located in Auckland, New Zealand. In total, 19 patients were recruited, 12 males and seven females, with an average age of 65 years (Appendix C, Table C1). Ethical approval was granted by the Southern Health and Disability Ethics Committee (14/STH/121). Written informed consent was obtained from all patients. Patients were scheduled to undergo radiotherapy as treatment for tumours of the head and neck region, administered five times per week, with accumulative doses between 50 – 70 Gy.

4.2.2 Sample collection

Unstimulated saliva samples and swabs were collected from 15 of the patients at the following radiation doses: 0 – 20 Gy, 21 – 40 Gy and 41 – 60 Gy (Appendix C, Table C1). Patients were asked to provide 1 mL of saliva at each interval. However, due to radiation-induced hyposalivation, this was rarely possible and only low volumes (< 100 μL) of saliva were usually obtained; some patients were unable to provide saliva samples at all during the higher two radiation dose intervals or collection was not possible for reasons beyond our control. Sterile rayon-tipped swabs (Copan Diagnostics Inc., CA, USA) were used to sample sites of oral mucositis ulcerations for all 19 patients (Appendix C, Table C1) by rolling/rubbing over the

affected surface. In the absence of oral mucositis, the buccal mucosa was swabbed. Swabs were preserved in RNA*later*® (Life Technologies, Auckland, New Zealand) and stored at -20°C, along with neat saliva samples.

4.2.3 Oral mucositis assessment

Oral mucositis severity was evaluated fortnightly by a qualified oral medicine specialist based on the World Health Organisation Oral Mucositis Assessment Scale (Miller et al., 1981), which grades changes in physical appearance (erythema, ulcerations) in conjunction with dietary intake ability (solids, liquids), as described in 1.5.3. The prevalence of oral mucositis was statistically assessed by treatment, gender and radiation interval using a Chi-squared test, with $\alpha = 0.05$. Prevalence by age was compared using Welch's t-test.

4.2.4 DNA extraction, amplification of target genes and sequencing preparation

4.2.4.1 DNA extraction

The Qiagen AllPrep DNA/RNA Isolation Kit was implemented for DNA extraction in this chapter for two reasons. Firstly, due to the collection of swab samples for this chapter. Within my research group, the Qiagen AllPrep DNA/RNA Isolation Kit was previously optimised for use with clinical swabs (Biswas, Hoggard, Jain, Taylor, & Douglas, 2015). Secondly, this extraction approach included RNA isolation for potential use in future applications.

Prior to extraction, a mechanical lysis step was performed with Lysing Matrix E bead tubes (MP Biomedicals, NSW, Australia) and RLT Plus lysis buffer, ruptured at 25 Hz for 40 s × 2 on the Qiagen Tissue Lyser II, in order to help release nucleic acids from the saliva and swab samples. Applicable to this study, genomic DNA was extracted from the lysate using the AllPrep DNA/RNA Isolation Kit (Qiagen, Germany) as per manufacturer's instructions, using a spin column for DNA isolation. PCR-grade sterile water was included as a negative extraction control.

4.2.4.2 16S rRNA gene amplification

Illumina-compatible primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 (described in 2.2.3.1) were used to amplify the V3 – V4 regions of the bacterial 16S ribosomal RNA (16S rRNA) gene, with PCR conditions described in 3.2.2.2.

4.2.4.3 ITS1 region amplification

Illumina-compatible primers ITS1F and ITS2 (described in 2.2.3.2) were used for amplification of the fungal ITS1 region, with PCR conditions described in 3.2.2.3.

4.2.4.4 Preparation of PCR amplicons for Illumina MiSeq

Duplicate PCR reactions were prepared for sequencing as described in 2.2.3.3 and submitted to the Auckland Genomics Centre for sequencing using the Illumina MiSeq platform. Sequence data were deposited with the NCBI SRA database under BioProject ID number PRJNA524720.

4.2.5 Bioinformatic analyses

16S rRNA and ITS1 sequencing data were processed independently using USEARCH v9.2 (Edgar, 2010) following similar pipelines to those described in 2.2.4.1 and 2.2.4.2: primer trimming, followed by a filtering and merging step to remove low quality reads. For 16S rRNA data, singletons and sequences < 350 bp were removed. ITS1 sequences with an abundance threshold of < 4 were removed (Dupuy et al., 2014), along with sequences < 100 bp. Operational taxonomic units (OTUs) were picked from merged sequences based on 97% sequence similarity as described in 2.2.4.1, while chimeric sequences were simultaneously removed. Taxonomy was assigned to bacterial sequences using the Human Oral Microbiome Database (v15.1.p9) (Chen et al., 2010); UNITE (v7) was used to assign fungal taxonomy to ITS1 sequences (Nilsson et al., 2018). Human-derived and chloroplast sequences were removed from the resulting bacterial and fungal OTU tables; the bacterial OTU table was split to create two tables based on sample type and independently rarefied prior to further analyses.

4.2.6 Analysis of the salivary bacterial microbiota during radiotherapy

Using QIIME (v1.9) (Caporaso et al., 2010), the salivary bacterial OTU table was rarefied to 14,591 sequences per sample and the relative abundance of each bacterial taxon-assigned OTU was independently compared across the three radiation intervals (0 – 20, 21 – 40 and 41 – 60 Gy) using paired, two-tailed *t*-tests with Bonferroni correction. Alpha diversity (observed species) was statistically compared and plotted in R (v3.4.2) (R Core Team, 2017). The rarefied biom file was uploaded to the online bioinformatics tool Calypso (v8.84) (Zakrzewski

et al., 2017) with no additional filtering and total sum normalisation to convert OTU counts to relative abundance (without data transformation). Bray-Curtis dissimilarity was visualised using non-metric multidimensional scaling (nMDS), partitioning of variation was assessed with the *Adonis* function. The core microbiota were identified based on the presence of an OTU or genus in at least 90% of samples at each radiation interval, with an average relative abundance of ≥ 1%. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) identified significant differences in relative abundances of OTUs with LDA scores of ≥ 3 in the salivary microbiota (Segata et al., 2011).

4.2.7 Analysis of bacterial community data from swabs

The bacterial OTU table was rarefied to 1,317 sequences per swab. Data were compared by grouping swabs with ≤ grade 1 oral mucositis (no ulceration) and swabs with ≥ grade 2 oral mucositis (ulcerations present). Site-specific comparisons of buccal mucosa and lateral tongue within the ≥ grade 2 oral mucositis subset were also performed. Relative abundances of bacterial OTUs and alpha diversity were compared between these groups using the methods described in 4.2.6. Pairwise, permutational multivariate analysis of variance (PERMANOVA) was conducted in PRIMER v6 software (Anderson, 2001) to statistically assess bacterial beta diversity. The rarefied bacterial swab biom file was uploaded to Calypso (with no additional filtering and total sum normalisation without data transformation) and analysed as described in 4.2.6. Spearman's correlations based on the 30 most abundant genera were conducted and visualised using a heat map to compare site-specific and mucositis-specific relative abundances in swabs; the lateral tongue swab taken at grade 1 was excluded from this analyses as it did not

fit the grouping criteria. The relative abundances of these 30 genera were also compared using ANOVA, with Tukey's post-hoc comparison. The discriminatory and predictive power of the buccal mucosal microbiota at 0 – 20 Gy on the development of oral mucositis was assessed using a support vector machine, using a leave one out cross-validation (based on all OTUs) in Calypso, using the default parameters. A random forest analysis of the 30 most abundant genera was applied to estimate relative importance through mean decreasing accuracy and area under the receiver operating characteristic curve (AUC) (Breiman, 2001) and was implemented in Calypso using the default parameters.

4.2.8 Quantification of bacterial load in swabs

Droplet Digital™ PCR (ddPCR) (Bio-Rad Laboratories Inc., CA, USA) was used to estimate bacterial load per swab extraction based on the number of 16S rRNA gene copies present. Primers 8F (5′ AGA GTT TGA TYM TGG CTC AG 3′) and 341R (5′ CCT ACG GGA GGC AGC AG 3′) targeting the V1 – V2 region of the bacterial 16S rRNA gene were used with the following reaction mix: 1× QX200™ ddPCR™ EvaGreen®, 227 nM of each primer, 1 µL of DNA template and PCR-grade water to a final volume of 22 µL. DG8™ Cartridges and QX200 Droplet Generation Oil for EvaGreen® were used for droplet generation. Thermal cycling was performed with the following conditions: enzyme activation (95°C for 5 min); 40 cycles of denaturation (95°C for 30 s) and combined annealing/extension step (60°C for 1 min); and signal stabilisation (4°C for 5 min, then 90°C for 5 min). Data were acquired on the DX200 Droplet Reader and analysed in QuantaSoft™ Analysis Pro to calculate the number of 16S rRNA gene copies per µL, log base 10. Data were statistically assessed using two-way

ANOVA (with Tukey's post-hoc test) to compare site-specific bacterial load by radiation interval and by the presence of ≥ grade 2 oral mucositis.

4.2.9 Predicted function of the bacterial community at sites of ulcerative mucositis

Functional predictions of rarefied swab bacterial communities were performed with Piphillin bioinformatic program (Iwai et al., 2016) using the KEGG reference database (October 2018 release) and a 97% sequence identity cut-off. The vegan package (v 2.4-4) in R was used to analyse and visualise KEGG orthology (KO) abundances based on Bray-Curtis dissimilarity (Oksanen et al., 2017). Differentially abundant predicted functions by mucositis class (≥ grade 2 or ≤ grade 1) were identified using LEfSe, with an applied threshold cut-off of ≥ 2 (Segata et al., 2011).

4.2.10 Analysis of fungal community data from swabs

Fungal sequence data were rarefied to 120 sequences per swab and alpha diversity estimates were performed in QIIME (v1.9). As data were variable and OTUs were predominately assigned to *Candida* species, the abundance and presence of this genus was compared between sites of ≥ grade 2 and ≤ grade 1 oral mucositis but no further analyses were performed.

4.3 Results

4.3.1 Oral mucositis prevalence

The prevalence of ≥ grade 2 oral mucositis increased with cumulative radiation dose, with significant increases observed between 0 – 20 and 21 – 40 Gy and between 0 – 20 and 41 – 60 Gy ($p < 0.05$). Patients receiving concomitant chemotherapy were more likely to develop oral mucositis: 3/4 compared with 5/15 of patients receiving radiotherapy alone ($p < 0.05$). Age and gender were not significant factors ($p > 0.05$). Prevalence by tumour location was observed as follows: oral cavity (3/3), oropharynx (4/8), parotid (0/5) and other (1/3).

4.3.2 Salivary microbiota during radiotherapy

The 38 saliva samples returned 2,237,155 bacterial sequences. After rarefication to 14,591, 312 OTUs from 153 bacterial genera were present in the 36 samples (from 15 patients). The salivary microbiota remained consistent during the course of radiotherapy, continuously dominated by *Streptococcus*, *Prevotella*, *Fusobacterium* and *Granulicatella*, which together comprised, on average, over 50% of the bacterial community at each of the radiation intervals (Figure 4.1).

No significant changes in the number of OTUs present were observed across the three radiation intervals, with 92 ± 35, 86 ± 46 and 92 ± 38 OTUs found at increasing radiation doses, respectively (Figure 4.2). Bacterial communities in the saliva samples (Figure 4.3) clustered

predominately by patient (R_2 = 65%, $p \leq 0.001$) and grouping by radiation interval was not statistically significant, suggesting that there was no obvious effect of radiotherapy on the bacterial communities.

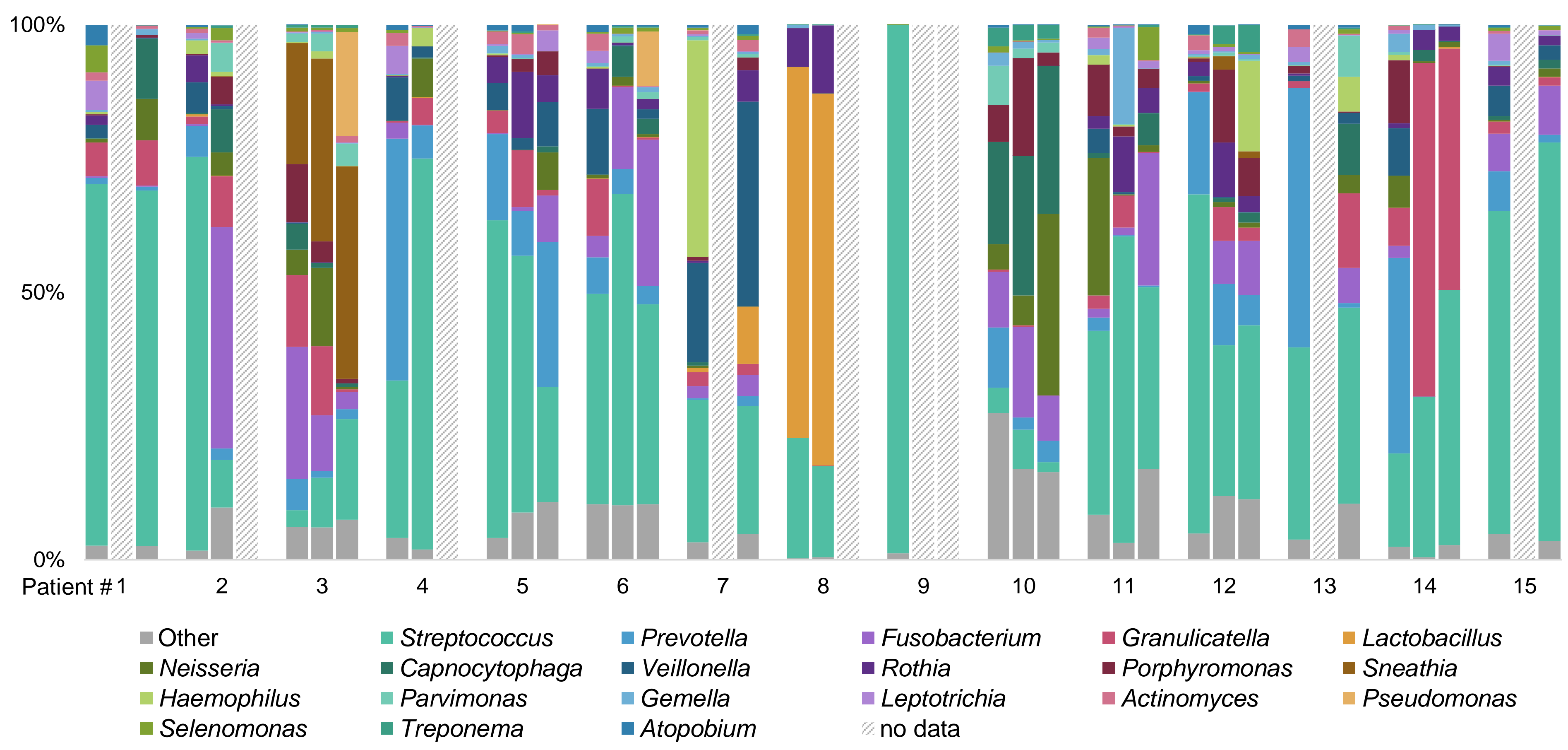


Figure 4.1. Genus-level summary of bacterial communities in saliva. Each group of three bars reflects the relative sequence abundances of bacterial communities in three separate saliva samples taken from a single patient sequentially at (from left to right) 0 – 20, 21 – 40 and 41 – 60 Gy. Data are shown for the 20 most abundant genera overall, rarefied to 14,591 sequences per sample. Bars with no data indicate that a saliva sample was unobtainable at the respective radiation interval.

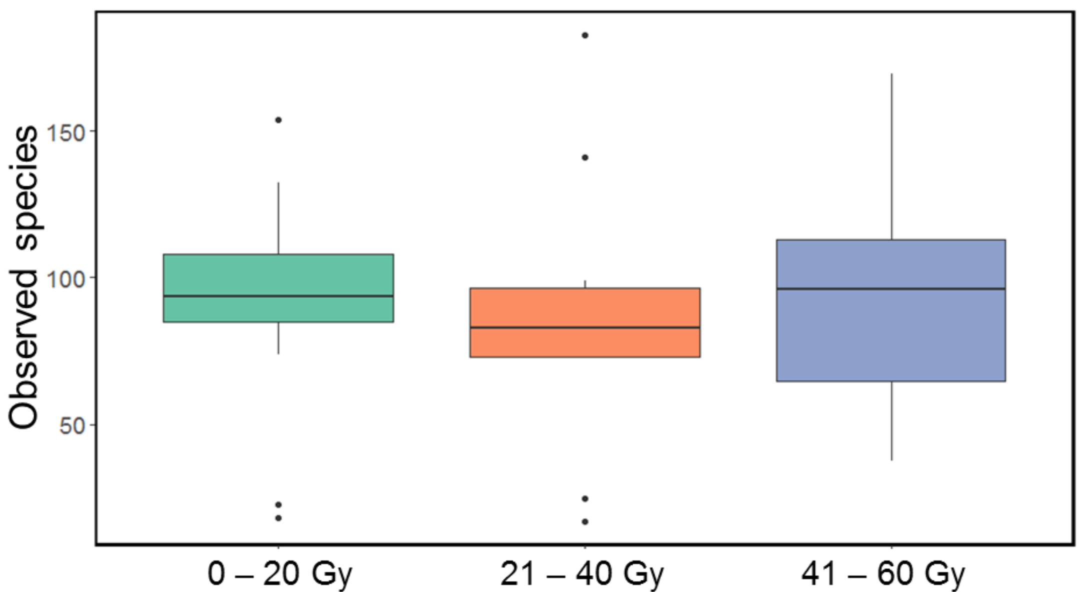


Figure 4.2. The number of 'observed species' is used to display salivary alpha diversity during increasing radiation intervals 0 – 20 (n = 15), 21 – 40 (n = 11) and 41 – 60 Gy (n = 12).

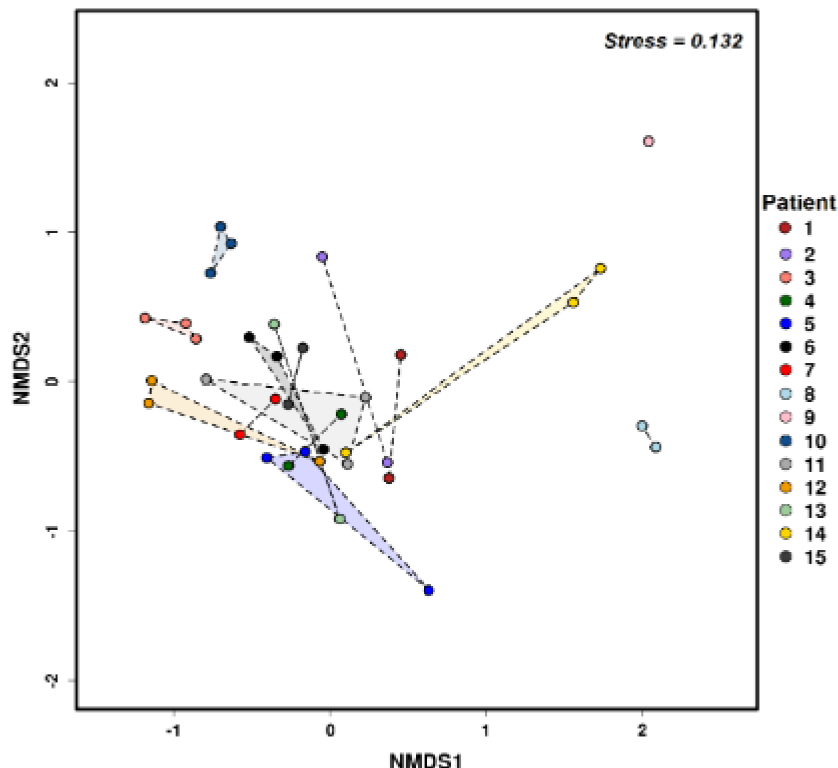


Figure 4.3. Beta diversity in saliva samples is demonstrated through a Bray-Curtis dissimilarity nMDS plot, with dashed lines connecting samples taken from the same patient at different radiation intervals.

4.3.3 Core salivary bacteria during radiotherapy

The core salivary microbiota during radiotherapy, defined as ≥ 90% occurrence at ≥ 1% average relative abundance, was dominated by an OTU assigned to *Streptococcus oralis* that was present in all samples as approximately one third of obtained sequences. Another OTU, assigned to *Fusobacterium nucleatum,* was present in much lower average relative abundances: 3% at 0 – 20 Gy, increasing to 9% at both higher radiation intervals. At the genus level, *Streptococcus*, *Fusobacterium* and *Capnocytophaga* comprised the core salivary microbiota during radiotherapy, as summarised in Table 4.1.

Table 4.1. Core microbiota of saliva by increasing radiation dose.

Radiation dose (Gy)	Mean relative abundance (%)			Occurrence (%)		
	0-20 (n = 15)	21-40 (n = 10)	41-60 (n = 11)	0-20 (n = 15)	21-40 (n = 10)	41-60 (n = 11)
OTUs						
Streptococcus oralis	32	31	33	100	100	100
Fusobacterium nucleatum	3	9	9	93	90	100
Genera						
Streptococcus	42	34	36	100	100	100
Fusobacterium	4	9	9	93	90	100
Capnocytophaga	2	4	6	93	100	100

4.3.4 Salivary microbiota and the development of oral mucositis

The 15 saliva samples taken at 0 – 20 Gy were used to investigate differences in bacterial community composition between patients who developed ≥ grade 2 oral mucositis later during radiotherapy and those who did not. LEfSe analysis revealed that OTUs assigned to *Capnocytophaga leadbetteri*, *Neisseria mucosa*, *Olsenella uli*, *Parvimonas micra* and *Tannerella forsythia* had a greater relative abundance in the saliva of the eight patients who developed ≥ grade 2 oral mucositis, although ANOVA indicated no significance difference ($p > 0.05$) (Appendix C, Figure C1).

4.3.5 Bacterial communities in buccal mucosa and lateral tongue swabs

From 19 patients, 47 swabs returned 1,167,831 bacterial sequences, which were classified into 368 OTUs and 163 genera when rarefied to 1,317 sequences per sample. *Streptococcus* was the most abundant genus on average, with a mean relative abundance of 38% across all swabs regardless of site and development of oral mucositis (Figure 4.4A) or the absence of oral mucositis (Figure 4.4B). *Neisseria*, *Fusobacterium* and *Prevotella* were prevalent, but in lower abundances than *Streptococcus*. Differences in the number of OTUs were not significant between swabs taken from sites of ≥ grade 2 oral mucositis (48 ± 24) compared with the remaining swabs (52 ± 29), or between mucositis grades, suggesting a comparable number of bacterial species colonised the mucosa at differing mucositis severities. No significant difference in observed species was detected by swab site. The majority of variation in bacterial community composition could be attributed to differences between patients (R_2 = 67%, p <

0.01; Figure 4.5). Differences due to ≥ grade 2 oral mucositis accounted for only 3% of variation in the model. Less than 1% of variation was attributable to swab site. PERMANOVA indicated that there was no significant shift in the microbiota within a patient attributable to the presence of ≥ grade 2 oral mucositis ulcerations, regardless of site.

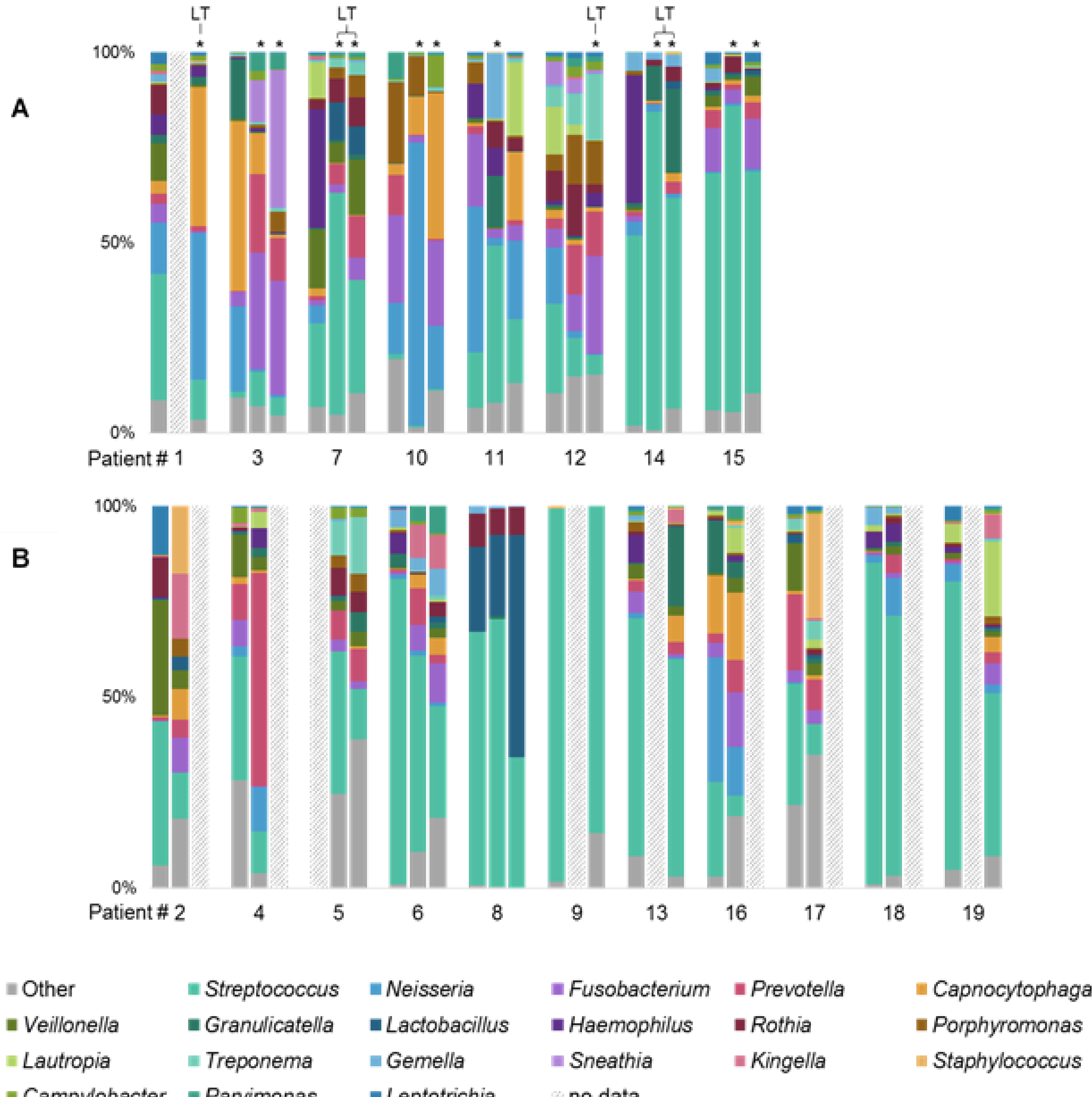


Figure 4.4. Summary of bacterial communities in swabs. In (A) and (B), each group of three bars reflects the relative sequence abundances of bacterial communities in three different swabs collected from a single patient sequentially at (from left to right) 0 – 20, 21 – 40 and 41 – 60 Gy. Data are shown the 20 most abundant genera (on average) rarefied to 1,317 sequences per sample. Bars with no data indicate that swabs were not collected at the respective radiation interval, or sequencing data failed to meet the rarefaction threshold. In (A) swabs taken from sites of ≥ grade 2 oral mucositis are identified by asterisks (*), those unmarked reflect ≤ grade 1 swabs; LT identifies lateral tongue swabs, unlabelled bars represent buccal mucosa swabs. In (B) each bar reflects the bacterial communities in a ≤ grade 1 buccal mucosa swab.

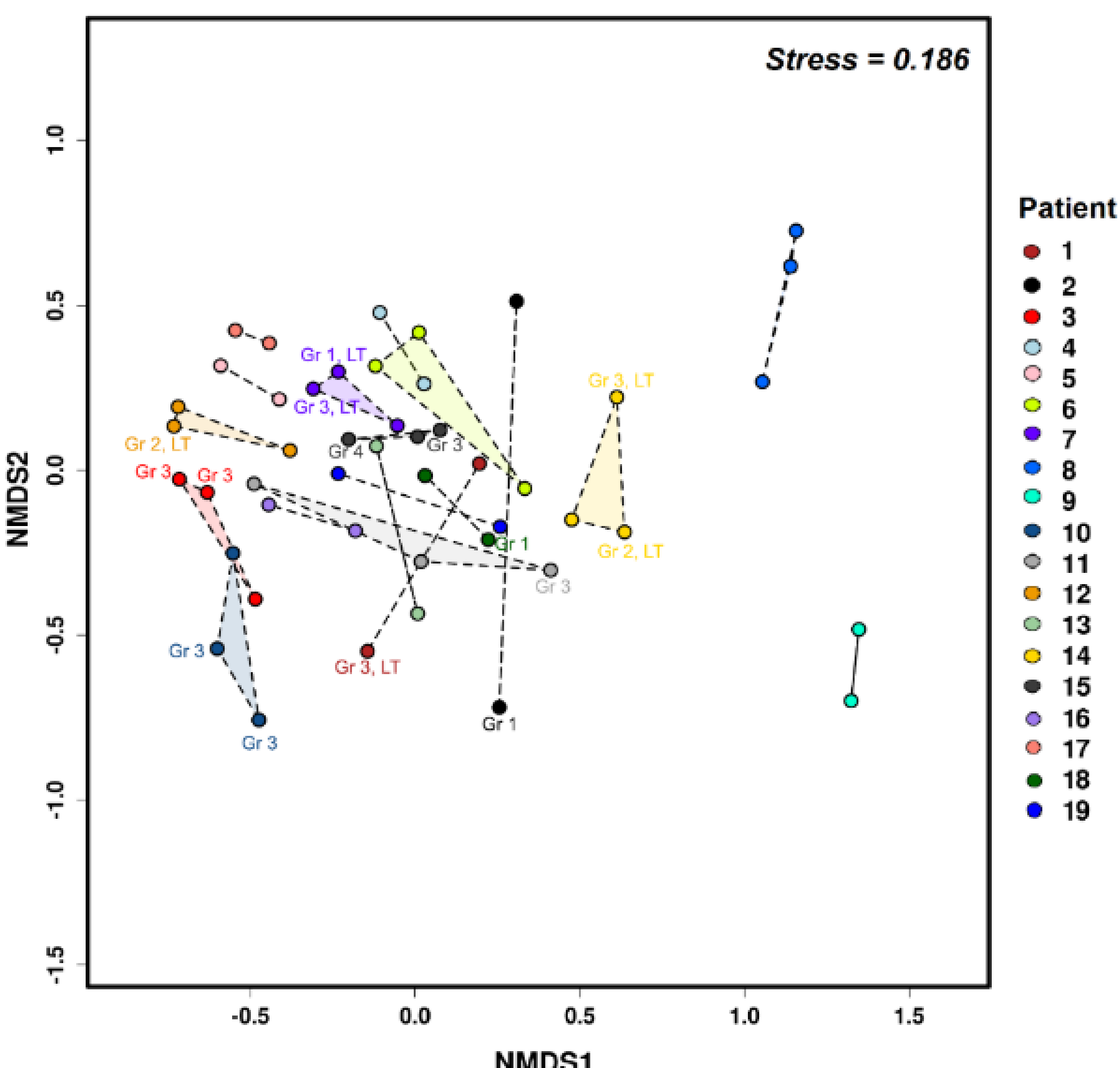


Figure 4.5. Beta diversity in swabs samples is demonstrated through a Bray-Curtis dissimilarity nMDS plot, with lines connecting samples taken from the same patient at different radiation intervals. Bacterial communities at sites of oral mucositis are labelled by grade, unlabelled points indicate the absence of oral mucositis. All points reflect buccal mucosa swabs, unless labelled LT (lateral tongue).

4.3.6 Bacterial genera correlated with ulcerative mucositis

Analysis of the 30 most abundant genera revealed several positive correlations with ≥ grade 2 oral on both the buccal and lateral tongue, while simultaneously negatively correlated with ≤ grade 1 buccal mucosa swabs: *Tannerella*, *Sneathia*, *Mycoplasma*, *Capnocytophaga*, *Bacteroidales* G2, *Porphyromonas* and *Eikenella* (Figure 4.6). Conversely, *Actinomyces*, *Veillonella*, *Lachnoanaerobaculum*, *Streptococcus*, *Lautropia*, *Kingella* and *Mollicutes* G1 were negatively correlated with ≥ grade 2 ulcerations on the buccal mucosa and lateral tongue but positively correlated with ≤ grade 1 buccal mucosa swabs (Figure 4.6). ANOVA indicated significant increases in the relative abundances of *Bacteroidales* G2, *Fusobacterium* and *Sneathia* within buccal mucosa swabs at sites of ≥ grade 2 oral mucositis ($p < 0.05$), while there was also a significant increase in *Bacteroidales* G2 in ≥ grade 2 buccal mucosal swabs compared to lateral tongue swabs ($p < 0.05$).

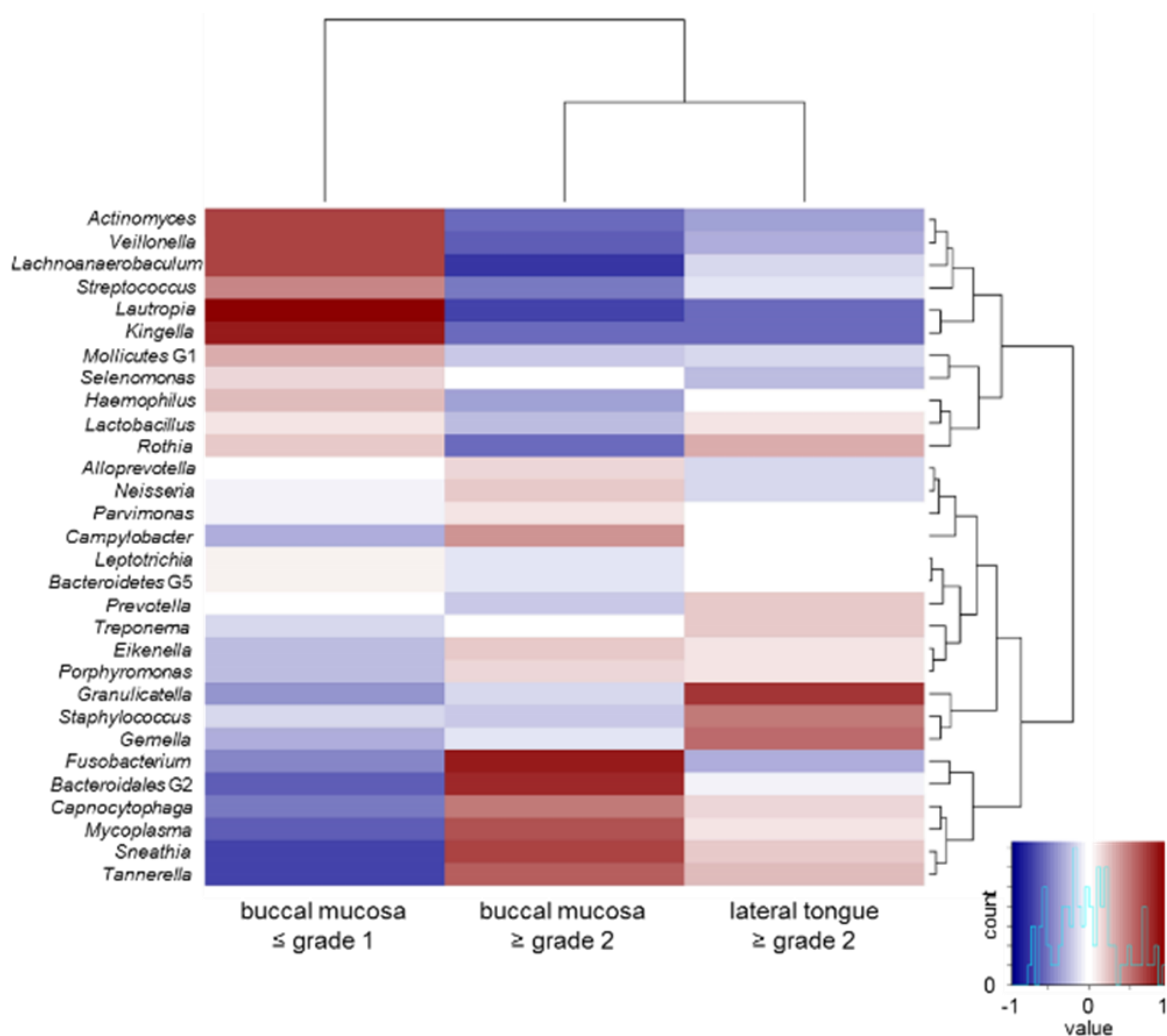


Figure 4.6. Correlation of bacterial genera with ulcerative oral mucositis. The heat map visualises correlations between the 30 most abundant bacterial genera (on average) identified in swabs and the site-specific presence or absence of ≥ grade 2 oral mucositis. Positive correlations are visualised in shades of red, negative correlations in shades of blue.

4.3.7 Predictive modelling or oral mucositis outcomes

The subset of 17 grade 0 buccal mucosa swabs taken at 0 – 20 Gy (Appendix C, Table C1) was used to assess the accuracy of predicting mucositis outcomes using bacterial community profiles, with all OTUs included. A classifier based on machine learning suggested that the buccal mucosa microbiota at 0 – 20 Gy was able to discriminate between patients who did and did not develop ≥ grade 2 oral mucositis with 65% accuracy. For the group of patients who did develop ≥ grade 2 mucositis, the sensitivity and specificity of the classifer was 43% and 60%, respectively. For patients who did not develop ulcerative mucositis, the sensitivity and specificity was 80% and 67%, respectively. Random forest analysis of the 30 most abundant genera revealed the relative importance of each genus in determining mucositis outcomes. *Streptococcus* was identified as the most important genus (AUC = 0.83) for predicting mucositis outcomes, with an average relative abundance of 54% in buccal mucosa swabs taken from patients who did not develop ≥ grade 2 oral mucositis. Patients who subsequently developed ≥ grade 2 oral mucositis reported a lower average relative abundance (26%) of *Streptococcus* (Appendix C, Table C2). Additionally, *Lactobacillus* and *Staphylococcus* were only found in swabs taken from patients who did not develop ≥ grade 2 oral mucositis (AUC = 0.80 and 0.35, respectively). *Fusobacterium*, *Haemophilus*, *Tannerella*, *Porphyromonas* and *Eikenella* were the most predictive genera with a greater average relative abundance in patients who developed ≥ grade 2 oral mucositis, with AUCs ranging from 0.69 – 0.79 (Appendix C, Table C2).

4.3.8 Estimation of bacterial load on the buccal mucosa

From the buccal mucosa swabs, bacterial load per swab was highest at the start of radiotherapy, falling (albeit not significantly) from 2.5 ± 1.0 log_{10} copies/μL (mean ± SD) per swab at 0 – 20 Gy to 2.1 ± 1.4 log_{10} copies/μL at 41 – 60 Gy. At buccal mucosal sites of ≥ grade 2 oral mucositis, the bacterial burden was higher than ≤ grade 1 buccal mucosa swabs and ≥ grade 2 lateral tongue swabs, although the differences were not significant (Figure 4.7).

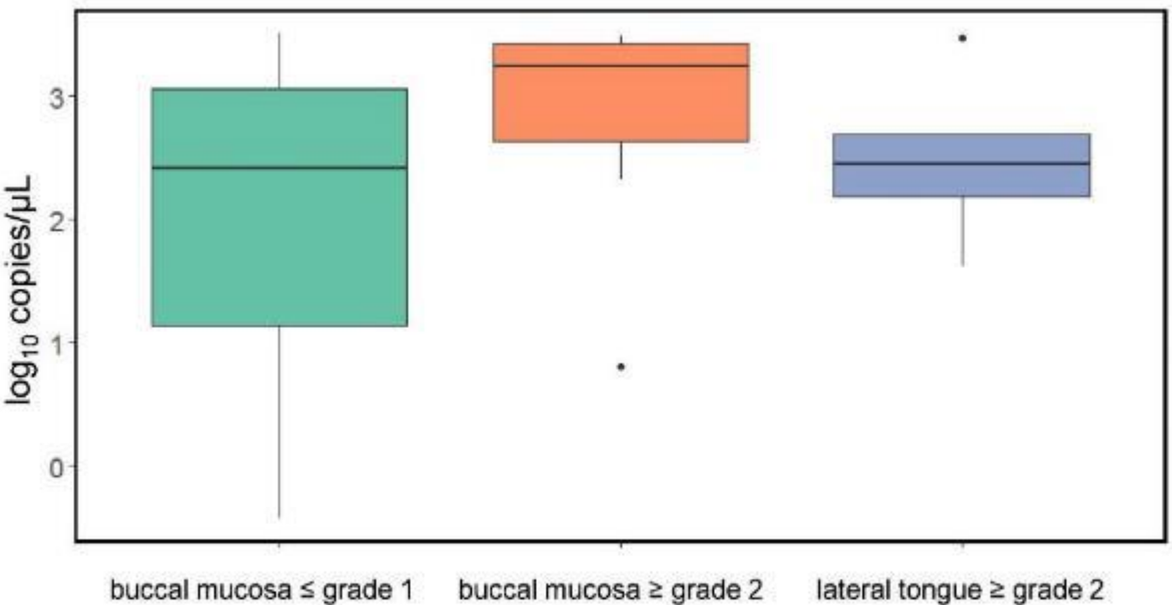


Figure 4.7. Site-specific bacterial load is demonstrated as boxplots reflecting the presence or absence of ≥ grade 2 oral mucositis. Bacterial load is expressed as on log_{10} of the 16S rRNA gene copy number. Buccal mucosa ≤ grade 1 (n = 34), buccal mucosa ≥ grade 2 (n = 7), lateral tongue ≥ grade 2 (n = 5). The solid line within each box indicates the median value for each group, and the whiskers extend to reflect the largest and smallest values based on the 1.5 times the inter-quartile range; outliers are plotted individually.

4.3.9 Functional prediction at sites of ulcerative mucositis

Visualisation of bacterial metagenome predicted function through nMDS at sites of ≥ grade 2 oral mucositis compared to ≤ grade 1 sites did not reveal any distinct clustering ($p > 0.05$). However, LEfSe analysis identified five KO functional orthologs that were more abundant at sites of ≥ grade 2 oral mucositis and two that were more abundant in ≤ grade 1 swabs. Predicted functions associated with ulcerative mucositis were involved in genetic information processing (translation, replication and repair). Of the two predicted functions associated with ≤ grade 1 sites, one was involved in metabolism of vitamin B6 and the second is a putative hydrolase of the haloacid dehalogenase superfamily.

4.3.10 Fungal communities in buccal mucosa and lateral tongue swabs

Sequencing of the ITS1 region in the 47 swabs produced 706,413 sequences that were rarefied to 120 sequences per sample, which excluded data from 10 swabs suggesting a low or absent fungal load at their collection site. Sequences were clustered into 39 OTUs, representing 35 genera. The fungal community was apparently one of very low diversity, with an average of 2.4 ± 1.2 OTUs at sites with ≥ grade 2 oral mucositis and 2.1 ± 1.2 for ≤ grade 1 sites.

Candida was the most abundant genus and included three OTUs identified individually as *C. albicans*, *C. dubliniensis* and *C. intermedia* (100% sequence similarity in NCBI BLASTn). *Candida* OTUs were abundant at sites both with and without oral mucositis: 7/9 (78%) swabs at sites of ≥ grade 2 mucositis had a range of 64 – 100% of the relative abundance comprised by *Candida*; 23/28 (82%), swabs taken from ≤ grade 1 sites had a relative abundance of

Candida between 56 – 100% (Figure 4.8). Other fungal genera detected in the swabs included *Cladosporium*, comprising 99% of the mycobiota in one swab, and detected in several other samples. *Cryptococcus* comprised 98% of the community in a swab from patient 11. *Rhodotorula*, an airborne contaminant that can be found in fruit juice, milk and on skin; and *Wallemia*, often found in low-moisture foods such as cakes and bread, featured in several patients at a single collection point, suggesting the presence of these genera may be transient.

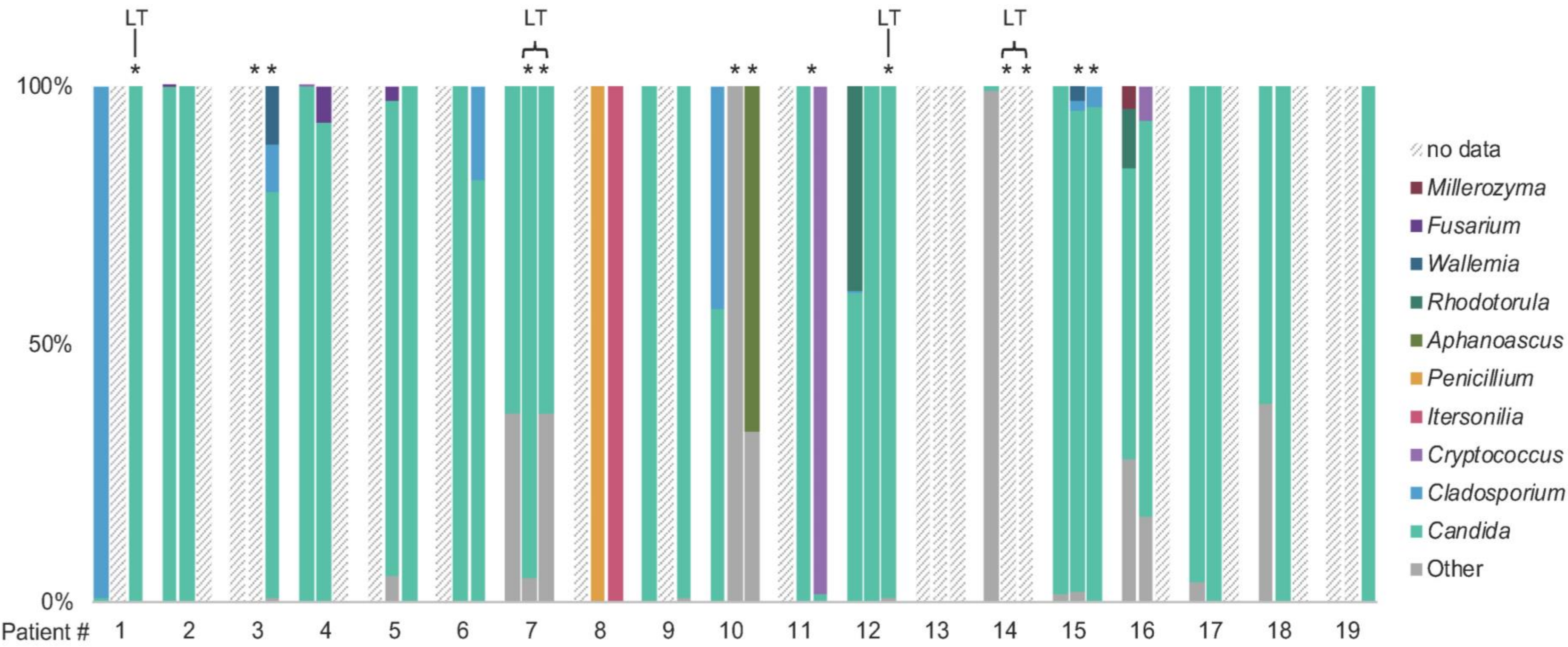


Figure 4.8. Summary of fungal communities in swabs. Each group of three bars reflects the fungal communities in three different swabs collected from a single patient sequentially at (from left to right) 0 – 20, 21 – 40 and 41 – 60 Gy. Data are shown for the 10 most abundant fungal genera (on average), rarefied to 120 sequences per sample. Swabs taken from sites of ≥ grade 2 oral mucositis are identified by asterisks (*), those unmarked reflect ≤ grade 1 swabs. Each bar reflects a buccal mucosa swab, unless labelled LT (lateral tongue). Bars with no data indicate that swabs were not collected at the respective radiation interval, or sequencing data failed to meet the rarefaction threshold.

4.4 Discussion

Oral mucositis is a significant complication for HNC patients undergoing radiotherapy, yet whether oral microbiota play a causal role in its onset and severity remains poorly understood. Despite hyposalivation, the core salivary microbiota remained relatively stable during radiotherapy and were consistently dominated by *Streptococcus*, *Fusobacterium* and *Capnocytophaga*. Therefore, this chapter suggests an association between the constituents of the salivary microbiota early in radiotherapy and oral mucositis outcomes, rather than radiotherapy-induced changes in the salivary microbiota affecting mucositis outcomes. Oral microbiota may intensify known patient- and treatment-related risk factors including age, concomitant chemotherapy, tumour location and tumour stage (Barasch & Peterson, 2003; Nishii et al., 2019). The data in this chapter presents a positive association between increased salivary relative abundances of *Capnocytophaga leadbetteri*, *Neisseria mucosa*, *Olsenella uli*, *Parvimonas micra* and *Tannerella forsythia* during the early stages of radiotherapy and the subsequent development of ulcerative oral mucositis. The latter three species are implicated in periodontitis (Dewhirst et al., 2001; Socransky et al., 1998) and their association with the development of oral mucositis may support general trends that patients with poor oral health do not tolerate radiotherapy as well as patients with good oral health (Khaw, Liberali, et al., 2014).

Analyses of the bacterial communities present on buccal mucosa and lateral tongue swabs taken from patients in this study support previously described relationships between the overabundance of anaerobes and oral ulcerations (Hou et al., 2018; Laheij et al., 2012; Zhu et al., 2017). *Tannerella*, *Sneathia*, *Mycoplasma*, *Capnocytophaga*, *Bacteroidales* G2, *Porphyromonas* and *Eikenella* were all positively correlated with the presence of grade ≥ 2 oral mucositis and negatively correlated with grade ≤ 1 swabs. Identification of *Tannerella*,

Porphyromonas and *Eikenella* as important genera predictive of oral mucositis in the random forest classification supports these correlation-based findings. These genera are obligate or facultative anaerobic Gram-negative bacilli (GNB), with *Tannerella* and *Porphyromonas* spp. established pathogens in the aetiology of periodontitis (Darveau, 2010; Socransky et al., 1998). Increases in *Proteobacteria*, particularly Gram-negative facultative anaerobes, are linked to mucositis severity (Zhu et al., 2017) and synchronous peak abundances of *Prevotella*, *Fusobacterium*, *Treponema* and *Porphyromonas* have been observed with the onset of severe mucositis (Hou et al., 2018). Conversely, genera positively correlated with the absence of mucositis ulcerations (grade ≤ 1), and simultaneously negatively correlated with grade ≥ 2 oral mucositis, had more diverse cellular morphologies including Gram-positive bacilli, Gram-positive cocci and Gram-negative cocci/coccobacilli. In the absence of a significant increase in bacterial load at sites of grade ≥ 2 oral mucositis, I speculate that the association of oral mucositis with anaerobic GNB may occur due to a dysbiosis of the microbiota at these sites. Future studies might consider calculating the ratio of the relative abundance of anaerobic GNB to that of core oral microbiota in order to measure the extent of dysbiosis.

Increased relative abundances of *Haemophilus, Fusobacterium, Tannerella*, *Porphyromonas* and *Eikenella* present on the buccal mucosa early in the course of radiotherapy (0 – 20 Gy) may also predispose patients to the development of ulcerative oral mucositis, based on the predictive modelling used in this study. The dysbiosis of mucositis ulcerations observed did not appear to alter the predicted function of the local microbial community in terms of mucositis-inducing pathways, although an increase in a predicted function associated with a hydrolase at sites without mucositis may enhance local detoxification.

The role of oral mycobiota in oral mucositis is difficult to interpret and requires further investigation using fungal culture or species-specific molecular approaches. The mostly likely candidate for exacerbating oral mucositis is *Candida* but I found no obvious differences in its

presence at sites with or without mucositis ulcerations. *Candida* spp. are found in the healthy oral mycobiota (Ghannoum et al., 2010; Vesty et al., 2017), but likely act as opportunistic pathogens in hosts compromised by radiotherapy. Candidiasis develops as a secondary infection and is often considered based on clinical appearance, although diagnosis can be complicated by the presence of oral mucositis (Sroussi et al., 2017).

Further validation of the findings in this chapter is needed using a larger cohort with a matched control population, an approach that would help delineate a microbial-based risk factor for oral mucositis in a clinical context. Furthermore, other factors that predispose patients to oral mucositis including low leukocyte or lymphocyte counts should be incorporated into future studies (Nishii et al., 2019).

This study suggests periopathogenic GNB may play a role in the pathogenesis of oral mucositis and supports the proposed relationship between oral mucositis and periodontitis, whereby patients are primed for an exaggerated inflammatory response during radiotherapy, manifest as oral mucositis (Khaw et al., 2014). However, whether a causal microbial element exists is difficult to establish, leaving it challenging to translate these findings to a clinical setting where meaningful improvements in patient care and comfort may evolve. While patients should be dentally fit prior to commencing radiotherapy, there is evidence to suggest oral hygiene interventions prior to and during cancer treatment may reduce the onset and severity of oral mucositis (Robertson, 2018; Saito et al., 2014). My findings support the need for interventions aimed at minimising microbial risk factors, for example implementing an oral hygiene protocol and the provision of oral health advice.

Chapter 5

Randomised, double-blind, placebo-controlled clinical trial of the oral probiotic *Streptococcus salivarius* M18 on head and neck cancer patients post-radiotherapy: a pilot study

5.1 Introduction

Head and neck cancer (HNC) describes a group of malignant tumours that often require radiotherapy as part of the treatment approach. Due to the close proximity of the salivary glands to the radiation portal, many HNC patients sustain permanent radiation-induced damage to the salivary glands during radiotherapy (Jensen et al., 2010; Vissink, Spijkervet, & Brennan, 2018). Subsequently, salivary gland function is reduced, resulting in chronic hyposalivation and xerostomia (dry mouth) that causes considerable discomfort, compromises mastication and speech, increases the risk of oral health complications and decreases the quality of life for many patients (Chambers et al., 2004; Jensen et al., 2010; Vissink, Jansma, Spijkervet, Burlage, & Coppes, 2003).

Hyposalivation and loss of salivary buffering capacity lead to a decrease in the pH of saliva and a shift to acidogenic and cariogenic bacteria, including an increase the in abundance of *Streptococcus mutans* and *Lactobacillus* spp. (Almståhl et al., 2018; Brown et al., 1975; Eliasson et al., 2006). These effects, combined with a decrease in the mechanical flushing mechanism of saliva to reduce plaque build-up, leave patients prone to post-radiotherapy gingivitis, periodontal disease and rampant dental decay (Chambers et al., 2004; Vissink et al., 2018; Yadav & Prakash, 2017).

Oral probiotics, primarily those containing lactobacilli, have been successfully shown to decrease the abundance of periopathogens in sub- and supra-gingival plaque, significantly improve plaque index and periodontal pocketing scores and reduce periodontal inflammation (Keller et al., 2018; Mayanagi et al., 2009; Riccia et al., 2007; Shimauchi et al., 2008; Staab, Eick, Knöfler, & Jentsch, 2009; Tekce et al., 2015; Teughels et al., 2013; Vivekananda, Vandana, & Bhat, 2010). Oral probiotic strain *S. salivarius* M18 has shown great promise and produces bacteriocin-like inhibitory substances (BLIS). *In vitro*, *S. salivarius* M18 (BLIS M18TM) has an inhibitory effect on the periodontal pathogens *Porphyromonas gingivalis* and *Prevotella intermedia* (Burton, Wescombe, et al., 2013) and decreases the expression of pro-inflammatory cytokines associated with periodontal disease, including interleukin (IL)-6 and IL-8 (Adam et al., 2011). *S. salivarius* M18 also inhibits mutans streptococci (Burton, Drummond, et al., 2013; James & Tagg, 1991; Wescombe, Hale, Heng, & Tagg, 2012) and, although dental decay is increasingly characterised by its polymicrobial nature, *S. mutans* is still considered its primary etiological agent (Banas & Drake, 2018; Simón-Soro & Mira, 2015). *S. mutans* establishes itself in the dental plaque biofilm where it ferments sucrose to produce lactic acid, which breaks down tooth enamel and leads to decay (Yadav & Prakash, 2017). Oral probiotic lozenges containing *S. salivarius* M18 successfully decreased the incidence of new dental caries in a high risk group of children (Di Pierro et al., 2015).

The few studies that have investigated the impact of oral probiotics on the oral microbiota show that bacterial communities are not substantially altered by oral probiotic interventions (Burton,

Wescombe, et al., 2013; Dassi et al., 2018; Keller et al., 2018). However, the potential for oral probiotics to modulate the oral microbiota has not been applied to post-radiotherapy HNC patients, who are susceptible to oral health deterioration. Therefore, this pilot study of *S. salivarius* M18 attempts to characterise and correlate probiotic-induced changes in oral health and the composition of the oral bacterial community in post-radiotherapy HNC patients.

5.2 Methods

5.2.1 Patient enrolment and randomisation

Seventeen patients were enrolled at Auckland City Hospital, New Zealand for this randomised, double-blinded, placebo-controlled study of the oral probiotic BLIS M18TM (BLIS Technologies Ltd, Dunedin, New Zealand). This pilot study was approved by the national Health and Disability Ethics Committee (16/STH/123) and gained institutional approval from the Auckland District Health Board (7396). All participants provided written informed consent. The trial was registered with the Australian New Zealand Clinical Trials Registry (372905).

Non-palliative, fully or partially dentate patients who had received ≥ 60 Gy of radiation to the head and neck region in the previous six months were eligible for inclusion. Patients requiring antibiotic treatment were excluded. Patients generally reported a low carbohydrate diet and did not require percutaneous endoscopic gastrostomy (PEG) feeding. Patients were randomly allocated into Group A (placebo) or Group B (probiotic) by block randomisation with a 1:1 ratio. Patients, the dental hygienist assessing oral health and the researcher responsible for randomisation were masked to the treatment/placebo allocation of the lozenges. Certificates of analysis with microbial specifications for each of the Group A and Group B lozenges were provided by the manufacturers and remained sealed during the trial period.

5.2.2 Study design and sample collection

This pilot study was conducted over a four-week intervention period with either oral probiotic or placebo lozenges. Pre- and post-intervention oral health assessments were performed, coinciding with the collection of plaque and saliva samples for microbial analyses (Figure 5.1). As a pilot study, the effect size was not calculated.

Pre-intervention, unstimulated saliva samples were collected in a sterile container; 1 mL of saliva was requested but patients usually provided less due to hyposalivation. A combination of sub- and supra-gingival plaque was collected pre-intervention from the posterior mandibular molars and, if insufficient plaque was available for collection, the lower incisors were sampled. Pre-intervention oral health was assessed as detailed below. Patients were given 30 of the randomly assigned Group A or B lozenges: Group A lozenges were a placebo; Group B lozenges contained 3.5 x 10^9 colony forming units (CFU) of *S. salivarius* M18 (BLIS M18™) per lozenge at the time of manufacture (BLIS Technologies Ltd, Dunedin, New Zealand, Appendix D1) . Patients were asked to suck one lozenge slowly until dissolved after brushing in the evening every day for four weeks. Oral hygiene advice during the trial complemented recommendations for post-radiotherapy oral care. Patients were advised to brush twice daily using a high fluoride toothpaste, with no post-brushing rinse and to clean interdentally. Four weeks post-intervention, plaque and saliva samples were collected and post-intervention oral health assessments were performed, as described above. Plaque samples, collected in RNA*later* (Life Technologies, Auckland, New Zealand), and neat saliva samples were stored at -20°C until further analysis.

5.2.3 Oral health assessments

The Community Periodontal Index of Treatment Needs (CPITN) was used to screen for gingival bleeding, calculus and periodontal pocketing (Cutress, Ainamo, & Sardo-Infirri, 1987). Dentition was divided into six sextants, and each sextant was scored between 1 and 4 by examining 10 index teeth, with the highest score reported. A score of 0 indicates no periodontal disease. Scores 1 – 4 respectively reflect increasing treatment needs: 1, gingival bleeding on probing; 2, calculus; 3, pocketing 4 – 5 mm; 4, pocketing ≥ 6 mm. Scores of 3 and 4 indicate periodontal disease. The O'Leary Plaque Index was used to quantify plaque on tooth surfaces (O'Leary, Drake, & Naylor, 2010). Each tooth was divided into four surfaces (buccal, distal, lingual and mesial), and the presence of plaque on each surface was recorded for all teeth present. The number of plaque-containing surfaces was divided by the total number of tooth surfaces available and reported as a percentage for each patient. All oral health assessments were performed by the same qualified oral hygienist.

5.2.4 Assessment of probiotic viability

After trial completion, one lozenge from each of Group A and B was separately dissolved in 10 mL of sterile, PCR-grade water. One microlitre of each suspension was inoculated on tryptic soy agar with sheep blood (Fort Richard, Auckland, New Zealand) and incubated at 35°C with 5% CO_2 for 32 h. Colonies were enumerated and a representative colony was identified by matrix assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry using the VITEK® MS system (bioMérieux, MediRay, Auckland, New Zealand).

5.2.5 DNA extraction and sequencing preparation

5.2.5.1 DNA extraction

One lozenge from each of groups A and B was dissolved separately in 1 mL of sterile, PCR-grade water. Aliquots of the clinical samples and the dissolved lozenges were mechanically lysed using Lysing Matrix E bead tubes (MP BioMedicals, NSW, Australia) and RLT Plus lysis buffer for 30 s × 2 using the Omni Bead Ruptor 24 (Omni International Inc., GA, USA). Purification of genomic DNA was achieved with the AllPrep DNA/RNA Isolation Kit (Qiagen, NRW, Germany), using a spin column for DNA isolation. Sterile, PCR-grade water was used as a negative extraction control.

5.2.5.2 16S rRNA gene amplification

The V3-V4 region of the bacterial 16S rRNA gene was amplified using Illumina-compatible primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 (Klindworth et al., 2013), as described in 2.2.3.1, with each PCR reaction containing: HotStar PCR Buffer (1×), 1 mM $MgCl_2$, 0.05 mM of each dNTP, 0.2 µM of each primer, 0.5 U HotStar DNA polymerase (Qiagen, NRW, Germany), 1 µL of DNA template and PCR-grade water to a final volume of 25 µL. *Escherichia coli* DNA and sterile water were used as positive and negative PCR controls, respectively. PCR was performed using the following thermocycling conditions: initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation (95°C for 30 s), annealing (55°C for 30 s) and extension (70°C for 40s), and a final extension step at 70°C for 3 min. A 1% agarose gel (w/v) containing SYBR Safe DNA Gel Stain (Invitrogen, CA, USA) was used to visualise PCR products under ultraviolet light.

5.2.5.3 Preparation of PCR amplicons for Illumina MiSeq

Duplicate PCR reactions were pooled and purified for sequencing, as described in 2.2.3.3, using AMPure XP magnetic beads (Beckman Coulter, MA, USA), and the DNA concentration was measured on the Qubit® 3.0 Fluorometer (Life Technologies, Auckland, New Zealand) using the High Sensitivity dsDNA kit (Invitrogen, CA, USA). Normalised samples were submitted to Auckland Genomics Ltd for sequencing on the Illumina MiSeq platform.

5.2.6 Bioinformatic analyses

Bacterial 16S rRNA gene sequence data from plaque and saliva samples were processed in USEARCH (v10) using the UNOISE algorithm to predict correct biological sequences (Edgar, 2016). This algorithm clusters sequences into zero-radius operational taxonomic units (ZOTUs), whereby closely related species are differentiated by distinct sequences (Edgar, 2016). This approach enables the high-level resolution of sequences to species-level and strain type identifications and has been applied in this chapter to closely examine *Streptococcus* species in the bacterial community data.

Firstly, primer-binding regions were trimmed before sequences were quality filtered and merged to remove sequences with a minimum merge length of < 350 bp and > 5 mismatches. Filtered sequences were clustered into ZOTUs using the '-unoise3'command, with a minimum cluster size of 8 (Edgar, 2016). The ZOTUs were mapped to the filtered sequence data with a minimum identity threshold of 99% in order to create a ZOTU table. Taxonomic prediction was performed using the RDP classifier (Wang et al., 2007) against the Human Oral Microbiome Database (v15.1) (Chen et al., 2010). ZOTUs observed in < 3 samples were

filtered from the final ZOTU table. *Streptococcus* ZOTUs unresolved to species-level were further classified using BLASTn, with species-level taxonomic classification based on ≥ 98% sequence similarity (Appendix D, Table D1). Alpha and beta diversity measurements were estimated in QIIME (v1.9) (Caporaso et al., 2010).

5.2.7 Network analyses

Microbial networks in plaque and saliva were inferred independently using SPIEC-EASI (SParse InversE Covariance Estimation for Ecological Association Inference), implemented using the Spiec-Easi R package (Kurtz et al., 2015). The SPIEC-EASI statistical method was developed for compositional data and has the advantage over correlation-based approaches of avoiding correlated but indirectly connected nodes, and has been verified against correlation-based analyses (Kurtz et al., 2015). First, unrarefied ZOTU tables were filtered and normalised to retain only ZOTUs with a minimum occurrence in 10% of samples. The SPIEC-EASI sparse neighbourhood and inverse covariance selection algorithms were used for data transformation and estimation of the interaction graph, based on Meinshausen-Buhlmann's neighbourhood selection method (Kurtz et al., 2015). The network was visualised in Cytoscape (v3.4.0) (Shannon et al., 2003), and nodes and edges that interacted with ZOTU1_*S. salivarius* were selected out. Inverse covariance was used to visually map the positive and negative edges of the selected network.

5.2.8 Statistical analyses

Patient demographics were compared between the probiotic and placebo groups using Fisher's exact test for categorical variables and a one-way analysis of variance (ANOVA) for continuous variables, $\alpha = 0.05$. Differences in pre- and post-intervention CPITN scores for each patient were used to categorise clinical responses as either an improvement (difference < 0) or no improvement (≥ 0) observed. Differences were calculated by subtracting the pre-intervention CPITN score from the post-intervention score, meaning that a negative result indicated an improvement in periodontal health, a positive result indicated a decline in periodontal health and zero indicated no change was detected. Fisher's exact test was used to statistically compare the clinical response categories between the probiotic and placebo groups. A two-way ANOVA with Kenward-Roger's approximation was conducted and visualised in R (v3.6.0) to compare O'Leary Plaque Index scores by time point (pre- and post-intervention), intervention group and individual. Bray-Curtis dissimilarity was used to calculate intra-patient variability pre- and post- intervention by sample type and was visualised through non-metric multidimensional scaling (nMDS), with differences in Bray-Curtis distance statistically compared using two-way ANOVA in R (v3.6.0) (R core team). Partitioning of the distance matrix by variable was assessed using the *Adonis* function. Changes in alpha diversity and the abundance of *Streptococcus* ZOTUs were visualised and statistically assessed by ANOVA with Tukey's post-hoc test for multiple comparison of means in R (v3.6.0) (R Core Team).

5.3 Results

5.3.1 Study cohort

In total, 17 patients were recruited for this pilot randomised control trial; four were excluded post-randomisation for reasons beyond our control including: antibiotic treatment, failure to comply and two patients were lost to follow-up (Figure 5.1). No significant pre-intervention differences were observed between the probiotic and placebo groups for the patient variables described in Table 5.1.

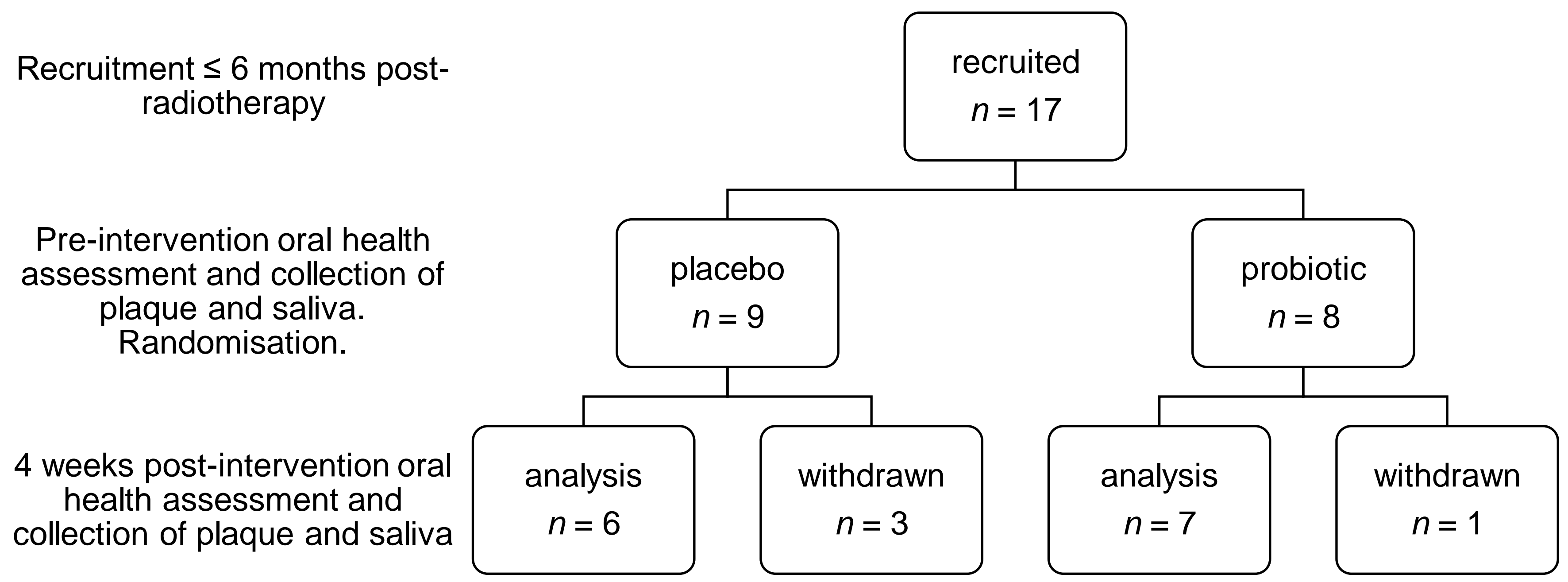


Figure 5.1. Study design.

Table 5.1. Patient demographics.

Variable[a]	**Placebo (*n* = 6)**	**Probiotic (*n* = 7)**	***p* value**
Gender, male:female	4:2	3:4	0.59
Age (years)	53.5 ± 17.1	53.3 ± 13.0	0.98
Total radiation dose (Gy)	65.5 ± 5.0	67.1 ± 4.5	0.56
Concomitant chemotherapy	3/6 (50%)	5/7 (71%)	0.59
Xerostomia	5/6 (83%)	5/7 (71%)	1.00
Weeks post radiotherapy	11.0 ± 8.6	6.9 ± 2.5	0.21
Total lozenges taken	26 ± 3.0	27 ± 3.0	0.42
Pre-intervention CPITN	3.2 ± 0.9	2.7 ± 0.9	0.42
Pre-intervention O'Leary PI	48 ± 30%	37 ± 25%	0.54

CPITN, Community Periodontal Index of Treatment Needs; PI, plaque index

[a] Categorical variables are reported as proportion yes/total (%), with the exception of gender, which is given as male:female ratio, no. (%). Continuous variables are summarised as mean ± standard deviation.

5.3.2 Comparison of pre- and post-intervention clinical measures of oral health

After the four week intervention, an improvement in CPITN was observed in three patients: 2/6 in the placebo group and 1/7 in the probiotic group, but the difference between the two groups was not significant ($p > 0.05$). The CPITN scores for all other patients remained stable. Post-intervention, an improvement in plaque index scores was observed in both groups, with the average percentage of tooth surfaces with plaque falling (albeit not significantly) from 37% to 26% in the probiotic group and from 48% to 32% in the placebo group (Figure 5.2). No significant reduction in tooth surfaces with plaque was observed between the probiotic and placebo groups ($p > 0.05$). O'Leary Plaque Index scores were not available for one patient in the probiotic group.

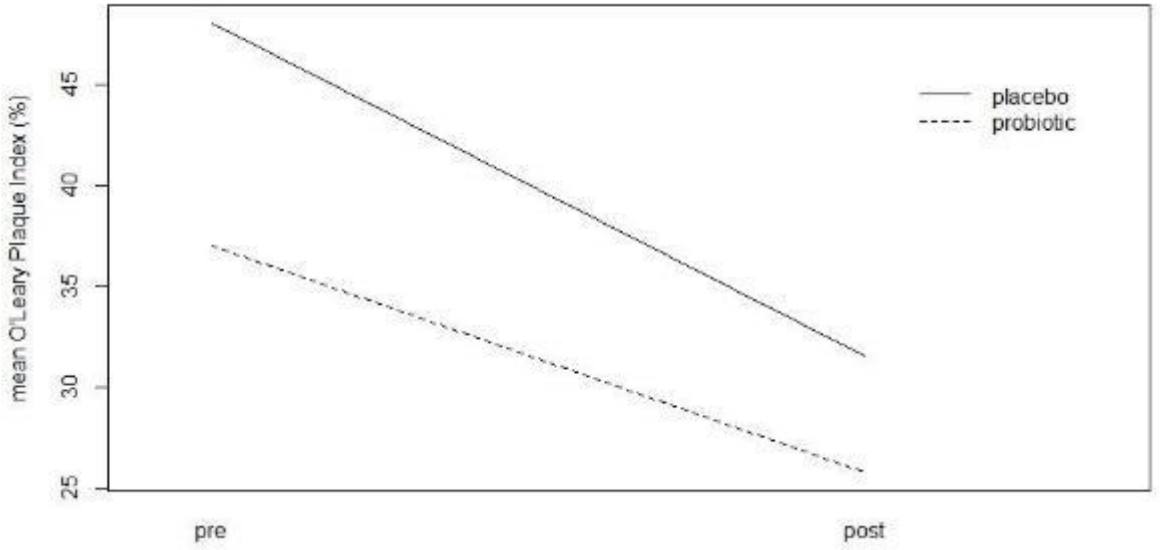


Figure 5.2. Interaction plot demonstrating the decline in O'Leary Plaque Index scores for both the probiotic and placebo groups post-intervention.

5.3.3 Probiotic viability

No growth was observed from the placebo lozenges (Group A, Figure 5.3); > 1 × 10^9 CFU/L of growth resembling alpha haemolytic streptococci was recovered from the culture of a BLIS M18™ lozenge obtained from a sealed study container (Group B, Figure 5.3), a representative colony was identified as belonging to the *S. salivarius* group.

Figure 5.3. No growth observed from placebo lozenge (left) and growth of small, alpha-haemolytic streptococci from the BLIS M18™ lozenge (right).

5.3.4 Bacterial community profiles

Post filtering and processing of the 52 patient samples, 1,117,173 bacterial 16S rRNA gene sequences were obtained and classified into 1,247 ZOTUs. Rarefaction to 3,639 sequences per sample was sufficient to capture the vast majority of bacterial diversity (Appendix D, Figure D1) and resulted in 1,102 ZOTUs classified into 117 genera in plaque samples and 931 ZOTUs classified into 106 genera in saliva samples. Of the 27,863 bacterial sequences obtained from the BLIS M18TM probiotic lozenge, 99.8% clustered with ZOTU1, which was assigned to *S. salivarius*.

The plaque microbiota was dominated by the anaerobic Gram-negative genera *Prevotella* and *Fusobacterium*, which comprised an average of 17% and 15% of the plaque bacterial community, respectively. The Gram-positive genus *Corynebacterium* was the next most abundant at 8% on average, however its presence varied between patients and was absent altogether in some plaque samples (Figure 5.4). *Streptococcus* followed at an average of 6% of the plaque bacterial community. In saliva samples, *Streptococcus* was the most abundant genus, accounting for 35% of the bacterial community, on average, across all samples. *Veillonella* and *Prevotella* were the next most abundant genera, comprising over 10% of the salivary microbiota at 12% and 10% on average, respectively (Figure 5.4).

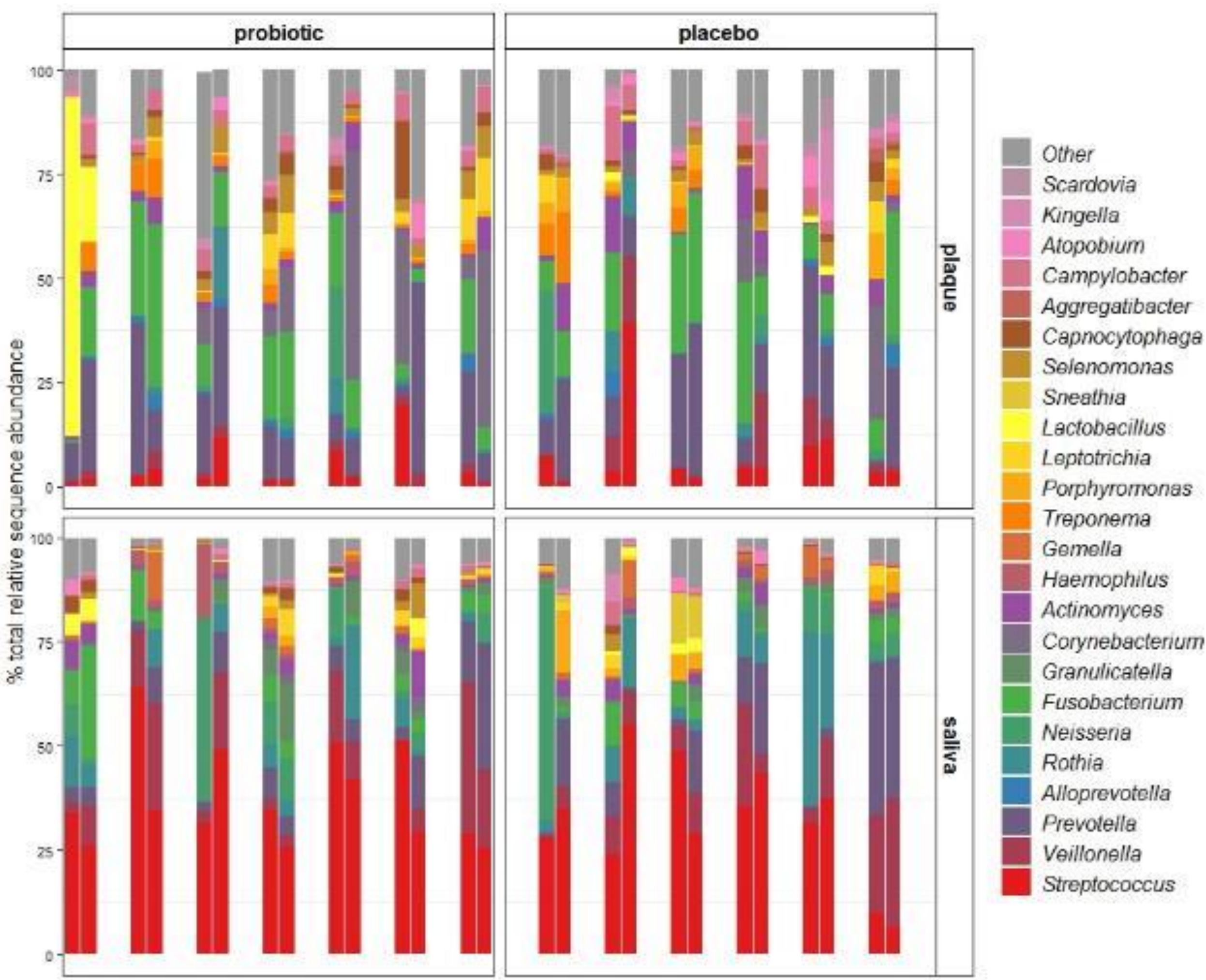


Figure 5.4. Genus-level summary of bacterial communities present in plaque and saliva for: plaque samples from the probiotic group (top left), plaque samples from the placebo group (top right), saliva samples from the probiotic group (bottom left), and saliva samples from the placebo group (bottom right). Each pair of bars represents one patient, with pre-intervention sample on the left and post-intervention sample on the right of the pair. Plaque and saliva samples are vertically aligned by corresponding patient.

5.3.5 Comparison of *Streptococcus* ZOTUs pre- and post-intervention

The total average relative sequence abundance of *Streptococcus* was significantly higher in saliva samples at 35 ± 2.6% (mean ± SE), compared to an average of 6 ± 1.6% in plaque samples ($p < 0.001$). In the probiotic group, the average relative abundance of *Streptococcus* decreased slightly post-intervention in both plaque and saliva samples, although this decrease was not significant ($p > 0.05$, Figure 5.5). Conversely, the average relative abundance of *Streptococcus* increased in the placebo group for both plaque and saliva samples but not significantly ($p > 0.05$, Figure 5.5).

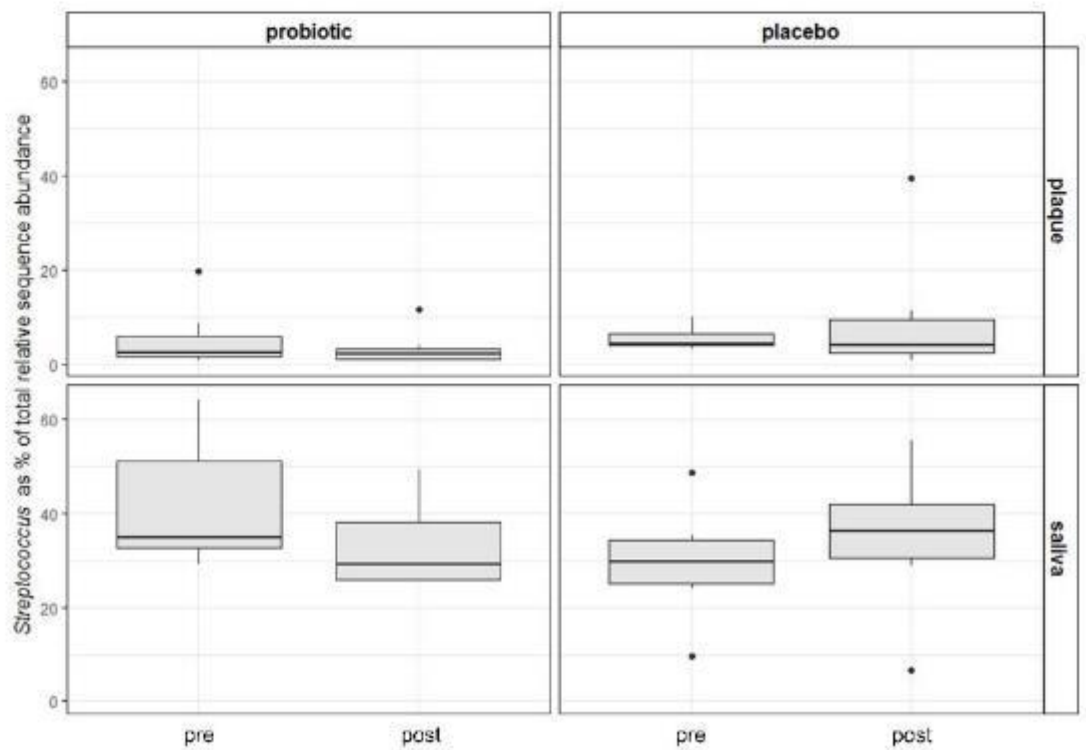


Figure 5.5. Average relative sequence abundance of *Streptococcus* in plaque and saliva bacterial communities pre- and post-intervention expressed as boxplots for: plaque samples from the probiotic group (top left), plaque samples from the placebo group (top right), saliva samples from the probiotic group (bottom left), and saliva samples from the placebo group (bottom right). The solid line within each box indicates the median value for each group, and the boxes extend to reflect the upper and lower quartiles.

The abundance of ZOTU1_*S. salivarius*, which was associated with the BLIS M18TM lozenges in this study, was examined relative to the total abundance of all *Streptococcus* ZOTUs for each sample both pre- and post-intervention, alongside changes in the abundance of ZOTUs assigned to mutans streptococci. The three other ZOTUs that were assigned to *S. salivarius* were included in the 'other streptococci' group (Appendix D, Table D1). ZOTUs assigned to mitis group streptococci (*S. oralis*, *S. sanguinis*, *S. parasanguinis*, *S. gordonii* and *S. cristatus*) and *S. intermedius* (anginosus group) were found in the highest abundances in 'other streptococci', on average, in most samples (Appendix D, Table D1).

The average abundance of ZOTU1_*S. salivarius* relative to total *Streptococcus* abundance was higher in saliva samples at 17 ± 3.0%, compared to 10 ± 3.7% in plaque, while the average abundance of mutans streptococci relative to total *Streptococcus* abundance was higher in plaque at 13 ± 4.2% compared to 2 ± 1.0% in saliva. The differences for each species between specimens was not significant ($p > 0.05$), and the majority of the *Streptococcus* abundance was grouped as 'other streptococci' for both plaque (77 ± 4.9%) and saliva (81 ± 3.3%). Within each of the probiotic and placebo groups, there were no consistent changes in either ZOTU1_*S. salivarius* or mutans streptococci, with the abundance of ZOTU1_*S. salivarius* increasing in saliva samples from patients in both groups (Figure 5.7). Interestingly, mutans streptococci tended to increase in post-intervention plaque samples from both groups (Figure 5.7). Overall, no significant changes in the average abundances of ZOTU1_*S. salivarius* or mutans streptococci (relative to total *Streptococcus* abundance) were observed pre- and post-intervention, within specimen type ($p > 0.05$, Figure 5.7).

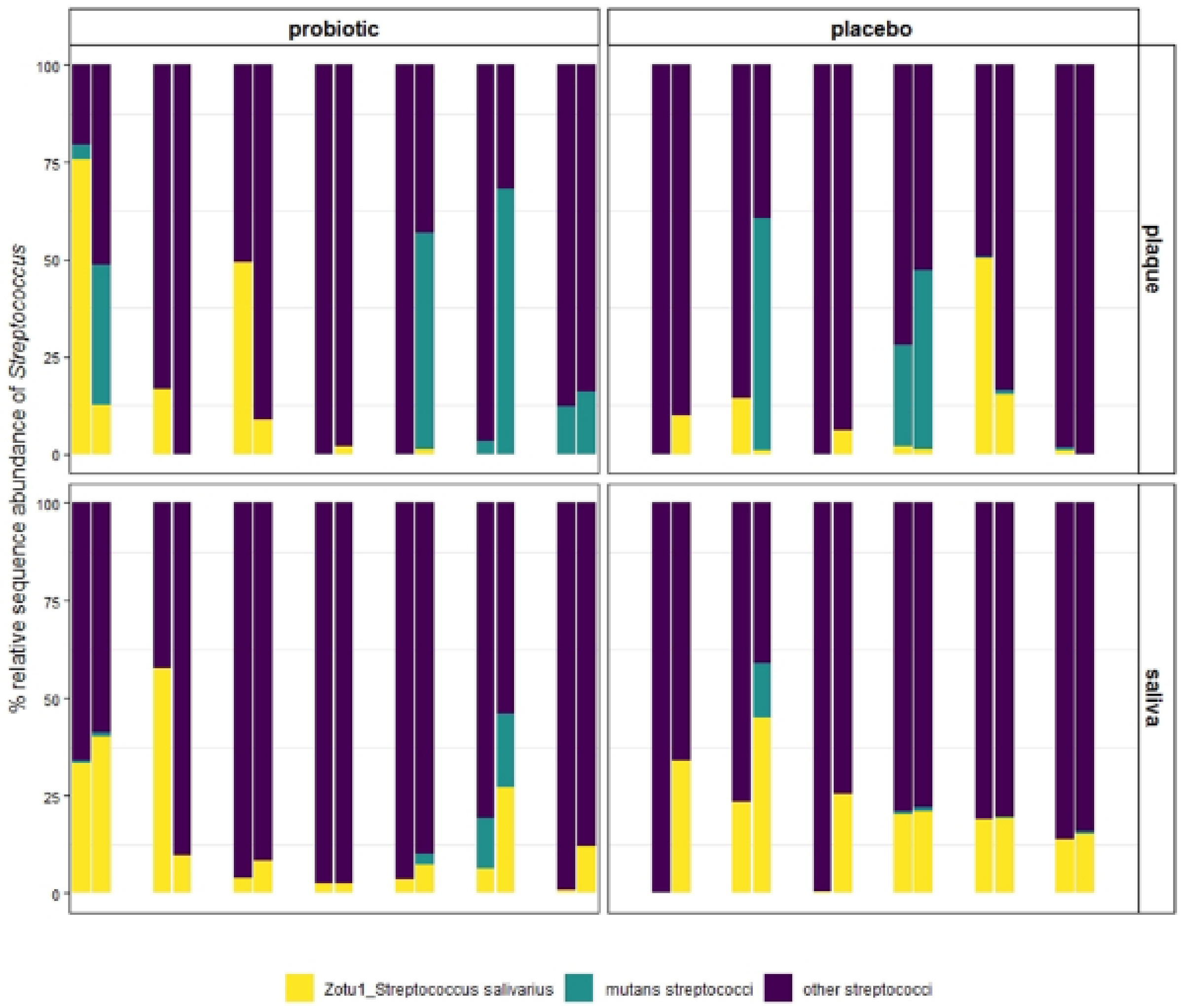


Figure 5.7. The relative abundance of 16S rRNA gene sequences affiliated with ZOTU1_*S. salivarius*, mutans streptococci and other streptococci relative to the total relative sequence abundance of all sequences assigned to *Streptococcus* for: plaque samples from the probiotic group (top left), plaque samples from the placebo group (top right), saliva samples from the probiotic group (bottom left), and saliva samples from the placebo group (bottom right). Each pair of bars represents one patient, with pre-intervention samples on the left and post-intervention on the right of the pair. Plaque and saliva samples are vertically aligned by corresponding patient.

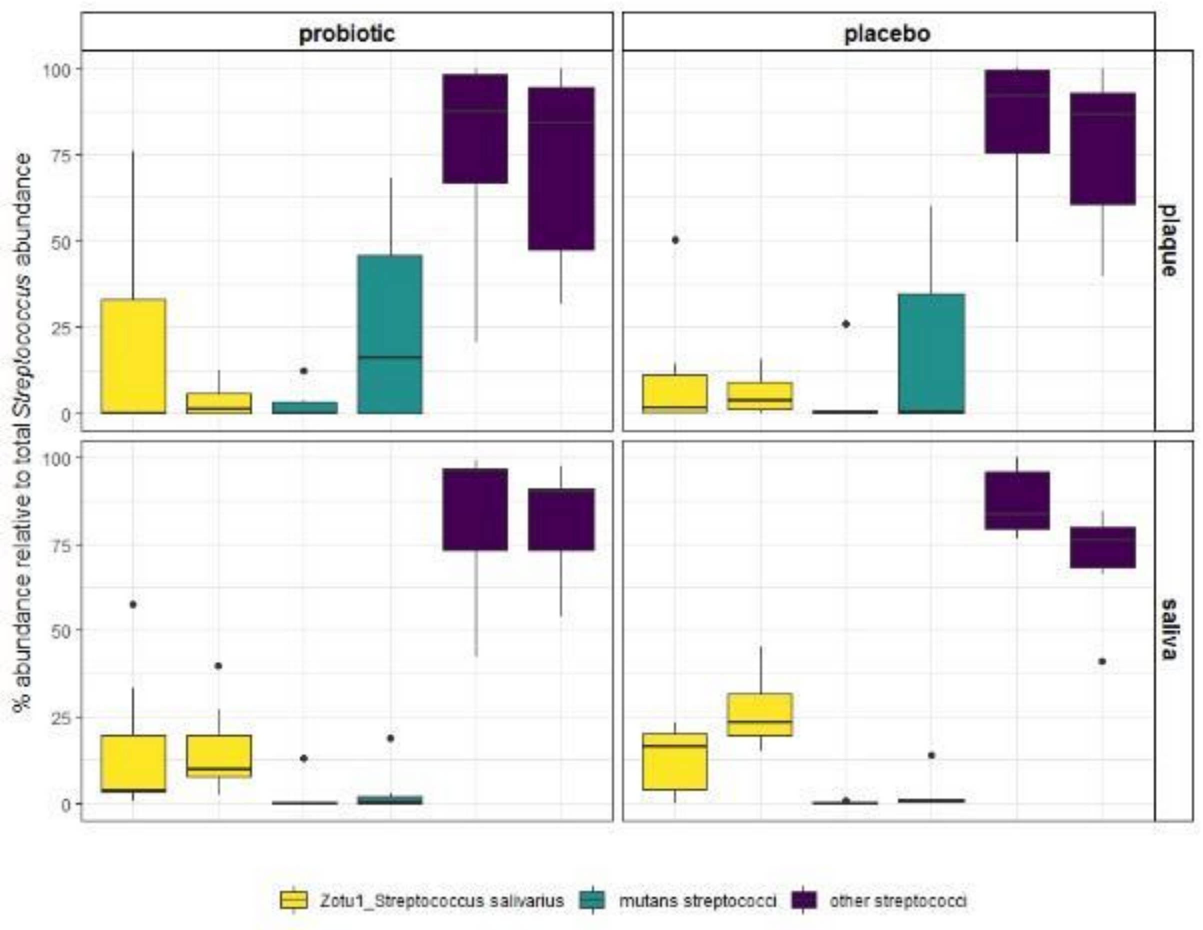


Figure 5.8. Boxplots expressing relative sequence abundance of ZOTU1_*S. salivarius*, mutans streptococci and all other streptococci, relative to the total relative sequence abundance of all *Streptococcus* sequences for: plaque samples from the probiotic group (top left), plaque samples from the placebo group (top right), saliva samples from the probiotic group (bottom left), and saliva samples from the placebo group (bottom right). Within each taxon group, results are paired as pre- (left) and post-intervention (right). The solid line within each box indicates the median value for each group, and the boxes extend to reflect the upper and lower quartiles.

5.3.6 Bacterial community diversity pre- and post-intervention

Alpha diversity, represented by the number of 'observed species' present, remained stable pre- and post-intervention for both groups (Figure 5.9). Overall, the number of observed species was the same for the combined plaque samples and the combined saliva samples, both with an average of 182 ± 12 observed species ($p > 0.05$). No significant changes in alpha diversity were observed in the plaque samples, with diversity falling slightly in the probiotic group from 176 ± 31 observed species pre-intervention to 172 ± 21 post-intervention, while rising from 186 ± 17 observed species pre-intervention to 196 ± 26 post-intervention in the placebo group ($p > 0.05$). Alpha diversity increased slightly post-intervention in saliva samples from both groups, from 177 ± 30 pre-intervention to 205 ± 25 observed species post-intervention in the probiotic group and from 165 ± 20 to 179 ± 23 observed species in the placebo group, however the pre- and post-intervention differences within or between intervention groups were not significant ($p > 0.05$).

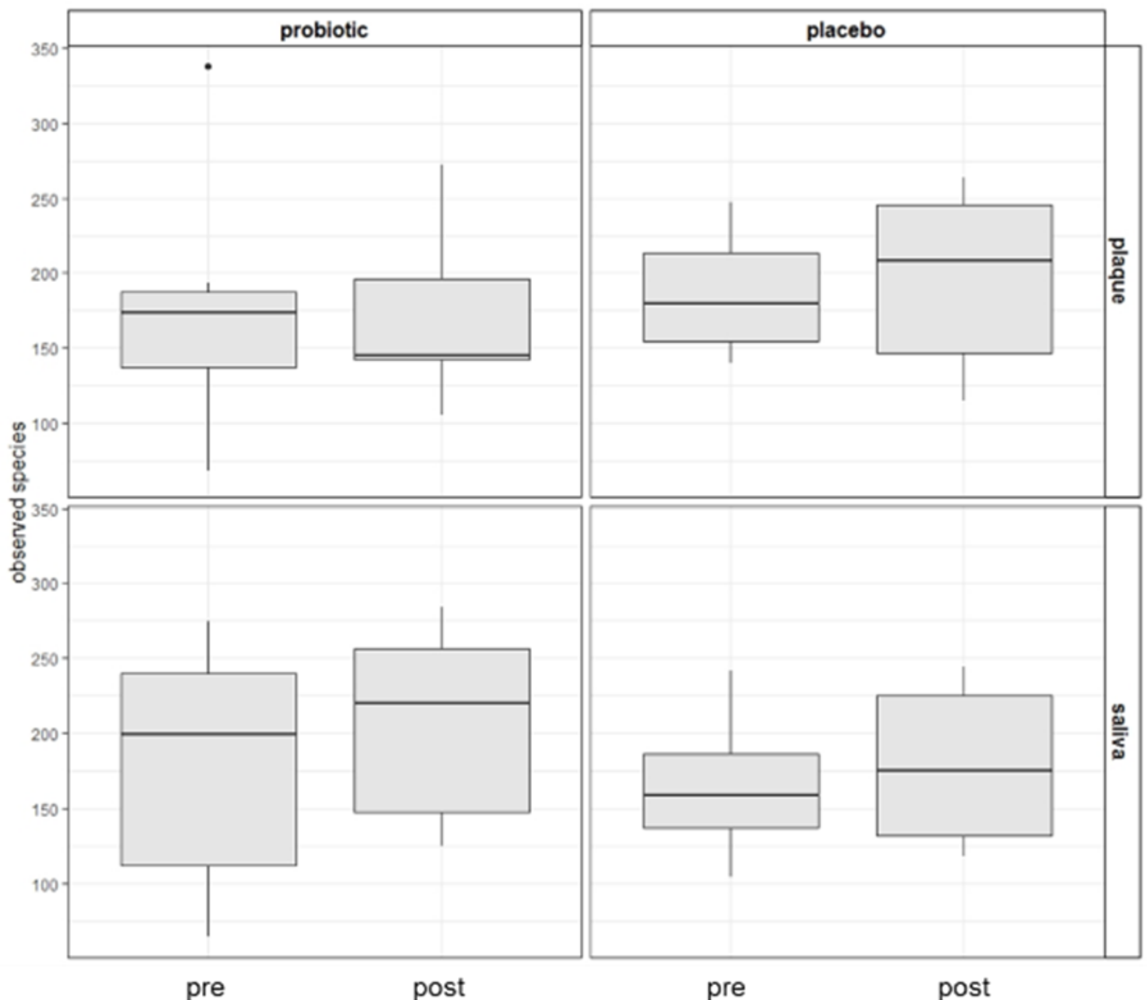


Figure 5.9. Alpha diversity expressed as boxplots using the 'observed species' metric. The solid line within each box indicates the median value for each group: plaque samples from the probiotic group (top left), plaque samples from the placebo group (top right), saliva samples from the probiotic group (bottom left), and saliva samples from the placebo group (bottom right). Boxes extend to reflect the upper and lower quartiles.

Visualisation of Bray-Curtis dissimilarity through nMDS revealed that samples significantly clustered by specimen type, accounting for 15% of variation in the model ($p < 0.001$, Figure 5.10). Intra-patient Bray-Curtis distances between pre- and post-intervention plaque samples from both groups were greater than the distances observed between pre- and post-intervention saliva samples from both groups, suggesting salivary microbiota were more stable in this study than the plaque microbiota ($p < 0.05$). No significant differences in pre- and post-intervention Bray-Curtis distance were observed within sample type between the probiotic and placebo groups ($p > 0.05$, Figure 5.11).

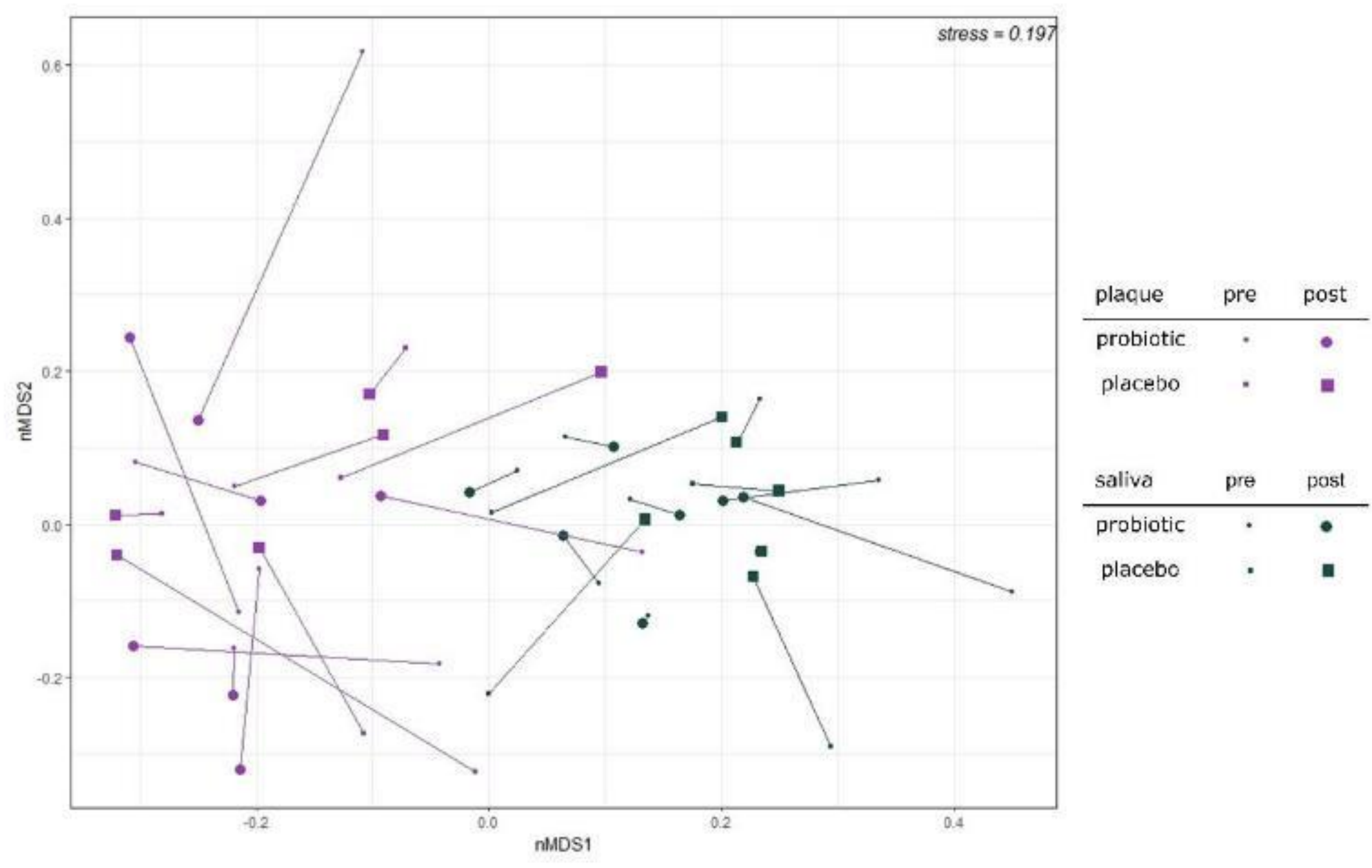


Figure 5.10. nMDS plot based on Bray-Curtis dissimilarity of all samples. Each point indicates one sample (according to the legend) and pairs of pre- and post-intervention samples for each patient are joined by vector lines, within sample type.

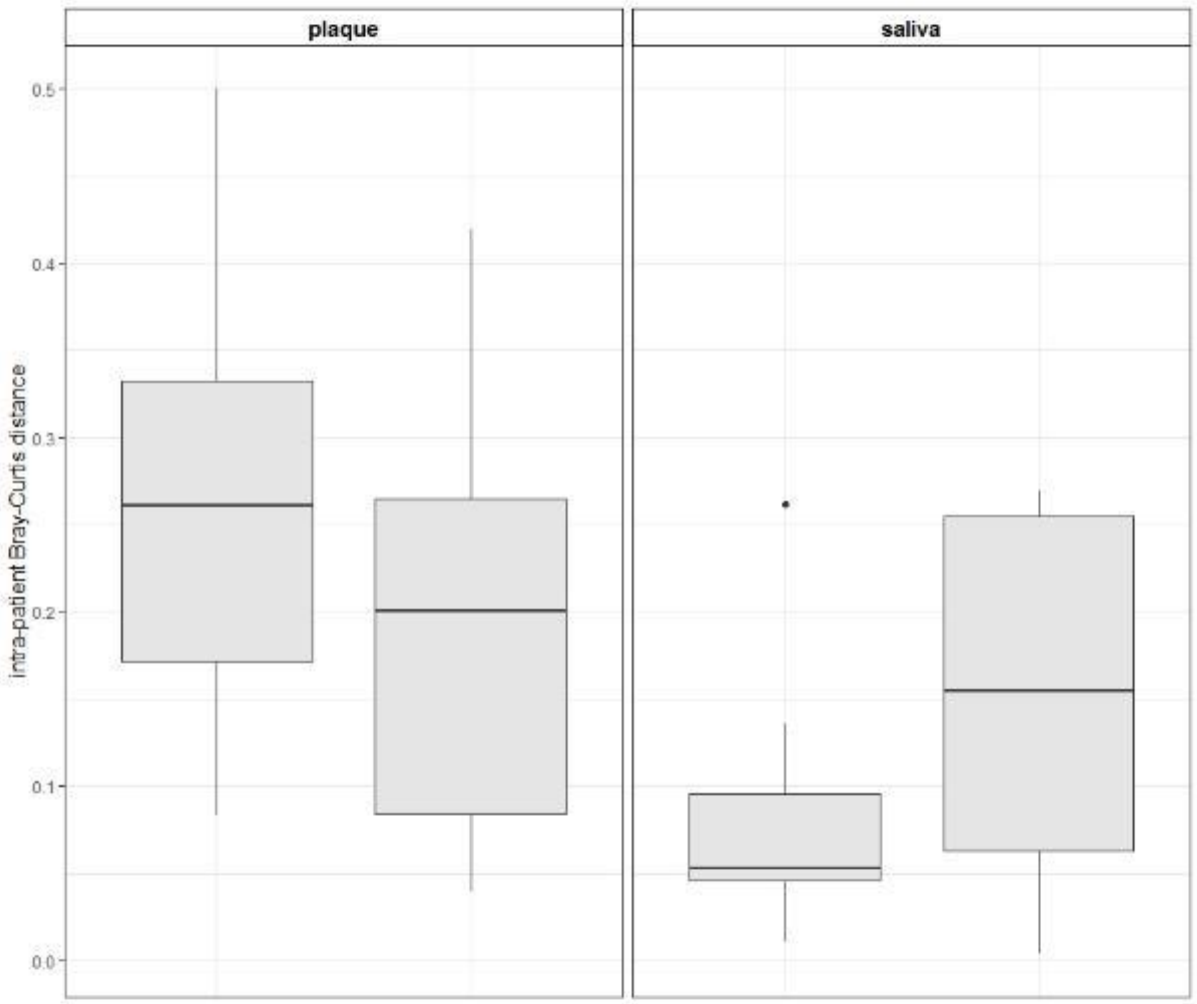


Figure 5.11. Boxplot of Bray-Curtis distance between patients pre- and post-intervention by intervention group for plaque (left) and saliva (right). The solid line within each box indicates the median value for each group, and the boxes extend to reflect the upper and lower quartiles.

5.3.7 Inference of bacterial community networks in plaque and saliva

The bacterial community network inferred from all plaque samples contained 8591 edges, of which 6450 were positive interactions and 2141 negative. ZOTU1_*S. salivarius*, associated with the BLIS M18TM lozenges, had a closeness centrality of 0.38 and its clustering coefficient was 0.04. In the plaque network, ZOTU1_ *S. salivarius* contained 22 direct edges and nine of these edges represented positive interactions, while the remaining 13 were negative interactions (Figure 5.12). Negative interactions occurred between ZOTU1_ *S. salivarius* and ZOTUs assigned to periopathogenic taxa including *Campylobacter rectus*, *Fretibacterium* spp., *Treponema maltophilum*, *T. socranskii* and *Selenomonas* spp. (Figure 5.12).

The combined saliva samples generated a lower density network than the plaque samples that contained 5070 edges, 3788 of which were positive interactions and 1282 negative. In the saliva network, ZOTU1_ *S. salivarius* had a closeness centrality of 0.35 and a clustering coefficient of 0.06. ZOTU1_ *S. salivarius* had fewer edges in the saliva network, forming 13 direct edges. Eight of these edges represented positive interactions (Figure 5.13), five of which were with other ZOTUs assigned to the *Streptococcus* genus, including ZOTU897 also assigned to *S. salivarius*.

ZOTU1039 (*S. cristatus*) and ZOTU1085 (assigned to closely related species *S. thermophilus*), were positively correlated with ZOTU1_ *S. salivarius* in both the plaque and saliva networks. Inverse covariance for the interactions in Figures 5.12 and 5.13 are described in Appendix D, Table D2.

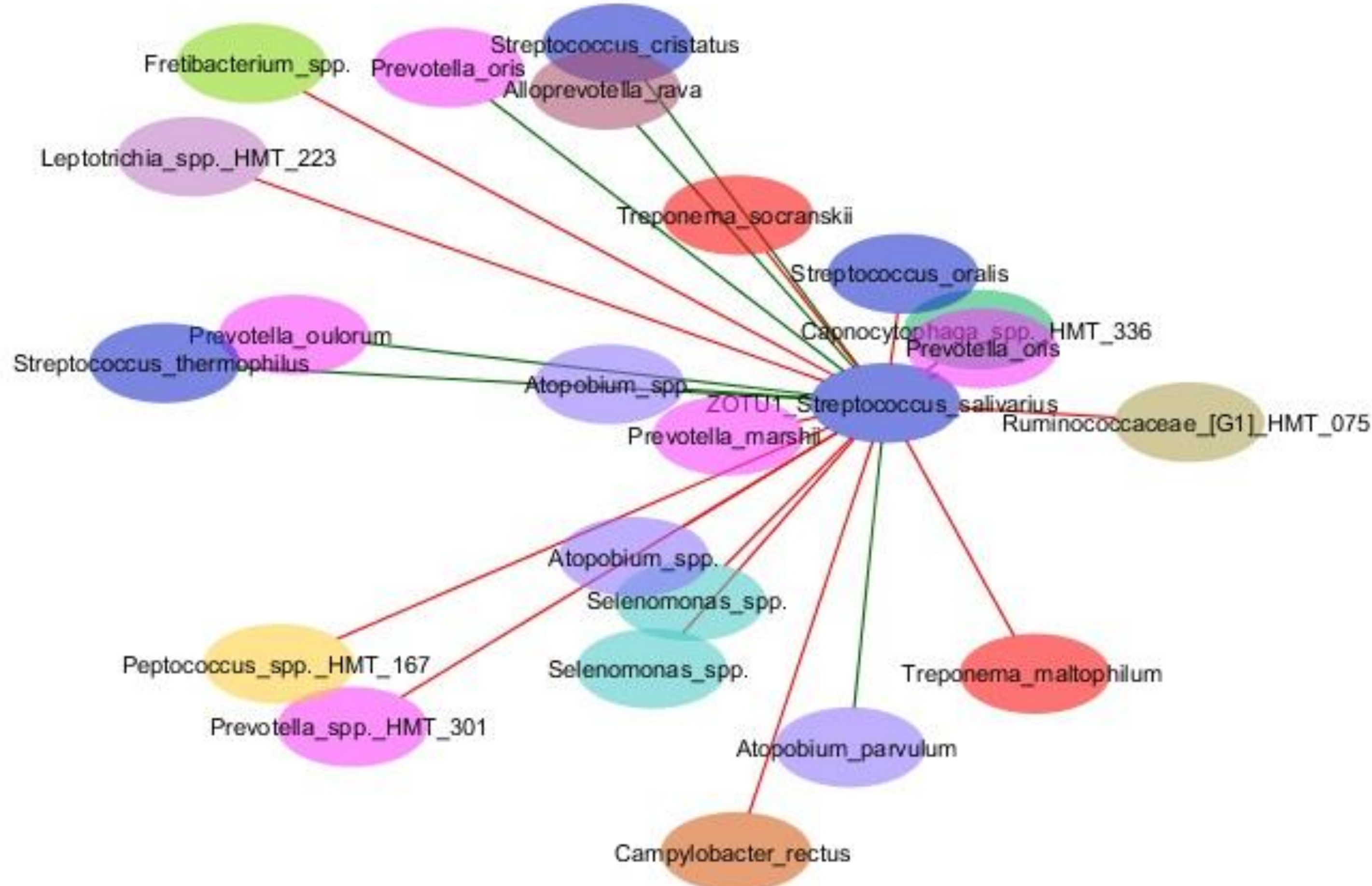


Figure 5.12. Selected nodes from the plaque bacterial network analysis based on interactions with ZOTU1_*S. salivarius*. Green and red edges represent positive and negative interactions, respectively; nodes are coloured by genus. HMT, human microbial taxon.

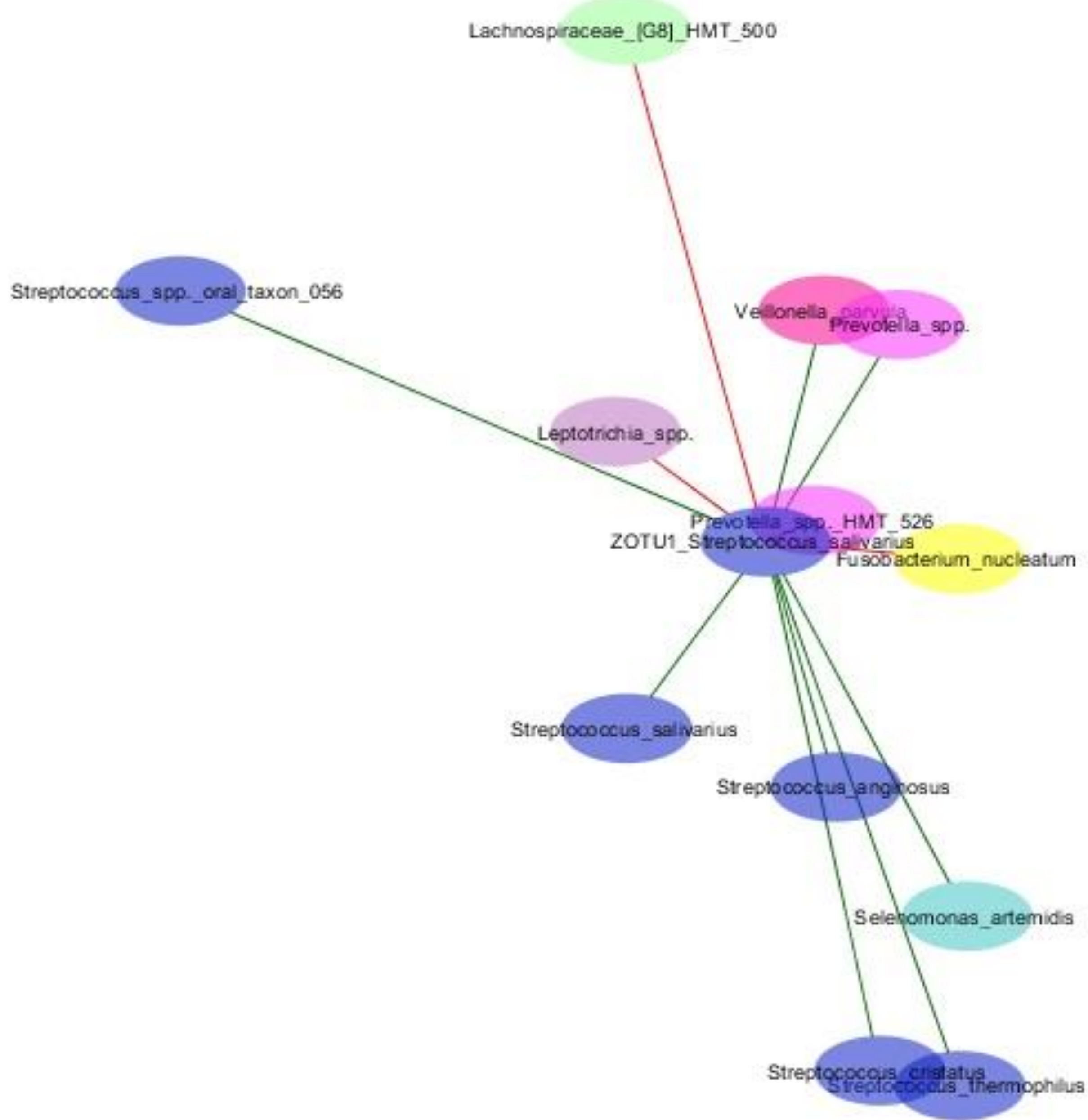


Figure 5.13. Selected nodes from the salivary bacterial network analysis based on interactions with ZOTU1_*S. salivarius*. Green and red edges represent positive and negative interactions, respectively; nodes are coloured by genus. HMT, human microbial taxon.

5.4 Discussion

In this pilot study, a four-week intervention with *S. salivarius* M18 in the form of an oral probiotic lozenge had minimal impact on the composition and diversity of the plaque and salivary microbiota in post-radiotherapy HNC patients. The findings in this chapter are consistent with previous trials that show probiotic streptococci do not effectively increase the overall abundance of oral streptococci and that the temporal persistence of probiotic streptococci is limited (Burton, Wescombe, et al., 2013; Dassi et al., 2018). *Streptococcus* forms part of the core resident oral microbiota (Segata et al., 2012; Vesty et al., 2017), making it difficult to detect meaningful changes in the relative abundance of this resident genus. Furthermore, detecting *S. salivarius* M18 and its effect on the oral microbiota requires strain-level analysis, attempted in this study using ZOTUs as a proxy for strain-level identification. Culture-based analyses have an advantage over sequencing-based studies in this context in that BLIS production can be detected phenotypically, thereby gaining a more accurate enumeration of *S. salivarius* M18 carriage and such methods have demonstrated that the persistence of *S. salivarius* M18 is dose-dependent (Burton, Wescombe, et al., 2013). Strain-specific Droplet Digital PCR or real-time qPCR could be considered as alternative molecular approaches to measure total abundance, rather than relative abundance.

The unique oral environment in HNC patients post-radiotherapy may reduce susceptibility to probiotic-induced changes in the oral microbiota. Hyposalivation and xerostomia, experienced by 10 of the 13 patients in this study, may compromise the ability of the probiotic to disseminate through the oral cavity. Radiotherapy-induced changes in the pH, volume and viscosity of saliva may increase the microbial concentration of saliva and limit its capacity to increase its microbial load, accounting for the lack of significant increase in *Streptococcus* relative abundance seen in the probiotic group post-intervention. Conducting a similar trial on a healthy

cohort may provide further insight into the capacity of *S. salivarius* M18 to alter oral bacterial communities. Data presented in this chapter also suggest that the probiotic was not able to penetrate the plaque biofilm, as there was no significant increase in the abundance of ZOTU1_*S. salivarius* in the post-intervention plaque samples from the probiotic group. Interestingly, the abundance of mutans streptococci relative to total *Streptococcus* abundance increased in several patients post-intervention in both groups.

Post-radiotherapy, the abundance of periopathogenic bacteria including *Tannerella forsythia* (Müller et al., 2019) and acid-tolerant microbes (Almståhl et al., 2018) increases. While this pilot study failed to find any significant abundance-based changes in *S. salivarius* or decay-associated mutans streptococci, other microbial mechanisms that affect post-radiotherapy oral health may be relevant. Based on bacterial network analyses, there may be motive to consider the use of BLIS M18TM clinically. In the saliva network, ZOTU1_*S. salivarius* positively interacted with other core microbiota, including *Prevotella*, *Veillonella* and several *Streptococcus* species but negatively interacted with *Fusobacterium nucleatum*, supporting previous observations that *Streptococcus* and *Fusobacterium* are negatively correlated in saliva (Vesty et al., 2018). In the plaque network, several negative interactions were detected between ZOTU1_*S. salivarius* and species from the periopathogenic genera *Campylobacter*, *Fretibacterium*, *Selenomonas* and *Treponema*. However, the data presented here is based on a pilot study and the long-term benefit of these interactions should be investigated in additional trials that include larger cohorts and a longer intervention period. Such studies may benefit from investigating the effect of *S. salivarius* M18 on salivary pH and the incidence of dental caries, in addition to changes in CPITN and plaque index scores.

An overall improvement in the O'Leary Plaque Index score was seen in both intervention groups in this study, suggesting clinical improvements were not driven by probiotic-induced changes in the oral microbiota. I speculate that the oral hygienist involvement may have

contributed to this improvement through oral hygiene advice that was provided in order to standardise the oral health regime for all participants in the trial. This advice may have resulted in the improvement of brushing and/or inter-dental cleaning routines for some patients compared to their pre-trial habits.

The use of oral probiotics to modulate host immune responses and microbial interactions through antagonism and co-aggregation is, in theory, a promising mechanism to improve oral health (Mahasneh & Mahasneh, 2017). However, these beneficial properties require further optimisation and exploitation before oral probiotics are clinically recommended as a complementary approach to improve post-radiotherapy oral health for HNC patients.

Chapter 6

General Discussion

Head and neck cancer (HNC) and treatment-associated side effects present significant physical, psychological and financial burdens to both patients and the healthcare system. The oral microbial contribution to carcinogenesis and the pathogenesis of treatment-induced sequelae remain theoretically linked but poorly described. This thesis investigated the relationship of the oral microbiota with HNC in three settings: as a pre-treatment risk factor; its contribution to the development of radiotherapy-induced oral mucositis; and manipulation post-radiotherapy with an oral probiotic. Targeted-amplicon sequencing captured the oral microbial communities in each of the chapters and the results were interpreted within the context of clinical patient outcomes. In this final chapter, the findings of this thesis are considered in light of international research and their potential for translation into a clinical setting.

6.1 Oncogenic potential of the oral microbiota

In the last five years, there has been increasing interest in characterising the link between oral microbiota and carcinogenesis (Healy & Moran, 2019). Of particular interest are those cases where head and neck squamous cell carcinoma is not explained by traditional risk factors such as alcohol consumption, human papilloma virus and tobacco use (Al-Hebshi, Borgnakke, & Johnson, 2019). Epidemiological studies associate poor oral health to oral carcinogenesis, leading to speculation that periopathogenic microbes may contribute to oral carcinogenesis (Yao, Zhou, Peng, Ji, & Liu, 2014). While defining taxon-specific shifts in the oral microbiota associated with HNC is challenging, a recent systematic review suggests that a theme of oral bacterial dysbiosis is evident in oral and oropharyngeal cancers (Gopinath et al., 2019).

In this thesis, the bacterial constituents of the HNC salivary microbiota and their associated inflammatory cytokine profiles did not effectively discriminate HNC patients from healthy controls. Rather, the bacterial data demonstrated that in HNC these biomarkers are more reflective of patients with compromised oral health. Therefore, while ill-defined, a causal link between periopathogenic bacteria and HNC cannot be ruled out.

Another important, yet understudied aspect of the oral microbiome is fungi. Very few studies have investigated the composition and functional role of fungi in either HNC patients or healthy controls. The data presented in this thesis suggested *Candida albicans* might differentiate the oral microbiota of HNC patients more effectively than its bacterial constituents. *C. albicans* is overrepresented in oral cancer biopsies (Perera et al., 2017) and is a potent producer of acetaldehyde (Gainza-Cirauqui et al., 2013). One exemplary study noted that *C. albicans* interacts with mucosal bacteria and drives dysbiosis in a mouse model (Bertolini et al., 2019). Rather than directly inducing carcinogenesis, it may be that dysbiosis of the oral microbiota accentuates the genotoxic effects of known carcinogens like alcohol and tobacco by generating carcinogenic levels of acetaldehyde. Consequently, future studies may benefit from measuring salivary acetaldehyde levels in conjunction with studying the oral metatranscriptome. Preliminary studies have reported increased gene expression of bacterial virulence factors in patients with oral squamous cell carcinoma, and this remains a promising area for research (Yost et al., 2018).

Ultimately, if dysbiosis of the oral microbiota was definitively associated with HNC, clinical interventions should focus on reducing its carcinogenic potential through the improvement of oral health and beneficial manipulation of the oral microbiota.

6.2 Oral microbiota and the pathogenesis of oral mucositis

Interactions between the host and oral microbiota play an important role in the pathogenesis of radiotherapy-induced oral mucositis. Dysbiosis prior to and during treatment likely intensifies mucosal inflammation and exaggerates oral mucositis (Bowen et al., 2019). The findings presented in Chapter 4 support this hypothesis by demonstrating that an increased abundance of anaerobic Gram-negative bacilli (GNB), including periopathogenic genera *Porphyromonas* and *Tannerella*, are positively correlated with sites of ulcerative oral mucositis. Very few studies have attempted to elucidate a link between oral microbes and the onset of oral mucositis, but the data presented in my thesis are consistent with previous findings that anaerobic GNB are implicated in ulcerative oral mucositis (Hou et al., 2018; Zhu et al., 2017). These results will help inform the development of microbial-based preventative measures that reduce the morbidity associated with this debilitating side effect. Implementation of such preventative measures has the potential to improve radiotherapy treatment tolerance, compliance and quality of life for HNC patients.

Currently, the Mucositis Study Group of the Multinational Association of Supportive Care in Cancer/International Society for Oral Oncology suggests implementation of multi-agent combination oral care protocols for the prevention of oral mucositis during radiotherapy treatment of head and neck cancer (Ariyawardana et al., 2019). Multi-agent combination oral care protocols include dental care, oral hygiene and bland rinses e.g. saline and sodium bicarbonate. While these guidelines do not directly affect the pathways involved in the pathogenesis of oral mucositis, they help maintain oral hygiene and comfort (McGuire et al., 2013). These measures may indirectly affect the oral microbiota by reducing the oral microbial load and thereby attenuating the host inflammatory response. The next step may be to incorporate modulation of the oral microbial composition with these guidelines to further

reduce inflammation. Probiotics reduce the severity of oral mucositis in nasopharyngeal carcinoma patients receiving chemoradiation (Chen et al., 2018); however, probiotics are less effective at limiting radiotherapy-induced oral mucositis in HNC patients (de Sanctis et al., 2019). Yet, in light of the findings in this thesis, probiotic-induced modulation of salivary microbiota should still be pursued as an approach to prevent oral mucositis and would complement oral hygiene recommendations for patients receiving radiotherapy to the head and neck region.

6.3 Probiotic-induced modulation of post-radiotherapy oral microbiota

The effects of oral probiotics can be considered in two categories: pathogen antagonism and modulation of the host immune response (Haukioja, 2010). In the first category, probiotic strains compete with pathogens for nutrients and sites of adhesion in the oral cavity. Furthermore, some bacterial strains (e.g. *Streptococcus salivarius* M18) produce antimicrobial compounds such as bacteriocin-like inhibitory substances (BLIS) that inhibit pathogenic bacteria (James & Tagg, 1991). Probiotics also enhance the host immune response and inhibit pathogen-induced production of inflammatory cytokines, thereby reducing periodontal inflammation and improving oral health (Riccia et al., 2007; Shimauchi et al., 2008).

Translating the benefits of oral probiotics in order to achieve a clinical improvement in the post-radiotherapy oral health of HNC patients relies on the probiotic strain's ability to colonise and persist in the oral cavity. The pilot study presented in Chapter 5 suggested that a four-week intervention with BLIS M18TM does not have a significant or obvious impact on the composition and diversity of the oral microbiota, in agreement with previous findings (Burton,

Wescombe, et al., 2013; Dassi et al., 2018). Oral probiotics containing strains of *Streptococcus* have limited temporal persistence in the oral microbiota and, post-intervention, levels of detection return to baseline (Burton, Wescombe, et al., 2013; Dassi et al., 2018). Therefore, the use of oral probiotics in a clinical setting needs further development and may require pre-intervention optimisation of the oral cavity (e.g. with medicated mouthwashes) to make the oral environment more receptive to probiotic manipulation.

Nonetheless, the use of oral probiotics does not negatively impact patient health and, while our understanding of their exact benefits and modes of action is currently limited, the theoretical benefits may be enough for clinicians and patients to consider their use as a supplementary means to improve post-radiotherapy oral health.

6.4 Future directions

In line with global predictions, the incidence of HNC is increasing in New Zealand (Lucas-Roxburgh et al., 2017; Marur & Forastiere, 2016). It is therefore essential to develop a better understanding of microbial-based risk factors for carcinogenesis and associated treatment-induced side effects.

The methods used in this thesis are largely focused on targeted amplicon sequencing in order to characterise oral microbial communities. While this approach has the benefit of identifying microbial biomarkers of disease and treatment outcomes, it is also important to consider the microbial community in the context of its functional capacity and how it interacts with its host. Future studies may benefit from incorporating metagenomic functional predictions in conjunction with analysis of the host transcriptome to fully characterise the host-microbial interactions that occur in HNC and its treatment. Data generated from such studies would benefit HNC and its treatment, as well as improve our understanding of the link between microbiota and other cancers. Characterising the microbial-mucosal interactions that occur during radiotherapy may be translatable to patients suffering chemotherapy-induced oral and gastrointestinal mucositis. Oral probiotic-induced beneficial modulation of the oral microbiota post-radiotherapy requires further investigation but has the promising potential to reduce the physical, psychological and ongoing financial burden of deteriorating oral health for HNC patients.

6.5 Concluding remarks

This thesis presents an analysis of the oral microbiota pre-, during and post-radiotherapy treatment of HNC. It begins with a novel comparison of four DNA-extraction methods in order to identify the best approach to simultaneously study oral bacterial and fungal communities. This information was then applied to describe the bacterial and fungal components of the HNC-associated salivary microbiota, highlighting the link between *C. albicans* and HNC. An important finding from Chapter 3 indicated that the salivary microbial and inflammatory biomarkers in HNC patients were more similar to patients with compromised oral health than healthy controls. Chapter 4 investigated microbial risk factors for radiotherapy-induced oral mucositis during treatment. The main finding from this section supported the hypothesis that poor oral health is linked to oral mucositis by describing a positive correlation between anaerobic Gram-negative bacilli and ulcerative mucositis. Chapter 5 presented the first clinical trial of the oral probiotic *S. salivarius* M18 in post-radiotherapy HNC patients.

The research presented in this thesis contributes significant new knowledge to our current understanding of the role of the oral microbiota in HNC. These results offer insights which may help mitigate the debilitating side-effects associated with radiotherapy treatment. I hope that this research contributes to the improvement of clinical outcomes and quality of life for HNC patients and will help inform future studies.

APPENDIX A

Table A1. DNA yield and quality measures (mean ± SEM) of triplicate data for extractions from *Cryptococcus neoformans* ATCC32045 and *Penicillium chrysogenum* ATCC10002 across four DNA extraction methods.

	***Cryptococcus neoformans* ATCC32045**			***Penicillium chrysogenum* ATCC10002**		
	DNA yield (ng/µL)	**A260/280 nm**	**A260/230 nm**	**DNA yield (ng/µL)**	**A260/280 nm**	**A260/230 nm**
MoBio PowerSoil® DNA Isolation Kit	7.483 ± 2.740	1.893 ± 0.066	0.500 ± 0.024	8.033 ± 3.228	1.940 ± 0.034	1.140 ± 0.706
Qiagen QIAamp® DNA Mini Kit	1.167 ± 0.101	1.666 ± 0.013	0.773 ± 0.408	0.667 ± 0.176	1.480 ± 0.268	0.022 ± 0.459
Zymo Bacterial/ Fungal MiniPrep	3.000 ± 0.477	1.979 ± 0.150	0.204 ± 0.093	4.467 ± 0.900	1.910 ± 0.051	0.334 ± 0.038
Phenol:chloroform-based isolation	10.78 ± 2.479	1.647 ± 0.068	3.213 ± 0.480	2.667 ± 0.328	1.648 ± 0.044	3.338 ± 1.208

APPENDIX B

Table B1. Head and neck cancer patient information.

Patient	Tumour location	[a]DMFT index			Dentures	[b]Periodontal disease	[c]Pre-RT extraction
		D	M	F			
1	left parotid	0	10	16	N		N
2	buccal mucosa	1	6	11	N	Y	Y
3	right tongue	0	4	15	N		N
4	left palate	0	7	5	N		N
5	left parotid	0	26	0	N		N
6	floor of mouth	1	5	6	N		N
7	lateral tongue	4	19	10	N		Y
8	floor of mouth	6	12	13	N	Y	Y
9	left tonsil	1	11	6	N		Y
10	base of tongue	0	17	0	Y	Y	Y
11	left tonsil	0	9	20	N		N
12	left tonsil	2	12	14	N		Y
13	base of tongue	0	11	13	N		N
14	left tonsil	0	6	21	N		N

[a] *Decayed (D), Missing (M), Filled (F) Teeth (DMFT) index describing number of affected teeth, based on orthopantomograms*

[b] *Evidence of periodontal disease, based on orthopantomograms*

[c] *Pre-radiotherapy extraction/s of compromised teeth required*

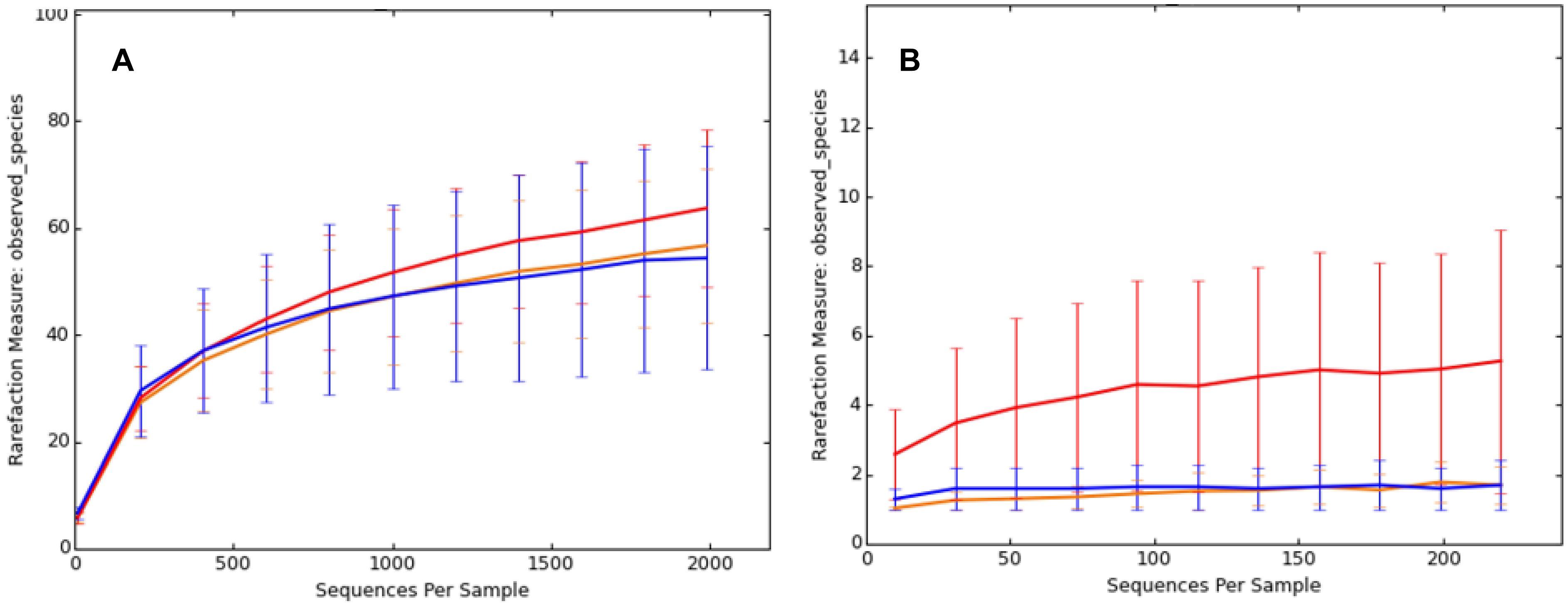


Figure B1. Alpha rarefaction analysis plots demonstrating the number of 'observed species' in saliva by disease group as a function of sequences per sample for (A) bacterial observed species and (B) fungal observed species.

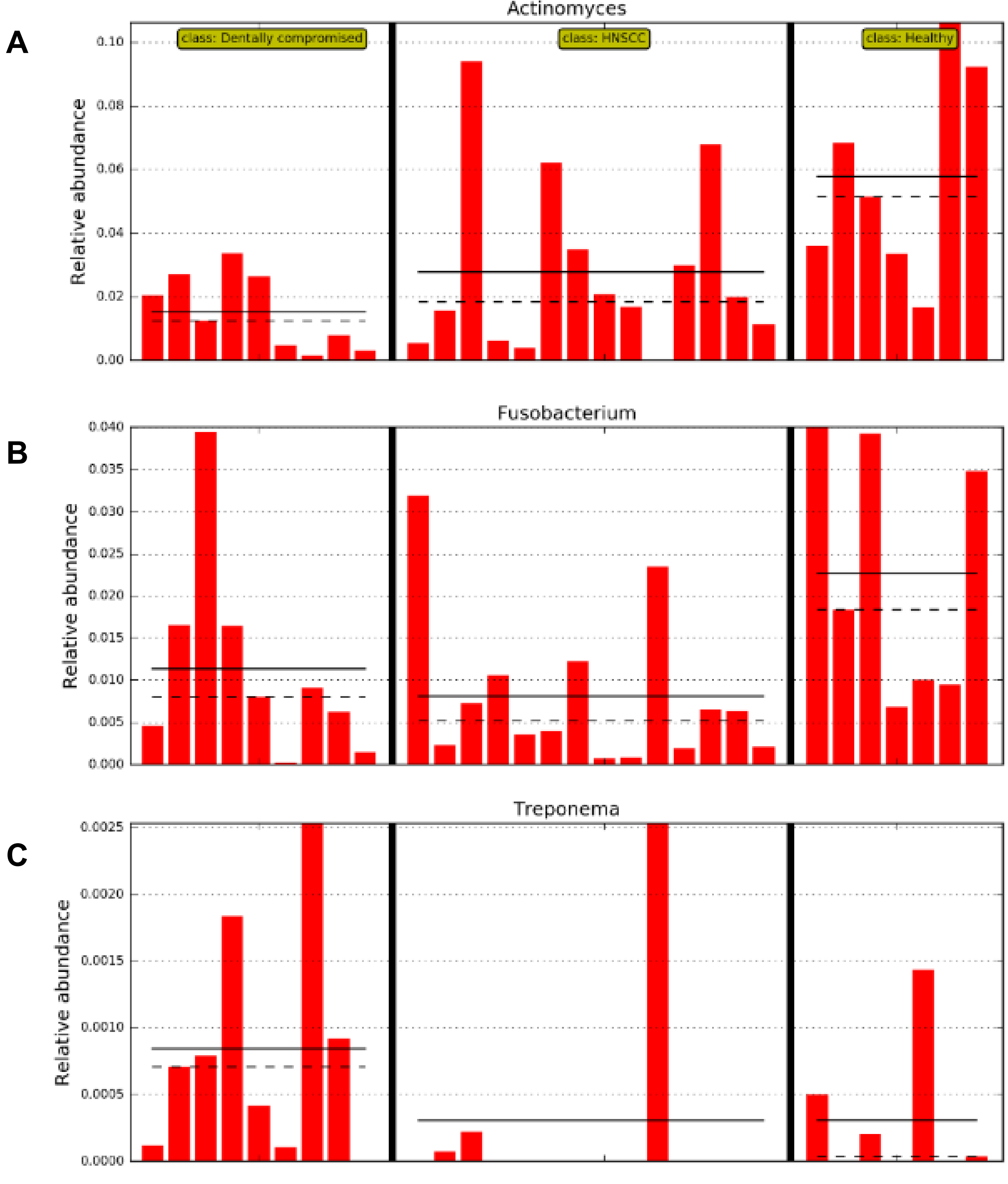


Figure B2. Differential relative abundances of bacterial genera associated with disease class through LEfSe analysis: (A) *Actinomyces*; (B) *Fusobacterium*; (C) *Treponema*. Mean and median relative abundances for each disease class are indicated by the solid and dashed lines, respectively.

APPENDIX C

Table C1. Patient information table.

Patient	Age	Sex	Smoker	Tumour site, p16 status[a]	Tumour stage[b]	RT/ CRT	Total Gy (Fx)[c]	Feeding route	Radiation dose (Gy)	OM grade	Saliva	Swab site
1	57	M	U	maxillary gingiva	pT4a N0 M0	RT	60 (30)	oral	0 – 20	0	✓	buccal mucosa
									21 – 40	n/a	n/a	n/a
									41 – 60	3	✓	lateral tongue
2	54	M	N	parotid	muco-epidermoid	RT	60 (30)	oral	0 – 20	0	✓	buccal mucosa
									21 – 40	1	✓	buccal mucosa
									41 – 60	n/a	n/a	n/a
3	64	F	N	tongue	pT2 N2b M0	RT	60 (30)	oral	0 – 20	0	✓	buccal mucosa
									21 – 40	3	✓	buccal mucosa
									41 – 60	3	✓	buccal mucosa
4	73	M	N	parotid	metastatic	RT	50 (20)	oral	0 – 20	0	✓	buccal mucosa
									21 – 40	0	✓	buccal mucosa
									41 – 60	n/a	n/a	n/a
5	45	F	N	nasopharynx	T3 N3b M0	RT	60 (35)	oral	0 – 20	0	✓	n/a
									21 – 40	0	✓	buccal mucosa
									41 – 60	0	✓	buccal mucosa
6	78	F	N	parotid	metastatic	RT	60 (30)	oral	0 – 20	0	✓	buccal mucosa
									21 – 40	0	✓	buccal mucosa
									41 – 60	0	✓	buccal mucosa
7	66	M	ex	tonsil, p16+	rT2 N2b M0	CRT	70 (35)	naso-gastric, > 40Gy	0 – 20	0	✓	buccal mucosa
									21 – 40	1	n/a	lateral tongue
									41 – 60	3	✓	lateral tongue
8	60	F	ex	tonsil, p16+	T1 N2b M0	RT	60 (30)	oral	0 – 20	0	✓	buccal mucosa
									21 – 40	0	✓	buccal mucosa
									41 – 60	0	✓	buccal mucosa
9	69	F	Y	soft palate	pT2 N1 Mx	RT	60 (30)	PEG, pre-RT	0 – 20	0	✓	buccal mucosa
									21 – 40	n/a	n/a	n/a
									41 – 60	0	n/a	buccal mucosa

Patient	Age	Sex	Smoker	Tumour site, p16 status[a]	Tumour stage[b]	RT/ CRT	Total Gy (Fx)[c]	Feeding route	Radiation dose (Gy)	OM grade	Saliva	Swab site
10	42	M	Y	floor of mouth	pT4a N0 M0	RT	60 (30)	PEG, > 48 Gy	0 – 20	0	✓	buccal mucosa
									21 – 40	3	✓	buccal mucosa
									41 – 60	3	✓	buccal mucosa
11	73	M	ex	neck	T1 N2c M0	RT	70 (35)	oral	0 – 20	0	✓	buccal mucosa
									21 – 40	3	✓	buccal mucosa
									41 – 60	0	✓	buccal mucosa
12	50	M	ex	oropharynx, p16+	T2 N2c M0	CRT	70 (35)	oral	0 – 20	0	✓	buccal mucosa
									21 – 40	0	✓	buccal mucosa
									41 – 60	2	✓	lateral tongue
13	53	M	N	base of tongue, p16+	T1 N2c M0	CRT	70 (35)	oral	0 – 20	0	✓	buccal mucosa
									21 – 40	n/a	n/a	n/a
									41 – 60	0	✓	buccal mucosa
14	74	F	N	tonsil, p16-	cT4a N2b	CRT	70 (35)	oral	0 – 20	0	✓	buccal mucosa
									21 – 40	2	✓	lateral tongue
									41 – 60	3	✓	lateral tongue
15	83	F	N	tonsil, p16-	T2 N0 M1	RT	65 (30)	oral	0 – 20	0	✓	buccal mucosa
									21 – 40	3	✓	buccal mucosa
									41 – 60	4	✓	buccal mucosa
16	88	M	N	parotid/neck	Tx N1 M0 Merkel cell	RT	60 (30)	oral	0 – 20	0	n/a	buccal mucosa
									21 – 40	0	n/a	buccal mucosa
									41 – 60	n/a	n/a	n/a
17	79	M	ex	pinna	Tx N2a M0 sarcomatoid	RT	50 (25)	oral	0 – 20	0	n/a	buccal mucosa
									21 – 40	0	n/a	buccal mucosa
									41 – 60	n/a	n/a	n/a
18	54	M	ex	oropharynx, p16+	T1 N2b M0	CRT	70 (35)	oral	0 – 20	1	n/a	buccal mucosa
									21 – 40	0	n/a	buccal mucosa
									41 – 60	n/a	n/a	n/a
19	78	M	N	parotid	Tx N2b M0	RT	60 (30)	oral	0 – 20	0	n/a	buccal mucosa
									21 – 40	n/a	n/a	n/a
									41 – 60	0	n/a	buccal mucosa

M, male; F, female; U, unknown; ex, ex-smoker; N, non-smoker; Y, current smoker; RT, radiotherapy; CRT, chemoradiation; PEG, percutaneous endoscopic gastrostomy; OM, oral mucositis

[a] *p16 is an indirect biomarker for tumours that harbour oncogenic human papilloma virus (HPV)*

[b] *TNM Classification of Malignant Tumours*

[c] *Gray (Gy) is the unit of absorbed ionising radiation dose, 1 gray = 1 joule/kilogram. Fx refers to fractions, the number of treatment sessions the total radiation dose was administered in.*

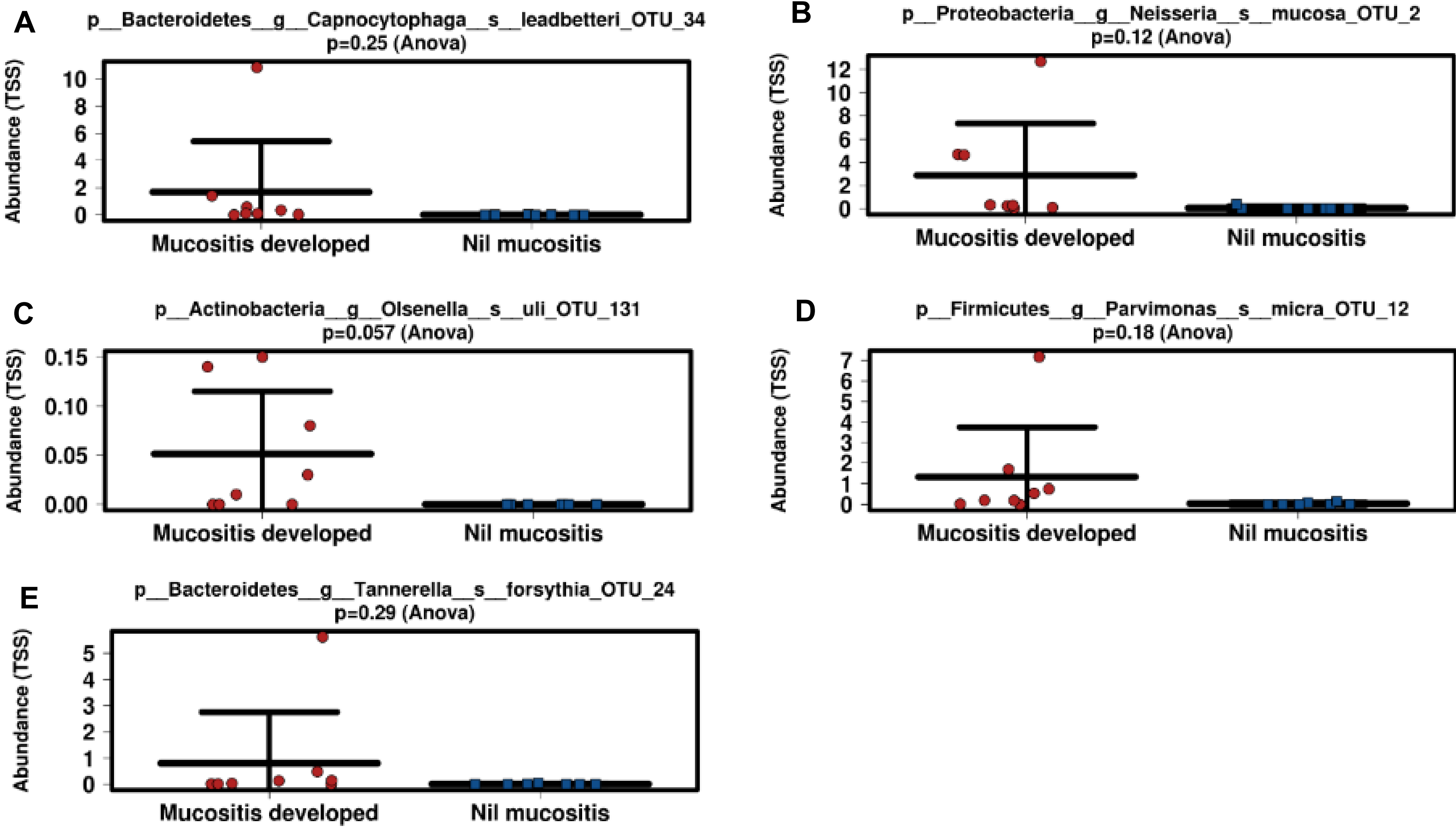


Figure C1. Differential abundances of bacterial OTUs in saliva associated with the subsequent development of grade ≥ 2 oral mucositis through LEfSe analysis. (A) *Capnocytophaga leadbetteri*; (B) *Neisseria mucosa*; (C) *Olsenella uli*; (D) *Parvimonas micra;* (E) *Tannerella forsythia.*

Table C2. Random forest analysis of the 30 most abundant bacterial genera in the 17 buccal mucosa swabs (grade 0) taken at 0 – 20 Gy.

Taxa	**MDA**	**AUC**	**AUC** (lower CI)	**AUC** (upper CI)	**OR** ≤ grade 1: ≥ grade 2	**Lower CI**	**Upper CI**	**Delta**	**Fold Change**	**Mean** ≤ grade 1	**Mean** ≥ grade 2
Streptococcus	18.0	0.83	0.61	1.00	2.50	0.34	23.97	1.14	2.09	53.62	25.60
Lactobacillus	13.0	0.80	0.64	0.96	1.50	0.12	36.88	0.70	314376.00	2.52	0.00
Fusobacterium	8.4	0.74	0.48	0.99	0.15	0.01	1.56	1.08	-3.10	2.80	8.67
Haemophilus	7.4	0.70	0.41	0.99	0.15	0.01	1.56	1.01	-5.60	1.94	10.88
Staphylococcus	7.0	0.35	0.20	0.50	2.57	0.25	60.02	0.87	14376.00	0.12	0.00
Tannerella	5.5	0.79	0.56	1.00	0.33	0.03	2.81	0.89	-4.27	0.12	0.52
Porphyromonas	5.0	0.69	0.41	0.97	0.15	0.01	1.56	1.01	-7.98	0.59	4.67
Eikenella	3.9	0.72	0.49	0.95	0.15	0.01	1.56	0.78	-5.09	0.03	0.15
Lachnoanaerobaculum	3.1	0.56	0.26	0.87	1.50	0.12	36.88	0.67	6.62	1.50	0.23
Actinomyces	2.0	0.69	0.44	0.95	9.00	1.00	207.88	1.22	6.25	1.97	0.32
Mycoplasma	0.7	0.57	0.29	0.85	0.28	0.01	3.62	0.70	-5.09	0.03	0.16
Sneathia	0.0	0.57	0.43	0.71	0.67	0.02	19.10	0.76	-109822.00	0.00	0.88
*Mollicutes*_G1	0.0	0.50	0.50	0.50	0.67	0.02	19.10	NA	1.00	0.00	0.00
Selenomonas	-0.4	0.52	0.23	0.81	1.07	0.13	10.56	0.60	2.65	0.55	0.21
*Bacteroidales*_G2	-3.1	0.66	0.43	0.88	0.67	0.02	19.10	0.47	-4.05	0.10	0.40

Taxa	MDA	AUC	AUC (lower CI)	AUC (upper CI)	OR ≤ grade 1: ≥ grade 2	Lower CI	Upper CI	Delta	Fold Change	Mean ≤ grade 1	Mean ≥ grade 2
Neisseria	-3.2	0.77	0.53	1.00	0.19	0.02	1.48	0.71	-2.49	5.69	14.19
Lautropia	-3.6	0.56	0.24	0.89	0.28	0.01	3.62	0.66	-3.76	0.84	3.14
Kingella	-3.8	0.64	0.37	0.91	6.00	0.66	136.79	0.26	1.57	0.27	0.17
Campylobacter	-4.1	0.61	0.32	0.90	0.32	0.04	2.33	0.33	1.46	0.87	0.60
Treponema	-4.6	0.66	0.40	0.92	0.28	0.01	3.62	0.53	-4.05	0.23	0.92
Gemella	-5.1	0.66	0.38	0.93	1.07	0.13	10.56	0.36	-1.67	0.85	1.42
Leptotrichia	-6.2	0.66	0.39	0.93	4.00	0.42	91.46	0.82	4.21	2.74	0.65
Veillonella	-6.5	0.59	0.30	0.88	4.00	0.42	91.46	0.53	2.32	7.02	3.02
Prevotella	-6.6	0.60	0.32	0.88	0.63	0.06	6.63	0.10	1.14	3.93	3.46
Unclassified	-6.7	0.60	0.28	0.92	0.57	0.07	4.43	0.50	-1.64	0.14	0.23
Alloprevotella	-7.6	0.66	0.39	0.94	0.63	0.06	6.63	0.53	-3.04	0.30	0.92
Parvimonas	-8.0	0.69	0.43	0.95	1.50	0.12	36.88	0.65	-6.23	0.19	1.18
Granulicatella	-8.1	0.62	0.34	0.90	1.50	0.12	36.88	0.13	-1.31	2.12	2.78
Rothia	-8.8	0.66	0.36	0.95	2.57	0.25	60.02	0.40	1.83	3.02	1.65
Capnocytophaga	-9.8	0.69	0.42	0.97	0.67	0.02	19.10	0.52	-3.31	2.26	7.49

MDA, mean decrease in accuracy; AUC, area under the curve; CI, confidence interval; OR, odds ration

APPENDIX D

Certificate of Analysis

Product: Strawberry Placebo Lozenge
Batch Number: 0043901
Date of Manufacture: June 2016
Use By: June 2018
Pack Format: Bottle of 30

Analysis	Result	Specification	Method
Listeria	Not Detected/25g	Not Detected/25g	Listeria Rapid Device Test: FDA BAM On Line
Coagulase producing Staphylococcus	Not Detected/g	Not Detected/g	ISO 6888-3:2003
Yeast and Moulds	<10 cfu/g	<100 cfu/g	British Pharmacopoeia
Streptococcus salivarius	<10 cfu/g	<10 cfu/g	Blis SOP 12
E.coli	Not Detected/g	Not Detected/g	Compendium 5th Edn 2015 Ch 9 (mod)
Salmonella	Not Detected/25g	Not Detected/25g	ISO 6579:2002 (E)

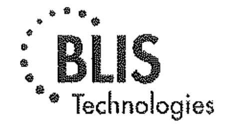
+At time of manufacture

Key to Terms:
cfu: Colony Forming Unit
NLT: Not Less Than

M. Drysdale

Signed: Melissa Drysdale Date: 16th May 2017
(Quality Manager)

Certificate of Analysis



Product: M18 Strawberry Lozenge 50mg
Batch Number: 0043801
Date of Manufacture: May 2017
Use By: May 2019
Pack Format: Bottle of 30

Analysis	Result	Specification	Method
Listeria	Not Detected/25g	Not Detected/25g	Listeria Rapid Device Test: FDA BAM On Line
Coagulase producing Staphylococcus	Not Detected/g	Not Detected/g	ISO 6888-3:2003
Yeast and Moulds	<10 cfu/g	<100 cfu/g	British Pharmacopoeia
Streptococcus salivarius	3.5 x 10^9 cfu/lozenge$^+$	NLT 2.5 x 10^9 cfu/lozenge$^+$	Blis SOP 12
E.coli	Not Detected/g	Not Detected/g	Compendium 5th Edn 2015 Ch 9 (mod)
Salmonella	Not Detected/25g	Not Detected/25g	ISO 6579:2002 (E)

$^+$At time of manufacture

Key to Terms:
cfu: Colony Forming Unit
NLT: Not Less Than

M. Drysdale

Signed: Melissa Drysdale Date: 16th May 2017
(Quality Manager)

Blis Technologies Ltd, 10 Birch Street,
P O Box 5804, Dunedin, 9016, New Zealand
Telephone: +64 3 4741338 Fax: +64 3 4749050 Email: info@blis.co.nz

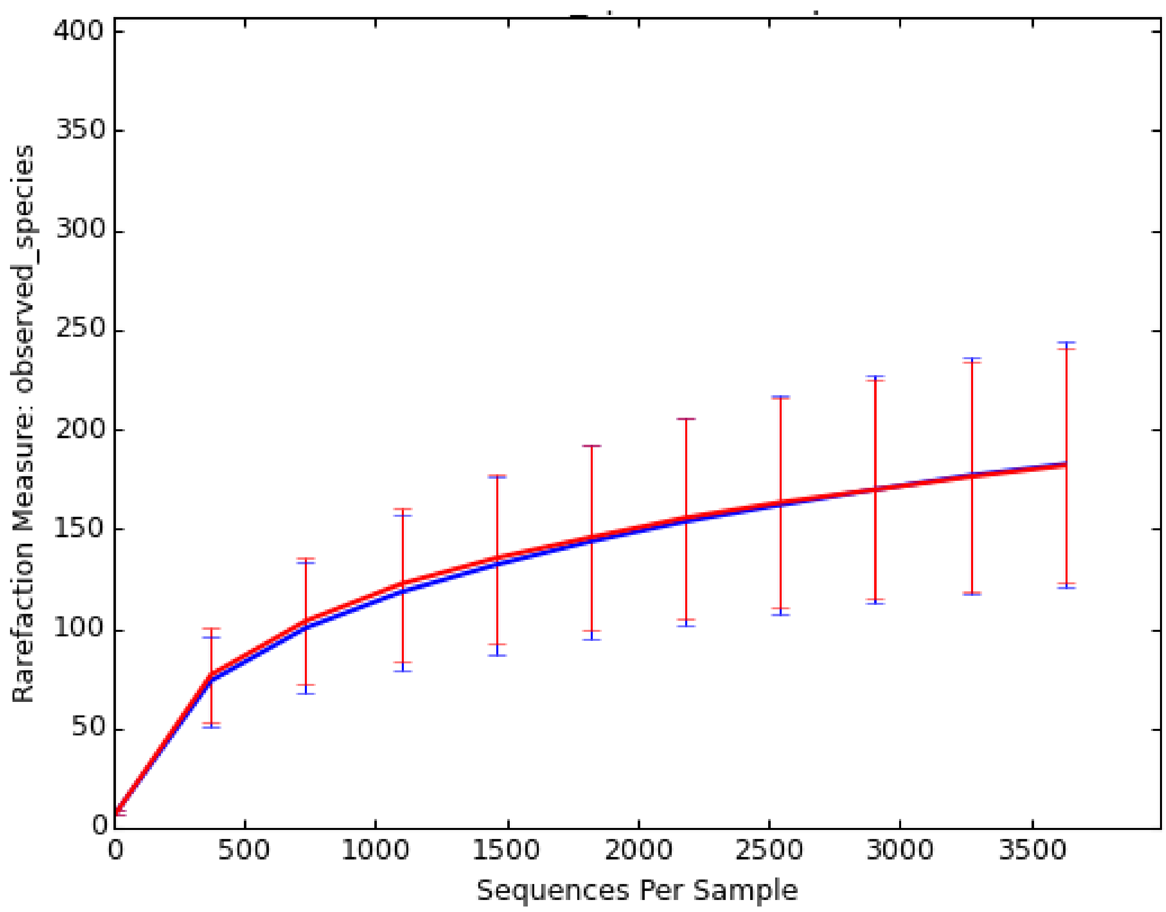


Figure D1. Alpha diversity rarefaction analysis based on the ‘observed species’ metric by sample type as a function of the number of sequences per sample. Average for plaque samples in red and saliva samples in blue.

Table D1. *Streptococcus* ZOTUs.

(A) *Streptococcus* ZOTUs in plaque samples from the BLIS cohort.

ZOTU classification (HOMD v15.1)	BLASTn[1]	2a[2]	2b	4a	4b	6a	6b	8a	8b	10a	10b	12a	12b	14a	14b	16a	16b
Zotu1_*Streptococcus salivarius*	n/a	22	9	15	0	0	0	30	38	0	1	0	1	0	0	0	0
Zotu2_*Streptococcus oralis_subsp._tigurinus_clade*_071	n/a	2	6	27	60	0	0	0	255	38	9	5	21	493	1	25	7
Zotu22_*Streptococcus parasanguinis_clade*_411	n/a	1	6	0	6	0	0	15	32	0	0	157	1	0	0	0	1
Zotu26_*Streptococcus mutans*	n/a	1	24	0	0	0	0	0	0	0	0	0	44	17	15	14	4
Zotu35_*Streptococcus salivarius*	n/a	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu37_*Streptococcus* species	*Streptococcus gordonii*	0	0	1	11	0	0	0	68	8	11	0	12	154	0	40	9

Zotu44_*Streptococcus species*	*Streptococcus sanguinis*	0	0	0	0	0	0	0	11	8	23	5	0	1	0	0	0
Zotu46_*Streptococcus intermedius*	n/a	2	4	22	69	8	68	6	10	1	0	4	1	3	1	32	0
Zotu59_*Streptococcus oralis_subsp._tigurinus_clade_*071	n/a	0	0	0	0	0	0	0	1	0	0	90	0	3	0	0	0
Zotu64_*Streptococcus* species	*Streptococcus parasanguinis*	0	6	0	0	0	0	0	5	0	0	6	0	3	0	0	0
Zotu93_*Streptococcus anginosus*	n/a	0	0	0	0	0	7	10	0	1	0	0	0	0	0	1	0
Zotu97_*Streptococcus constellatus*	n/a	0	8	9	0	93	22	0	0	1	2	0	0	1	5	0	0
Zotu133_*Streptococcus* species	*Streptococcus cristatus*	0	1	0	0	0	0	0	0	0	3	0	0	11	0	0	2
Zotu168_*Streptococcus anginosus*	n/a	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0

Zotu180_*Streptococcus* species	*Streptococcus* species	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Zotu201_*Streptococcus* species	*Streptococcus peroris/lactarius*	0	0	0	0	0	0	0	0	0	0	32	0	0	0	0	0
Zotu267_*Streptococcus* species	*Streptococcus cristatus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu299_*Streptococcus oralis_subsp._tigurinus_clade*_071	n/a	0	1	4	0	0	0	0	0	0	0	1	0	1	0	0	0
Zotu335_*Streptococcus* species	*Streptococcus anginosus*	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu340_*Streptococcus oralis_subsp._tigurinus_clade*_071	n/a	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0
Zotu341_*Streptococcus* species	*Streptococcus* species oral taxon 056	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0

Zotu355_*Streptococcus* species	*Streptococcus salivarius*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu517_*Streptococcus anginosus*	n/a	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu615_*Streptococcus* species	*Streptococcus* species	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu668_*Streptococcus sobrinus*	n/a	1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu774_*Streptococcus parasanguinis_clade*_411	n/a	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu779_*Streptococcus* species	*Streptococcus oralis*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu820_*Streptococcus* species	*Streptococcus anginosus* group	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu882_*Streptococcus* species	*Streptococcus* species	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Zotu897_*Streptococcus* species	*Streptococcus salivarius*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu933_*Streptococcus* species	*Streptococcus* species	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1039_*Streptococcus* species	*Streptococcus cristatus*	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1085_*Streptococcus* species	*Streptococcus thermophilus*	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1130_*Streptococcus* species	*Streptococcus pneumoniae*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1174_*Streptococcus* species	*Streptococcus anginosus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1200_*Streptococcus* species	*Streptococcus gordonii*	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Zotu1214_*Streptococcus mutans*	n/a	0	1	0	0	0	0	0	0	0	0	0	1	6	0	0	0

Zotu1218_*Streptococcus* species	*Streptococcus* species	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1243_*Streptococcus* species	*Streptococcus cristatus*	0	0	3	0	0	0	0	1	0	0	0	0	2	0	0	0
Zotu1252_*Streptococcus mutans*	n/a	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1290_*Streptococcus* species	*Streptococcus* species	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
Zotu1300_*Streptococcus parasanguinis_clade*_411	n/a	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1309_*Streptococcus* species	no match/low cover	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1329_*Streptococcus* species	*Streptococcus* species	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1346_*Streptococcus* species	*Streptococcus oralis*	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1348_*Streptococcus* species	no match/low cover	0	0	0	0	0	0	0	1	0	0	2	0	1	0	0	0

Zotu1412_*Streptococcus* species	no match/low cover	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Zotu1415_*Streptococcus* species	*Streptococcus* species	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Zotu1427_*Streptococcus* species	no match/low cover	0	0	0	0	0	0	0	0	1	0	0	0	14	0	0	2
Zotu1430_*Streptococcus* species	*Streptococcus parasanguinis*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1431_*Streptococcus* species	*Streptococcus cristatus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1444_*Streptococcus* species	*Streptococcus gordonii*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1466_*Streptococcus* species	*Streptococcus gordonii*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

HOMD, Human Oral Microbiome Database (v15.1); Zotu, zero-radius OTU

[1]BLASTn classification based on ≥ 98% sequence similarity

[2]a, pre-intervention; b, post-intervention

(B) *Streptococcus* ZOTUs in plaque samples from the placebo cohort.

ZOTU classification (HOMD v15.1)	BLASTn[1]	3a	3b	7a	7b	9a	9b	13a	13b	15a	15b	17a	17b
Zotu1_*Streptococcus salivarius*	n/a	0	3	20	14	0	4	3	2	181	63	1	0
Zotu2_*Streptococcus oralis_subsp._tigurinus_clade*_071	n/a	77	6	20	68	70	37	9	42	2	4	79	115
Zotu22_*Streptococcus parasanguinis_clade*_411	n/a	0	0	0	262	0	0	0	0	34	11	1	0
Zotu26_*Streptococcus mutans*	n/a	0	0	0	812	0	0	40	71	1	4	1	0
Zotu35_*Streptococcus salivarius*	n/a	0	0	0	0	0	0	0	0	0	0	0	0
Zotu37_*Streptococcus* species	*Streptococcus gordonii*	104	0	9	11	39	0	7	3	25	195	14	8
Zotu44_*Streptococcus species*	*Streptococcus sanguinis*	52	6	4	2	1	7	1	4	3	16	11	10
Zotu46_*Streptococcus intermedius*	n/a	1	13	60	88	36	6	93	32	57	77	3	0

Zotu59_*Streptococcus oralis_subsp._tigurinus_clade*_071	n/a	0	0	0	1	0	0	0	0	0	0	0	0
Zotu64_*Streptococcus* species	*Streptococcus parasanguinis*	0	1	0	31	0	0	0	0	3	6	0	0
Zotu93_*Streptococcus anginosus*	n/a	0	0	21	44	1	1	0	0	2	1	0	0
Zotu97_*Streptococcus constellatus*	n/a	0	1	0	0	7	5	0	0	0	0	0	1
Zotu133_*Streptococcus* species	*Streptococcus cristatus*	8	0	1	2	0	0	0	0	0	0	1	2
Zotu168_*Streptococcus anginosus*	n/a	0	0	0	1	1	0	0	0	1	1	0	0
Zotu180_*Streptococcus* species	*Streptococcus* species	1	0	0	0	0	0	0	0	0	0	0	0
Zotu201_*Streptococcus* species	*Streptococcus peroris/lactarius*	0	0	0	10	1	0	0	0	0	0	0	0

Zotu267_*Streptococcus* species	*Streptococcus cristatus*	0	0	0	0	0	0	0	0	0	0	0	1
Zotu299_*Streptococcus oralis_subsp._tigurinus_clade*_071	n/a	10	0	1	5	0	3	0	0	0	0	2	3
Zotu335_*Streptococcus* species	*Streptococcus anginosus*	0	0	2	2	0	0	0	0	11	4	0	0
Zotu340_*Streptococcus oralis_subsp._tigurinus_clade*_071	n/a	0	0	0	3	0	0	0	0	0	0	0	0
Zotu341_*Streptococcus* species	*Streptococcus* species oral taxon 056	0	0	0	3	0	1	0	0	15	10	0	0
Zotu355_*Streptococcus* species	*Streptococcus salivarius*	0	0	1	0	0	0	0	0	6	3	0	0
Zotu517_*Streptococcus anginosus*	n/a	0	0	0	0	0	0	0	0	0	0	0	0
Zotu615_*Streptococcus* species	*Streptococcus* species	0	0	0	0	0	0	0	0	0	0	0	0

Zotu668_*Streptococcus sobrinus*	n/a	0	0	0	0	0	0	0	0	0	0	0	0
Zotu774_*Streptococcus parasanguinis_clade*_411	n/a	0	0	0	2	0	0	0	0	1	0	0	0
Zotu779_*Streptococcus* species	*Streptococcus oralis*	0	0	0	0	0	0	0	0	0	0	0	0
Zotu820_*Streptococcus* species	*Streptococcus anginosus* group	0	0	0	0	0	0	0	0	0	0	0	0
Zotu882_*Streptococcus* species	*Streptococcus* species	0	0	0	1	0	0	0	0	0	0	0	0
Zotu897_*Streptococcus* species	*Streptococcus salivarius*	0	0	0	0	0	0	0	0	0	0	0	0
Zotu933_*Streptococcus* species	*Streptococcus* species	0	0	1	0	0	0	0	0	1	0	0	0
Zotu1039_*Streptococcus* species	*Streptococcus cristatus*	0	0	1	1	0	0	0	0	7	2	0	0

Zotu1085_*Streptococcus* species	*Streptococcus thermophilus*	0	0	0	0	0	0	0	0	2	0	0	0
Zotu1130_*Streptococcus* species	*Streptococcus pneumoniae*	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1174_*Streptococcus* species	*Streptococcus anginosus*	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1200_*Streptococcus* species	*Streptococcus gordonii*	0	0	0	1	0	0	0	0	5	5	0	0
Zotu1214_*Streptococcus mutans*	n/a	0	0	0	38	0	0	0	0	0	0	0	0
Zotu1218_*Streptococcus* species	*Streptococcus* species	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1243_*Streptococcus* species	*Streptococcus cristatus*	0	0	0	1	0	2	0	0	2	4	1	0
Zotu1252_*Streptococcus mutans*	n/a	0	0	0	5	0	0	0	0	0	0	0	0
Zotu1290_*Streptococcus* species	*Streptococcus* species	0	0	0	2	0	0	0	0	0	0	0	0

Zotu1300_*Streptococcus parasanguinis_clade*_411	n/a	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1309_*Streptococcus* species	no match/low cover	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1329_*Streptococcus* species	*Streptococcus* species	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1346_*Streptococcus* species	*Streptococcus oralis*	0	0	0	1	0	0	1	1	0	0	0	0
Zotu1348_*Streptococcus* species	no match/low cover	0	0	0	3	0	0	0	0	0	0	0	0
Zotu1412_*Streptococcus* species	no match/low cover	11	0	0	0	0	0	0	0	0	0	0	0
Zotu1415_*Streptococcus* species	*Streptococcus* species	0	0	0	2	0	0	0	0	0	0	0	0
Zotu1427_*Streptococcus* species	no match/low cover	0	0	0	2	0	0	0	0	0	0	1	0
Zotu1430_*Streptococcus* species	*Streptococcus parasanguinis*	0	0	0	0	0	0	0	0	0	0	0	0

Zotu1431_*Streptococcus* species	*Streptococcus cristatus*	0	0	0	2	0	0	0	0	0	0	0	1
Zotu1444_*Streptococcus* species	*Streptococcus gordonii*	0	0	0	13	0	0	0	0	0	0	0	0
Zotu1466_*Streptococcus* species	*Streptococcus gordonii*	0	0	0	0	0	0	0	0	1	0	0	0

HOMD, Human Oral Microbiome Database (v15.1); Zotu, zero-radius OTU

[1]BLASTn classification based on ≥ 98% sequence similarity

[2]a, pre-intervention; b, post-intervention

(C) *Streptococcus* ZOTUs in saliva samples from the BLIS cohort.

ZOTU classification (HOMD v15.1)	BLASTn[1]	2a[2]	2b	4a	4b	6a	6b	8a	8b	10a	10b	12a	12b	14a	14b	16a	16b
Zotu1_*Streptococcus salivarius*	n/a	412	379	1340	120	0	33	42	145	32	22	65	111	116	288	7	110
Zotu2_*Streptococcus oralis_subsp._tigurinus_clade*_071	n/a	198	90	163	709	0	15	69	482	804	136	1	664	417	149	200	275
Zotu22_*Streptococcus parasanguinis_clade*_411	n/a	11	25	17	7	0	0	86	275	27	41	1512	177	21	7	227	55
Zotu26_*Streptococcus mutans*	n/a	5	7	0	0	0	0	0	0	0	0	0	26	207	161	0	1
Zotu35_*Streptococcus salivarius*	n/a	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Zotu37_*Streptococcus* species	*Streptococcus gordonii*	17	13	18	36	0	2	1	78	81	76	1	104	271	49	52	42

Zotu44_*Streptococcus species*	*Streptococcus sanguinis*	212	103	4	3	0	0	0	166	65	223	4	14	160	127	5	3
Zotu46_*Streptococcus intermedius*	n/a	132	125	152	76	7	106	2	9	4	10	0	10	80	11	8	11
Zotu59_*Streptococcus oralis_subsp._tigurinus_clade*_071	n/a	0	0	2	29	0	1	809	36	64	258	67	37	35	5	145	139
Zotu64_*Streptococcus* species	*Streptococcus parasanguinis*	10	6	36	62	0	10	55	142	45	37	63	71	225	15	259	70
Zotu93_*Streptococcus anginosus*	n/a	0	1	0	0	0	21	0	0	0	1	0	0	3	0	1	0
Zotu97_*Streptococcus constellatus*	n/a	30	9	2	3	31	54	0	3	1	0	7	2	45	19	5	6
Zotu133_*Streptococcus* species	*Streptococcus cristatus*	21	11	9	27	0	0	0	92	55	29	0	45	47	12	28	19
Zotu168_*Streptococcus anginosus*	n/a	0	0	0	0	0	6	0	0	0	0	0	0	2	0	0	0

Zotu180_*Streptococcus* species	*Streptococcus* species	0	0	0	0	0	0	3	3	1	5	2	3	2	1	6	3
Zotu201_*Streptococcus* species	*Streptococcus peroris/ lactarius*	2	1	3	2	0	0	53	62	11	26	81	49	2	2	61	32
Zotu267_*Streptococcus* species	*Streptococcus cristatus*	2	1	0	0	0	0	0	6	0	8	0	0	6	7	0	0
Zotu299_*Streptococcus oralis_subsp._tigurinus_clade_*071	n/a	28	15	36	22	0	0	2	103	37	30	3	63	48	24	19	25
Zotu335_*Streptococcus* species	*Streptococcus anginosus*	13	16	101	6	0	2	0	2	0	0	0	2	2	2	0	2
Zotu340_*Streptococcus oralis_subsp._tigurinus_clade_*071	n/a	12	7	27	27	0	0	0	20	5	4	2	16	11	10	1	22

Zotu341_*Streptococcus* species	*Streptococcus* species oral taxon 056	26	41	87	10	0	0	1	25	9	10	19	12	22	23	2	13
Zotu355_*Streptococcus* species	*Streptococcus salivarius*	13	23	80	4	0	6	0	2	0	1	0	3	1	3	1	2
Zotu517_*Streptococcus anginosus*	n/a	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu615_*Streptococcus* species	*Streptococcus* species	9	3	47	10	0	1	2	17	4	0	0	13	5	21	5	16
Zotu668_*Streptococcus sobrinus*	n/a	37	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu774_*Streptococcus parasanguinis_clade*_411	n/a	1	0	5	0	0	0	2	2	0	1	5	3	4	0	0	1
Zotu779_*Streptococcus* species	*Streptococcus oralis*	0	1	12	7	0	0	0	7	3	1	0	6	1	3	0	13

Zotu820_*Streptococcus* species	*Streptococcus anginosus* group	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu882_*Streptococcus* species	*Streptococcus* species	3	6	0	0	0	0	0	3	0	1	0	0	2	2	0	0
Zotu897_*Streptococcus* species	*Streptococcus salivarius*	2	1	7	3	0	0	1	1	0	0	0	3	3	3	0	3
Zotu933_*Streptococcus* species	*Streptococcus* species	0	1	3	0	0	0	0	7	0	3	1	4	2	2	6	2
Zotu1039_*Streptococcus* species	*Streptococcus cristatus*	15	21	118	41	0	1	8	45	10	2	0	33	18	38	2	42
Zotu1085_*Streptococcus* species	*Streptococcus thermophilus*	15	17	24	1	0	0	0	18	1	3	4	1	11	18	0	4

Zotu1130_*Streptococcus* species	*Streptococcus pneumoniae*	0	0	0	12	0	0	0	0	0	0	0	1	2	0	2	2
Zotu1174_*Streptococcus* species	*Streptococcus anginosus*	0	0	3	2	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1200_*Streptococcus* species	*Streptococcus gordonii*	0	1	0	1	0	0	0	0	0	0	0	1	8	8	0	0
Zotu1214_*Streptococcus mutans*	n/a	0	1	0	0	0	0	0	0	0	0	0	12	29	17	0	0
Zotu1218_*Streptococcus* species	*Streptococcus* species	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1243_*Streptococcus* species	*Streptococcus cristatus*	2	6	18	18	0	3	0	4	0	0	0	3	18	2	4	3
Zotu1252_*Streptococcus mutans*	n/a	0	2	0	0	0	0	0	0	0	0	0	1	6	21	0	0

Zotu1290_*Streptococcus* species	*Streptococcus* species	0	0	0	0	0	0	0	0	0	0	0	0	7	6	0	0
Zotu1300_*Streptococcus parasanguinis_clade*_411	n/a	0	1	4	2	0	0	2	5	0	0	1	1	2	1	1	6
Zotu1309_*Streptococcus* species	no match/low cover	0	2	0	1	0	0	0	13	0	2	9	17	1	0	7	1
Zotu1329_*Streptococcus* species	*Streptococcus* species	0	0	2	0	0	0	1	4	1	0	2	1	1	0	0	1
Zotu1346_*Streptococcus* species	*Streptococcus oralis*	3	1	5	11	0	3	1	1	1	2	0	2	6	0	0	0
Zotu1348_*Streptococcus* species	no match/ low cover	2	2	0	3	0	0	0	4	1	0	0	6	6	1	1	0

Zotu1412_*Streptococcus* species	no match/ low cover	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Zotu1415_*Streptococcus* species	*Streptococcus* species	0	0	0	0	0	0	0	0	0	0	0	0	3	2	0	0
Zotu1427_*Streptococcus* species	no match/ low cover	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
Zotu1430_*Streptococcus* species	*Streptococcus parasanguinis*	0	1	0	1	0	0	6	3	0	1	1	0	1	0	0	3
Zotu1431_*Streptococcus* species	*Streptococcus cristatus*	0	0	0	0	0	3	0	0	0	0	0	0	6	0	1	0
Zotu1444_*Streptococcus* species	*Streptococcus gordonii*	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0

Zotu1466_*Streptococcus* species	*Streptococcus gordonii*	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0

HOMD, Human Oral Microbiome Database (v15.1); Zotu, zero-radius OTU

[1]BLASTn classification based on ≥ 98% sequence similarity

[2]a, pre-intervention; b, post-intervention

(D) *Streptococcus* ZOTUs in saliva samples from the placebo cohort.

ZOTU classification (HOMD v15.1)	**BLASTn[1]**	**3a[2]**	**3b**	**7a**	**7b**	**9a**	**9b**	**13a**	**13b**	**15a**	**15b**	**17a**	**17b**
Zotu1_*Streptococcus salivarius*	n/a	0	428	204	907	8	268	262	330	213	264	48	36
Zotu2_*Streptococcus oralis_subsp._tigurinus_clade*_071	n/a	109	206	238	232	505	221	346	338	143	43	136	122
Zotu22_*Streptococcus parasanguinis_clade*_411	n/a	11	46	90	132	514	118	204	276	76	220	22	10
Zotu26_*Streptococcus mutans*	n/a	0	0	0	229	0	0	7	13	0	1	0	1
Zotu35_*Streptococcus salivarius*	n/a	0	0	0	0	0	0	0	0	0	0	0	0
Zotu37_*Streptococcus* species	*Streptococcus gordonii*	86	45	37	23	90	53	51	45	289	240	16	11
Zotu44_*Streptococcus species*	*Streptococcus sanguinis*	580	6	76	54	28	38	11	23	45	65	11	5
Zotu46_*Streptococcus intermedius*	n/a	7	6	117	79	6	2	13	6	31	16	0	1

Zotu59_*Streptococcus oralis_subsp._tigurinus_clade*_071	n/a	6	98	0	6	45	14	9	21	4	8	24	7
Zotu64_*Streptococcus* species	*Streptococcus parasanguinis*	36	128	5	29	144	59	45	170	57	183	29	15
Zotu93_*Streptococcus anginosus*	n/a	0	0	48	18	0	0	0	0	0	0	0	0
Zotu97_*Streptococcus constellatus*	n/a	5	5	0	0	69	39	0	0	0	0	3	2
Zotu133_*Streptococcus* species	*Streptococcus cristatus*	31	22	10	17	67	33	29	36	15	12	13	2
Zotu168_*Streptococcus anginosus*	n/a	0	0	2	1	0	0	0	0	0	0	0	0
Zotu180_*Streptococcus* species	*Streptococcus* species	1	1	0	0	2	2	3	1	0	5	0	0
Zotu201_*Streptococcus* species	*Streptococcus peroris/lactarius*	3	21	5	15	123	20	44	45	6	7	4	0

Zotu267_*Streptococcus* species	*Streptococcus cristatus*	7	1	0	2	0	0	0	0	0	3	0	0
Zotu299_*Streptococcus oralis_subsp._tigurinus_clade*_071	n/a	73	16	14	20	104	28	71	47	17	8	13	8
Zotu335_*Streptococcus* species	*Streptococcus anginosus*	0	4	3	17	0	4	0	1	3	3	0	0
Zotu340_*Streptococcus oralis_subsp._tigurinus_clade*_071	n/a	3	27	2	20	11	8	30	20	8	2	2	4
Zotu341_*Streptococcus* species	*Streptococcus* species oral taxon 056	0	33	0	32	6	30	23	40	59	94	5	0
Zotu355_*Streptococcus* species	*Streptococcus salivarius*	0	5	1	17	0	8	2	0	5	2	0	0
Zotu517_*Streptococcus anginosus*	n/a	0	0	1	0	0	0	0	0	0	0	0	0
Zotu615_*Streptococcus* species	*Streptococcus* species	0	28	1	22	7	11	24	31	10	8	5	1

Zotu668_*Streptococcus sobrinus*	n/a	0	0	0	0	0	0	0	0	0	0	0	0
Zotu774_*Streptococcus parasanguinis_clade*_411	n/a	0	5	0	8	0	4	4	6	4	15	1	0
Zotu779_*Streptococcus* species	*Streptococcus oralis*	0	14	0	5	0	6	9	13	3	1	3	1
Zotu820_*Streptococcus* species	*Streptococcus anginosus* group	0	0	0	0	0	0	0	0	0	0	0	0
Zotu882_*Streptococcus* species	*Streptococcus* species	0	0	0	0	0	0	0	0	0	2	1	0
Zotu897_*Streptococcus* species	*Streptococcus salivarius*	0	4	1	3	0	5	1	4	2	2	1	0
Zotu933_*Streptococcus* species	*Streptococcus* species	2	3	1	3	12	3	2	6	0	1	1	1
Zotu1039_*Streptococcus* species	*Streptococcus cristatus*	0	65	9	47	2	44	50	57	53	49	9	6
Zotu1085_*Streptococcus* species	*Streptococcus thermophilus*	0	6	3	8	1	12	13	12	11	25	2	1

Zotu1130_*Streptococcus* species	*Streptococcus pneumoniae*	0	0	0	0	2	2	0	0	0	0	0	0
Zotu1174_*Streptococcus* species	*Streptococcus anginosus*	0	0	0	0	0	0	0	0	1	0	0	0
Zotu1200_*Streptococcus* species	*Streptococcus gordonii*	0	2	0	2	0	0	3	3	21	16	1	1
Zotu1214_*Streptococcus mutans*	n/a	0	0	0	14	0	0	0	2	0	0	0	0
Zotu1218_*Streptococcus* species	*Streptococcus* species	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1243_*Streptococcus* species	*Streptococcus cristatus*	1	3	4	1	21	2	3	0	1	3	1	0
Zotu1252_*Streptococcus mutans*	n/a	0	0	0	37	0	0	0	2	0	0	1	0
Zotu1290_*Streptococcus* species	*Streptococcus* species	0	0	0	1	0	0	0	0	0	0	0	0
Zotu1300_*Streptococcus parasanguinis_clade*_411	n/a	0	24	0	0	0	4	4	7	4	10	1	0

Zotu1309_*Streptococcus* species	no match/low cover	5	0	0	3	4	4	11	5	16	24	0	0
Zotu1329_*Streptococcus* species	*Streptococcus* species	0	3	0	0	0	5	3	7	1	3	0	0
Zotu1346_*Streptococcus* species	*Streptococcus oralis*	0	0	0	1	0	1	2	2	0	0	0	0
Zotu1348_*Streptococcus* species	no match/low cover	0	0	0	3	0	2	6	2	32	21	0	0
Zotu1412_*Streptococcus* species	no match/low cover	54	0	0	0	0	0	0	0	0	0	0	0
Zotu1415_*Streptococcus* species	*Streptococcus* species	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1427_*Streptococcus* species	no match/low cover	0	0	0	0	0	0	0	0	0	0	0	0
Zotu1430_*Streptococcus* species	*Streptococcus parasanguinis*	0	11	0	4	0	1	3	6	4	8	1	1

Zotu1431_*Streptococcus* species	*Streptococcus cristatus*	0	0	0	0	2	2	0	0	1	0	0	0
Zotu1444_*Streptococcus* species	*Streptococcus gordonii*	0	1	0	0	0	0	0	0	0	0	0	0
Zotu1466_*Streptococcus* species	*Streptococcus gordonii*	0	0	0	0	0	0	0	0	4	0	0	0

HOMD, Human Oral Microbiome Database (v15.1); Zotu, zero-radius OTU

[1]BLASTn classification based on ≥ 98% sequence similarity

[2]a, pre-intervention; b, post-intervention

Table D2. Interactions with ZOTU1_*Streptococcus salivarius*.

(A) Plaque

ZOTU	Taxonomic classification (HOMD v15.1)/BLASTn	Inverse covariance
Zotu1039	*Streptococcus cristatus*	0.376445
Zotu50	*Atopobium parvulum*	0.31431
Zotu217	*Prevotella oris*	0.140236
Zotu782	*Capnocytophaga* spp. HMT 336	0.073003
Zotu68	*Atopobium* spp.	0.04168
Zotu744	*Prevotella oulorum*	0.039413
Zotu1085	*Streptococcus thermophilus*	0.027808
Zotu145	*Prevotella oris*	0.005125
Zotu1380	*Alloprevotella rava*	0.000449
Zotu589	*Selenomonas* spp.	-0.00357
Zotu1037	*Treponema maltophilum*	-0.01185
Zotu1366	*Prevotella marshii*	-0.01484
Zotu90	*Treponema socranskii*	-0.02036
Zotu265	*Atopobium* spp.	-0.02814
Zotu238	*Fretibacterium* spp.	-0.0299

ZOTU	Taxonomic classification (HOMD v15.1)/BLASTn	Inverse covariance
Zotu160	*Campylobacter rectus*	-0.03213
Zotu211	*Ruminococcaceae* [G1] HMT 075	-0.03419
Zotu253	*Leptotrichia* spp. HMT 223	-0.0366
Zotu611	*Selenomonas* spp.	-0.03835
Zotu2	*Streptococcus oralis* subsp. *tigurinus*	-0.04169
Zotu530	*Prevotella* spp. HMT 301	-0.09091
Zotu586	*Peptococcus* spp. HMT 167	-0.10722

Zotu, zero-radius OTU; HOMD, Human Oral Microbiome Database (v15.1); HMT, human microbial taxon

(B) Saliva

ZOTU	Taxonomic classification (HOMD v15.1)/BLASTn	Inverse covariance
Zotu1039	*Streptococcus cristatus*	0.308078
Zotu897	*Streptococcus salivarius*	0.306017
Zotu1085	*Streptococcus thermophilus*	0.19516
Zotu341	*Streptococcus* spp. oral taxon 056	0.146117
Zotu63	*Veillonella parvula*	0.050931
Zotu335	*Streptococcus anginosus* group	0.044688
Zotu344	*Selenomonas artemidis*	0.039257
Zotu83	*Prevotella* spp.	0.03785
Zotu1412*	No match/low cover	-0.01215
Zotu384	*Prevotella* spp. HMT 526	-0.01419
Zotu349	*Lachnospiraceae* [G8] HMT 500	-0.01599
Zotu79	*Fusobacterium nucleatum* subsp. *vincentii*	-0.09284
Zotu103	*Leptotrichia* spp.	-0.12163

Zotu, zero-radius OTU; HOMD, Human Oral Microbiome Database (v15.1); HMT, human microbial taxon

* Not included in network

Bibliography

Aas, J. A., Barbuto, S. M., Alpagot, T., Olsen, I., Dewhirst, F. E., & Paster, B. J. (2007). Subgingival plaque microbiota in HIV positive patients. *Journal of Clinical Periodontology*, *34*(3), 189–195. doi:10.1111/j.1600-051X.2006.01034.x

Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., & Dewhirst, F. E. (2005). Defining the normal bacterial flora of the oral cavity. *Journal of Clinical Microbiology*, *43*(11), 5721–5732. doi:10.1128/JCM.43.11.5721-5732.2005

Abusleme, L., Hong, B. Y., Dupuy, A. K., Strausbaugh, L. D., & Diaz, P. I. (2014). Influence of DNA extraction on oral microbial profiles obtained via 16S rRNA gene sequencing. *Journal of Oral Microbiology*, *6*(1), 23990. doi:10.3402/jom.v6.23990

Adam, E., Jindal, M., Seney, S., Summers, K., Hamilton, D. W., Hatibovic-Kofman, S., & Cadieux, P. A. (2011, March). *Streptococcus salivarius* K12 and M18 probiotics reduce periodontal pathogen-induced inflammation. Presented at International Association for Dental Research 9th General Session and Exhibition, San Diego, CA, USA. Paper no. 150126

Al-Dasooqi, N., Sonis, S. T., Bowen, J. M., Bateman, E., Blijlevens, N., Gibson, R. J., … Lalla, R. V. (2013). Emerging evidence on the pathobiology of mucositis. *Supportive Care in Cancer, 21*(7), 2075–2083. doi:10.1007/s00520-013-1900-x

Al-Hebshi, N. N., Borgnakke, W. S., & Johnson, N. W. (2019). The microbiome of oral squamous cell carcinomas: a functional perspective. *Current Oral Health Reports, 6*(2), 145–160. doi:10.1007/s40496-019-0215-5

Almståhl, A., Finizia, C., Carlén, A., Fagerberg-Mohlin, B., & Alstad, T. (2018). Mucosal microflora in head and neck cancer patients. *International Journal of Dental Hygiene,*

16(4), 459–466. doi:10.1111/idh.12348

Anderson, M.J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, *26*(1), 32–46. doi:10.1046/j.1442-9993.2001.01070.x

Anderson, M. J., Gorley, R. N., & Clarke, K. R. (2008). PERMANOVA+ for Primer: guide to software and statistical methods. Primer-E Limited. doi:10.13564/j.cnki.issn.1672-9382.2013.01.010

Ariyawardana, A., Cheng, K. K. F., Kandwal, A., Tilly, V., Al-Azri, A. R., Galiti, D., … Elad, S. (2019). Systematic review of anti-inflammatory agents for the management of oral mucositis in cancer patients and clinical practice guidelines. *Supportive Care in Cancer, 27*(10), 3985–3995. doi:10.1007/s00520-019-04888-w

Bakri, M. M., Hussaini, H. M., Holmes, A., Cannon, R. D., & Rich, A. M. (2010). Revisiting the association between candidal infection and carcinoma, particularly oral squamous cell carcinoma. *Journal of Oral Microbiology*, *2*(1), 5780. doi:10.3402/jom.v2i0.5780

Banas, J. A., & Drake, D. R. (2018). Are the mutans streptococci still considered relevant to understanding the microbial etiology of dental caries? *BMC Oral Health*, *18*(1), 1–8. doi:10.1186/s12903-018-0595-2

Bandara, H. M. H. N., Panduwawala, C. P., & Samaranayake, L. P. (2018). Biodiversity of the human oral mycobiome in health and disease. *Oral Diseases*, *25*(2), 363–371. doi:10.1111/odi.12899

Barasch, A., & Peterson, D. E. (2003). Risk factors for ulcerative oral mucositis in cancer patients: unanswered questions. *Oral Oncology*, *39*(2), 91–100. doi:10.1016/S1368-8375(02)00033-7

Belstrøm, D., Fiehn, N.-E., Nielsen, C. H., Kirkby, N., Twetman, S., Klepac-Ceraj, V., …

Holmstrup, P. (2013). Differences in bacterial saliva profile between periodontitis patients and a control cohort. *Journal of Clinical Periodontology*, *41*(2), 104–112. doi:10.1111/jcpe.12190

Belstrøm, D., Holmstrup, P., Bardow, A., Kokaras, A., Fiehn, N. E., & Paster, B. J. (2016). Temporal stability of the salivary microbiota in oral health. *PLoS One*, *11*(1), e0147472. doi:10.1371/journal.pone.0147472

Belstrøm, D., Sembler-Møller, M. L., Grande, M. A., Kirkby, N., Cotton, S. L., Paster, B. J., & Holmstrup, P. (2017). Microbial profile comparisons of saliva, pooled and site-specific subgingival samples in periodontitis patients. *PLoS One*, *12*(8), e0182992. doi:10.1371/journal.pone.0182992

Bertolini, M., Ranjan, A., Thompson, A., Diaz, P. I., Sobue, T., Maas, K., & Dongari-Bagtzoglou, A. (2019). *Candida albicans* induces mucosal bacterial dysbiosis that promotes invasive infection. *PLoS Pathogens*, *15*(4), e1007717. doi:10.1371/journal.ppat.1007717

Biswas, K., Hoggard, M., Jain, R., Taylor, M. W., & Douglas, R. G. (2015). The nasal microbiota in health and disease: variation within and between subjects. *Frontiers in Microbiology*, *6*, 134. doi:10.3389/fmicb.2015.00134

Bowen, J., Bossi, P., Wardill, H., Sebille, Y. Van, Bateman, E., & Correa, M. E. (2019). The pathogenesis of mucositis : updated perspectives and emerging targets. *Supportive Care in Cancer, 27*(10), 4023–4033. doi:10.1007/s00520-019-04893-z

Breiman, L. (2001). Random Forests. *Machine Learning*, *45*(1), 5–32. doi:10.1023/A:1010933404324

Bressan, V., Stevanin, S., Bianchi, M., Aleo, G., Bagnasco, A., & Sasso, L. (2016). The

effects of swallowing disorders, dysgeusia, oral mucositis and xerostomia on nutritional status, oral intake and weight loss in head and neck cancer patients: a systematic review. *Cancer Treatment Reviews*, *45*, 105–119. doi:10.1016/j.ctrv.2016.03.006

Brown, L. R., Dreizen, S., Handler, S., & Johnston, D. A. (1975). Effect of radiation-induced xerostomia on human oral microflora. *Journal of Dental Research*, *54*(4), 740–750. doi:10.1177/00220345750540040801

Burton, J. P., Drummond, B. K., Chilcott, C. N., Tagg, J. R., Thomson, W. M., Hale, J. D. F., & Wescombe, P. A. (2013). Influence of the probiotic *Streptococcus salivarius* strain M18 on indices of dental health in children: a randomized double-blind, placebo-controlled trial. *Journal of Medical Microbiology*, *62(6)*, 875–884. doi:10.1099/jmm.0.056663-0

Burton, J. P., Wescombe, P. A., Macklaim, J. M., Chai, M. H. C., MacDonald, K., Hale, J. D. F., … Cadieux, P. A. (2013). Persistence of the oral probiotic *Streptococcus salivarius* M18 is dose dependent and megaplasmid transfer can augment their bacteriocin production and adhesion characteristics. *PLoS One*, *8*(6), e65991. doi:10.1371/journal.pone.0065991

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., … Knight, R. (2010). QIIME allows analysis of high- throughput community sequencing data. *Nature Methods*, *7*(5), 335–336. doi:10.1038/nmeth0510-335

Cekici, A., Kantarci, A., Hasturk, H., & Van Dyke, T. E. (2014). Inflammatory and immune pathways in the pathogenesis of periodontal disease. *Periodontology 2000*, *64*(1), 57–80. doi:10.1111/prd.12002

Chambers, M. S., Garden, A. S., Kies, M. S., & Martin, J. W. (2004). Radiation-induced xerostomia in patients with head and neck cancer: pathogenesis, impact on quality of

life, and management. *Head and Neck*, *26*(9), 796–807. doi:10.1002/hed.20045

Chattopadhyay, I., & Panda, M. (2019). Recent trends of saliva omics biomarkers for the diagnosis and treatment of oral cancer. *Journal of Oral Biosciences, 61*(2)*, 84–94*. doi:10.1016/j.job.2019.03.002

Chaturvedi, A. K., Engels, E. A., Anderson, W. F., & Gillison, M. L. (2008). Incidence trends for human papillomavirus-related and -unrelated oral squamous cell carcinomas in the United States. *Journal of Clinical Oncology*, *26*(4), 612–619. doi:10.1200/JCO.2007.14.1713

Chen, T., Dong, Q., Wang, H., Xia, C., Li, J., Qiu, Y., … Su, Y. (2018). A randomized, double-blind, placebo-controlled trial of probiotics to reduce the severity of oral mucositis induced by chemoradiotherapy for patients with nasopharyngeal carcinoma. *Cancer*, *125*(7), 1081–1090. doi:10.1002/cncr.31907

Chen, T, Yu, W. H., Izard, J., Baranova, O. V., Lakshmanan, A., & Dewhirst, F. E. (2010). The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. *Database : The Journal of Biological Databases and Curation*, *2010*, 1–10. doi:10.1093/database/baq013

Cheng, Y.-S., Rees, T., & Wright, J. (2014). A review of research on salivary biomarkers for oral cancer detection. *Clinical and Translational Medicine*, *3*(1), 3. doi:10.1186/2001-1326-3-3

Cutress, T. W., Ainamo, J., & Sardo-Infirri, J. (1987). The community periodontal index of treatment needs (CPITN) procedure for population groups and individuals. *International Dental Journal*, *37*(4), 222–233. PMID: 3481626.

Darveau, R. P. (2010). Periodontitis: a polymicrobial disruption of host homeostasis. *Nature*

Reviews Microbiology, *8*(7), 481–490. doi:10.1038/nrmicro2337

Darveau, R. P., Hajishengallis, G., & Curtis, M. A. (2012). *Porphyromonas gingivalis* as a potential community activist for disease. *Journal of Dental Research*, *91*(9), 816–820. doi:10.1177/0022034512453589

Dassi, E., Ferretti, P., Covello, G., Speccher, A., Migazzi, A., Bosco, B., … Segata, N. (2018). The short-term impact of probiotic consumption on the oral cavity microbiome. *Scientific Reports*, *8*(1), 2–9. doi:10.1038/s41598-018-28491-x

De Sanctis, V., Merlotti, A., De Felice, F., Trignani, M., Dell'Oca, I., Lastrucci, L., … Paiar, F. (2019). Intensity modulated radiation therapy and oral mucosa sparing in head and neck cancer patients: a systematic review on behalf of Italian Association of Radiation Oncology – Head and neck working group. *Critical Reviews in Oncology/Hematology*, *139*(January), 24–30. doi:10.1016/j.critrevonc.2019.04.022

de Sanctis, Vitaliana, Belgioia, L., Cante, D., la Porta, M. R., Caspiani, O., Guarnaccia, R., … Russi, E. G. (2019). *Lactobacillus brevis* CD2 for prevention of oral mucositis in patients with head and neck tumors: a multicentric randomized study. *Anticancer Research*, *39*(4), 1935–1942. doi:10.21873/anticanres.13303

Dewhirst, F. E., Paster, B. J., Tzellas, N., Coleman, B., Downes, J., Spartt, D. A., & Wade, W. G. (2001). Characterization of novel human oral isolates and cloned 16S rDNA sequences that fall in the family *Coriobacteriaceae*: description of *Olsenella* gen. nov., reclassification of *Lactobacillus uli* as *Olsenella uli* comb. nov. and description of *Olsenella profu*. *International Journal of Systematic and Evolutionary Microbiology*, *51*(5), 1797–1804. doi:10.1099/00207713-51-5-1797

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C. R. R., Yu, W. H., … Wade, W. G. (2010). The human oral microbiome. *Journal of Bacteriology*, *192*(19), 5002–

5017. doi:10.1128/JB.00542-10

Di Pierro, F., Zanvit, A., Nobili, P., Risso, P., & Fornaini, C. (2015). Cariogram outcome after 90 days of oral treatment with *Streptococcus salivarius* M18 in children at high risk for dental caries: results of a randomized, controlled study. *Clinical, Cosmetic and Investigational Dentistry*, *7*, 107–113. doi:10.2147/CCIDE.S93066

Diaz, P. I., Hong, B. Y., Dupuy, A. K., & Strausbaugh, L. D. (2017). Mining the oral mycobiome: methods, components, and meaning. *Virulence*, *8*(3), 313–323. doi:10.1080/21505594.2016.1252015

Diaz, P. I., Strausbaugh, L. D., & Dongari-Bagtzoglou, A. (2014). Fungal-bacterial interactions and their relevance to oral health: linking the clinic and the bench. *Frontiers in Cellular and Infection Microbiology*, *4*, 101. doi:10.3389/fcimb.2014.00101

Dirix, P., Nuyts, S., & Van Den Bogaert, W. (2006). Radiation-induced xerostomia in patients with head and neck cancer: a literature review. *Cancer*, *107*(11), 2525–2534. doi:10.1002/cncr.22302

Dreizen, S., Daly, T. E., Drane, J. B., & Brown, L. R. (1977). Oral complications of cancer radiotherapy. *Postgraduate Medicine*, *61*(2), 85–92. doi:10.1080/00325481.1977.11712115

Dupuy, A. K., David, M. S., Li, L., Heider, T. N., Peterson, J. D., Montano, E. A., … Strausbaugh, L. D. (2014). Redefining the human oral mycobiome with improved practices in amplicon-based taxonomy: discovery of *Malassezia* as a prominent commensal. *PLoS One*, *9*(3), e90899. doi:10.1371/journal.pone.0090899

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460–2461. doi:10.1093/bioinformatics/btq461

Edgar, R.C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature Methods, 10*, 996-998. doi: 10.1038/nmeth.2604

Edgar, R. C. (2016). UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. *BioRxiv,* 081257. doi:10.1101/081257

Eliasson, L., Carlén, A., Almståhl, A., Wikström, M., & Lingström, P. (2006). Dental plaque pH and micro-organisms during hyposalivation. *Journal of Dental Research*, *85*(4), 334–338. doi:10.1177/154405910608500410

Epstein, J. B., Emerton, S., Kolbinson, D. A., Le, N. D., Phillips, N., Stevenson-Moore, P., & Osoba, D. (1999). Quality of life and oral function following radiotherapy for head and neck cancer. *Head and Neck*, *21*(1), 1–11. doi:10.1002/(SICI)1097-0347(199901)21:1<1::AID-HED1>3.0.CO;2-4

Epstein, J. B., Lunn, R., Le, N., & Stevenson-Moore, P. (1998). Periodontal attachment loss in patients after head and neck radiation therapy. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*, *86*(6), 673–677. doi:10.1016/S1079-2104(98)90202-5

Fechney, J. M., Browne, G. V., Prabhu, N., Irinyi, L., Meyer, W., Hughes, T., … Adler, C. J. (2019). Preliminary study of the oral mycobiome of children with and without dental caries. *Journal of Oral Microbiology*, *11*(1), 1536182. doi:10.1080/20002297.2018.1536182

Gainza-Cirauqui, M. L., Nieminen, M. T., Novak Frazer, L., Aguirre-Urizar, J. M., Moragues, M. D., & Rautemaa, R. (2013). Production of carcinogenic acetaldehyde by *Candida albicans* from patients with potentially malignant oral mucosal disorders. *Journal of Oral Pathology and Medicine*, *42*(3), 243–249. doi:10.1111/j.1600-0714.2012.01203.x

Galvão-Moreira, L. V., & Da Cruz, M. C. F. N. (2016). Oral microbiome, periodontitis and risk of head and neck cancer. *Oral Oncology*, *53*, 17–19. doi:10.1016/j.oraloncology.2015.11.013

Gardes, M., & Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes, application to the identification of mycorrihiza and rusts. *Molecular Ecology*, *2*(2), 113–118. doi:10.1111/J.1365-294x.1993.Tb00005.X

Gasche, J. A., Hoffmann, J., Boland, C. R., & Goel, A. (2011). Interleukin-6 promotes tumorigenesis by altering DNA methylation in oral cancer cells. *International Journal of Cancer*, *129*(5), 1053–1063. doi:10.1002/ijc.25764

Ghannoum, M. A., Jurevic, R. J., Mukherjee, P. K., Cui, F., Sikaroodi, M., Naqvi, A., & Gillevet, P. M. (2010). Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS Pathogens*, *6*(1), e1000713. doi:10.1371/journal.ppat.1000713

Gholizadeh, P., Eslami, H., Yousefi, M., Asgharzadeh, M., Aghazadeh, M., & Kafil, H. S. (2016). Role of oral microbiome on oral cancers, a review. *Biomedicine and Pharmacotherapy*, *84*, 552–558. doi:10.1016/j.biopha.2016.09.082

Gopinath, D., Menon, R. K., Banerjee, M., Su Yuxiong, R., Botelho, M. G., & Johnson, N. W. (2019). Culture-independent studies on bacterial dysbiosis in oral and oropharyngeal squamous cell carcinoma: a systematic review. *Critical Reviews in Oncology/Hematology*, *139*, 31–40. doi:10.1016/j.critrevonc.2019.04.018

Guerrero-Preston, R., Godoy-Vitorino, F., Jedlicka, A., Rodríguez-Hilario, A., González, H., Bondy, J., … Sidransky, D. (2016). 16S rRNA amplicon sequencing identifies microbiota associated with oral cancer, Human Papilloma Virus infection and surgical treatment. *Oncotarget*, *7*(32), 51320. doi:10.18632/oncotarget.9710

Ha, N. H., Woo, B. H., Kim, D. J., Ha, E. S., Choi, J. I., Kim, S. J., … Park, H. R. (2015). Prolonged and repetitive exposure to *Porphyromonas gingivalis* increases aggressiveness of oral cancer cells by promoting acquisition of cancer stem cell properties. *Tumor Biology*, *36*(12), 9947–9960. doi:10.1007/s13277-015-3764-9

Hajishengallis, G., Darveau, R., & Curtis, M. (2012). The keystone pathogen hypothesis. *Nature Reviews Microbiology*, *10*(10), 717–725. doi:10.1038/nrmicro2873

Halwachs, B., Madhusudhan, N., Krause, R., Nilsson, R. H., Moissl-Eichinger, C., Högenauer, C., … Gorkiewicz, G. (2017). Critical issues in mycobiota analysis. *Frontiers in Microbiology*, *8*, 180. doi:10.3389/fmicb.2017.00180

Hamada, S., & Slade, H. D. (1980). Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiological Reviews*, *44*(2), 331–384. PMID:6446023.

Hameed, M., Zafar, K., & Ghafoor, R. (2018). Management of oral complications in irradiated head and neck cancer patients: literature review. *International Journal of Surgery: Short Reports*, *3*(1), 15. doi:10.4103/ijssr.ijssr_6_18

Haukioja, A. (2010). Probiotics and oral health. *European Journal of Dentistry*, *4*(3), 348–355. PMID:20613927.

Healy, C. M., & Moran, G. P. (2019). The microbiome and oral cancer: more questions than answers. *Oral Oncology*, *89*, 30–33. doi:10.1016/j.oraloncology.2018.12.003

Hibbett, D. S., & Taylor, J. W. (2013). Fungal systematics: is a new age of enlightenment at hand? *Nature Reviews Microbiology*, *11*(2), 129–133. doi:10.1038/nrmicro2963

Hoggard, M., Vesty, A., Wong, G., Montgomery, J. M., Fourie, C., Douglas, R. G., … Taylor, M. W. (2018). Characterizing the human mycobiota: a comparison of small subunit rRNA, ITS1, ITS2, and large subunit rRNA genomic targets. *Frontiers in*

Microbiology, *9*, 2208. doi:10.3389/fmicb.2018.02208

Hong, C. H. L., Napeñas, J. J., Hodgson, B. D., Stokman, M. A., Mathers-Stauffer, V., Elting, L. S., … Brennan, M. T. (2010). A systematic review of dental disease in patients undergoing cancer therapy. *Supportive Care in Cancer*, *18*(8), 1007–1021. doi:10.1007/s00520-010-0873-2

Hooper, L., Littman, D., & Macpherson, A. (2012). Interactions between the microbiota and the immune system. *Science*, *336*(6086), 1268–1273. doi:10.1126/science.1223490

Hooper, S. J., Wilson, M. J., & Crean, S. J. (2009). Exploring the link between microorganisms and oral cancer: a systematic review of the literature. *Head & Neck*, *31*(9), 1228–1239. doi:10.1002/hed.21140

Hou, J., Zheng, H. M., Li, P., Liu, H. Y., Zhou, H. W., & Yang, X. J. (2018). Distinct shifts in the oral microbiota are associated with the progression and aggravation of mucositis during radiotherapy. *Radiotherapy and Oncology, 129*(1), 44–51. doi:10.1016/j.radonc.2018.04.023

Human Microbiome Project Consortium. (2012). Structure, function and diversity of the healthy human microbiome. *Nature*, *486*(7402), 207–214. https://doi.org/10.1038/nature11234

Iwai, S., Weinmaier, T., Schmidt, B. L., Albertson, D. G., Poloso, N. J., Dabbagh, K., & DeSantis, T. Z. (2016). Piphillin: improved prediction of metagenomic content by direct inference from human microbiomes. *PLoS One*, *11*(11), e0166104. doi:10.1371/journal.pone.0166104

Jaedicke, K. M., Preshaw, P. M., & Taylor, J. J. (2016). Salivary cytokines as biomarkers of periodontal diseases. *Periodontology 2000*, *70*(1), 164–183. doi:10.1111/prd.12117

James, S. M., & Tagg, J. R. (1991). The prevention of dental caries by BLIS-mediated inhibition of mutans streptococci. *New Zealand Dental Journal*, *87*(389), 80–83. PMID:1956623.

Jawad, H., Hodson, N. A., & Nixon, P. J. (2015). A review of dental treatment of head and neck cancer patients, before, during and after radiotherapy: part 1. *British Dental Journal*, *218*(2), 65–68. doi:10.1038/sj.bdj.2015.28

Jensen, S. B., Pedersen, A. M. L., Vissink, A., Andersen, E., Brown, C. G., Davies, A. N., … Brennan, M. T. (2010). A systematic review of salivary gland hypofunction and xerostomia induced by cancer therapies: prevalence, severity and impact on quality of life. *Supportive Care in Cancer*, *18*(8), 1039–1060. doi:10.1007/s00520-010-0827-8

Joyston-Bechal, S., Hayes, K., Davenport, E. S., & Hardie, J. M. (1992). Caries incidence, mutans streptococci and lactobacilli in irradiated patients during a 12-month preventive programme using chlorhexidine and fluoride. *Caries Research*, *26*(5), 384–390. doi:10.1159/000261473

Kageyama, S., Takeshita, T., Asakawa, M., Shibata, Y., Takeuchi, K., Yamanaka, W., & Yamashita, Y. (2017). Relative abundance of total subgingival plaque-specific bacteria in salivary microbiota reflects the overall periodontal condition in patients with periodontitis. *PloS One*, *12*(4), e0174782. doi:10.1371/journal.pone.0174782

Kagnoff, M. F., & Eckmann, L. (1997). Epithelial cells as sensors for microbial infection. *Journal of Clinical Investigation*, *100*(1), 6–10. doi:10.1172/JCI119522

Katz, J., Onate, M. D., Pauley, K. M., Bhattacharyya, I., & Cha, S. (2011). Presence of *Porphyromonas gingivalis* in gingival squamous cell carcinoma. *International Journal of Oral Science*, *3*(4), 209–215. doi:10.4248/IJOS11075

Keijser, B. J. F., Zaura, E., Huse, S. M., Van Der Vossen, J. M. B. M., Schuren, F. H. J., Montijn, R. C., … Crielaard, W. (2008). Pyrosequencinq analysis of the oral microflora of healthy adults. *Journal of Dental Research*, *87*(11), 1016–1020. doi:10.1177/154405910808701104

Keller, M. K., Brandsborg, E., Holmstrøm, K., & Twetman, S. (2018). Effect of tablets containing probiotic candidate strains on gingival inflammation and composition of the salivary microbiome: a randomised controlled trial. *Beneficial Microbes*, *9*(3), 487–494. doi:10.3920/BM2017.0104

Khaw, A., Liberali, S., Logan, R., Keefe, D., & Bartold, P. M. (2014). Influence of periodontitis on the experience of oral mucositis in cancer patients undergoing head and neck radiotherapy: a pilot study. *Supportive Care in Cancer*, *22*(8), 2119–2125. doi:10.1007/s00520-014-2186-3

Khaw, A., Logan, R., Keefe, D., & Bartold, M. (2014). Radiation-induced oral mucositis and periodontitis - proposal for an inter-relationship. *Oral Diseases*, *20*(3), e7–e18. doi:10.1111/odi.12199

Kielbassa, A. M., Hinkelbein, W., Hellwig, E., & Meyer-Lückel, H. (2006). Radiation-related damage to dentition. *Lancet Oncology*, *7*(4), 326–335. doi:10.1016/S1470-2045(06)70658-1

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, *41*(1), e1. doi:10.1093/nar/gks808

Kõljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F. S., Bahram, M., … Larsson, K. H. (2013). Towards a unified paradigm for sequence-based identification of

fungi. *Molecular Ecology*, *22*(21), 5271–5277. doi:10.1111/mec.12481

Krom, B. P., Kidwai, S., & Cate, J. M. ten. (2014). *Candida* and other fungal species: forgotten players of healthy oral microbiota. *Journal of Dental Research*, *93*(5), 445–451. doi:10.1177/0022034514521814

Kurtz, Z. D., Müller, C. L., Miraldi, E. R., Littman, D. R., Blaser, M. J., & Bonneau, R. A. (2015). Sparse and Compositionally Robust Inference of Microbial Ecological Networks. *PLoS Computational Biology*, *11*(5), e1004226. doi:10.1371/journal.pcbi.1004226

Laheij, A. M. G. A., & de Soet, J. J. (2014). Can the oral microflora affect oral ulcerative mucositis? *Current Opinion in Supportive and Palliative Care*, *8*(2), 180–187. doi:10.1097/SPC.0000000000000053

Laheij, A. M. G. A., de Soet, J. J., von dem Borne, P. A, Kuijper, E. J., Kraneveld, E. A., van Loveren, C., & Raber-Durlacher, J. E. (2012). Oral bacteria and yeasts in relationship to oral ulcerations in hematopoietic stem cell transplant recipients. *Supportive Care in Cancer*, *20*(12), 3231–3240. doi:10.1007/s00520-012-1463-2

Lamont, R. J., Koo, H., & Hajishengallis, G. (2018). The oral microbiota: dynamic communities and host interactions. *Nature Reviews Microbiology*, *16*, 745-759. doi:10.1038/s41579-018-0089-x

Lazarevic, V., Gaïa, N., Girard, M., François, P., & Schrenzel, J. (2013). Comparison of DNA extraction methods in analysis of salivary bacterial communities. *PLoS One*, *8*(7), e67699. doi:10.1371/journal.pone.0067699

Limon, J. J., Skalski, J. H., & Underhill, D. M. (2017). Commensal fungi in health and disease. *Cell Host & Microbe*, *22*(2), 156–165. doi:10.1016/j.chom.2017.07.002

Cheng, Y.-S., Jordan, L., Gorugantula, L. M., Schneiderman, E., Chen, H.-S., & Rees, T. (2014). Salivary interleukin-6 and -8 in patients with oral cancer and patients with chronic oral inflammatory diseases. *Journal of Periodontology*, *85*(7), 956–965. doi:10.1902/jop.2013.130320

Loesche, W. J. (1986). Role of *Streptococcus mutans* in human dental decay. *Microbiological Reviews*, *50*(4), 353–380. PMID:3540569.

Lucas-Roxburgh, R., Benschop, J., Lockett, B., Van Den Heever, U., Williams, R., & Howe, L. (2017). The prevalence of human papillomavirus in oropharyngeal cancer in a New Zealand population. *PLoS One*, *12*(10), e0186424. doi:10.1371/journal.pone.0186424

Mager, D. L., Ximenez-Fyvie, L. A., Haffajee, A. D., & Socransky, S. S. (2003). Distribution of selected bacterial species on intraoral surfaces. *Journal of Clinical Periodontology*, *30*(7), 644–654. doi:10.1034/j.1600-051X.2003.00376.x

Mahasneh, S., & Mahasneh, A. (2017). Probiotics: a promising role in dental health. *Dentistry Journal*, *5*(4), 26. doi:10.3390/dj5040026

Maria, O. M., Eliopoulos, N., & Muanza, T. (2017). Radiation-induced oral mucositis. *Frontiers in Oncology*, *7*, 89. doi:10.3389/fonc.2017.00089

Markitziu, A., Zafiropoulos, G., Tsalikis, L., & Cohen, L. (2006). Gingival health and salivary function in head and neck-irradiated patients. *Oral Surgery, Oral Medicine, Oral Pathology*, *73*(4), 427–433. doi:10.1016/0030-4220(92)90319-l

Marsh, P. D. (1994). Microbial ecology of dental plaque and its significance in health and disease. *Advances in Dental Research*, *8*(2), 263–271. doi:10.1177/08959374940080022001

Marsh, P. D., Do, T., Beighton, D., & Devine, D. A. (2016). Influence of saliva on the oral

microbiota. *Periodontology 2000*, *70*(1), 80–92. doi:10.1111/prd.12098

Marur, S., & Forastiere, A. A. (2016). Head and neck squamous cell carcinoma: update on epidemiology, diagnosis, and treatment. *Mayo Clinic Proceedings*, *91*(3), 386–396. doi:10.1016/j.mayocp.2015.12.017

Mayanagi, G., Kimura, M., Nakaya, S., Hirata, H., Sakamoto, M., Benno, Y., & Shimauchi, H. (2009). Probiotic effects of orally administered *Lactobacillus salivarius* WB21-containing tablets on periodontopathic bacteria: a double-blinded, placebo-controlled, randomized clinical trial. *Journal of Clinical Periodontology*, *36*(6), 506–513. doi:10.1111/j.1600-051X.2009.01392.x

McGuire, D. B., Fulton, J. S., Park, J., Brown, C. G., Correa, M. E. P., Eilers, J., … Lalla, R. V. (2013). Systematic review of basic oral care for the management of oral mucositis in cancer patients. *Supportive Care in Cancer*, *21*(11), 3165–3177. doi:10.1007/s00520-013-1942-0

Mercadante, V., Al Hamad, A., Lodi, G., Porter, S., & Fedele, S. (2017). Interventions for the management of radiotherapy-induced xerostomia and hyposalivation: a systematic review and meta-analysis. *Oral Oncology*, *66*, 64–74. doi:10.1016/j.oraloncology.2016.12.031

Miller, A. B., Hoogstraten, B., Staquet, M., & Winkler, A. (1981). Reporting results of cancer treatment. *Cancer*, *47*(1), 207–214. doi:10.1002/1097-0142(19810101)47:1<207::AID-CNCR2820470134>3.0.CO;2-6

Morita, E., Narikiyo, M., Yano, A., Nishimura, E., Igaki, H., Sasaki, H., … Kawabe, R. (2003). Different frequencies of *Streptococcus anginosus* infection in oral cancer and esophageal cancer. *Cancer Science*, *94*(6), 492–496. doi:10.1111/j.1349-7006.2003.tb01471.x

Mukherjee, P. K., Chandra, J., Retuerto, M., Sikaroodi, M., Brown, R. E., Jurevic, R., … Ghannoum, M. A. (2014). Oral mycobiome analysis of HIV-infected patients: identification of *Pichia* as an antagonist of opportunistic fungi. *PLoS Pathogens*, *10*(3), e1003996. doi:10.1371/journal.ppat.1003996

Müller, V. J., Belibasakis, G. N., Bosshard, P. P., Wiedemeier, D. B., Bichsel, D., Rücker, M., & Stadlinger, B. (2019). Change of saliva composition with radiotherapy. *Archives of Oral Biology*, *106*, 104480. doi:10.1016/j.archoralbio.2019.104480

Nilsson, R. H., Larsson, K.-H., Taylor, A. F. S., Bengtsson-Palme, J., Jeppesen, T. S., Schigel, D., … Abarenkov, K. (2018). The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classification. *Nucleic Acids Research, 47*(D1), D259–D264. doi:10.1093/nar/gky1022

Nilsson, R. H., Kristiansson, E., Ryberg, M., Hallenberg, N., & Larsson, K. H. (2008). Intraspecific ITS variability in the Kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification. *Evolutionary Bioinformatics*, *2008*(4), 193–201. doi:10.4137/EBO.S653

Nishihara, T., & Koseki, T. (2004). Microbial etiology of periodontitis. *Periodontology 2000*, *36*(1), 14–26. doi:10.1111/j.1600-0757.2004.03671.x

Nishii, M., Soutome, S., Kawakita, A., Yutori, H., Iwata, E., & Akashi, M. (2019). Factors associated with severe oral mucositis and candidiasis in patients undergoing radiotherapy for oral and oropharyngeal carcinomas : a retrospective multicenter study of 326 patients. *Supportive Care in Cancer*, 1–7. doi:10.1007/s00520-019-04885-z

O'Leary, T. J., Drake, R. B., & Naylor, J. E. (2010). The plaque control record. *Journal of Periodontology*, *43*(1), 38–38. doi:10.1902/jop.1972.43.1.38

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, P. L., McGlinn, D., Minchin, P. R., … Wagner, H. (2017). Vegan: Community Ecology Package. *R Package Version*. Retrieved from https://cran.r-project.org/package=vegan

Perera, M., Al-hebshi, N. N., Perera, I., Ipe, D., Ulett, G. C., Speicher, D. J., … Johnson, N. W. (2017). A dysbiotic mycobiome dominated by *Candida albicans* is identified within oral squamous-cell carcinomas. *Journal of Oral Microbiology*, *9*(1), 1385369. doi:10.1080/20002297.2017.1385369

Perera, M., Al-Hebshi, N. N., Speicher, D. J., Perera, I., & Johnson, N. W. (2016). Emerging role of bacteria in oral carcinogenesis: a review with special reference to perio-pathogenic bacteria. *Journal of Oral Microbiology*, *1*(8), 1–10. doi:10.3402/jom.v8.32762

Peters, B. A., Wu, J., Hayes, R. B., & Ahn, J. (2017). The oral fungal mycobiome: Characteristics and relation to periodontitis in a pilot study. *BMC Microbiology*, *17*(1), 1–11. doi:10.1186/s12866-017-1064-9

Petersen, P. E., & Ogawa, H. (2012). The global burden of periodontal disease: towards integration with chronic disease prevention and control. *Periodontology 2000*, *60*(1), 15–39. doi:10.1111/j.1600-0757.2011.00425.x

Polgárová, K., Behuliak, M., & Celec, P. (2010). Effect of saliva processing on bacterial DNA extraction. *New Microbiologica*, *33*(4), 373–379. doi:10.3402/jom.v7.27429

Porter, S. R., Fedele, S., & Habbab, K. M. (2010). Xerostomia in head and neck malignancy. *Oral Oncology*, *46*(6), 460–463. doi:10.1016/j.oraloncology.2010.03.008

Price, M.N., Dehal, P.S., & Arkin, A.P. (2009). FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Molecular biology and*

evolution, 26(7), 1641–1650. doi:10.1093/molbev/msp077

Pushalkar, S., Mane, S. P., Ji, X., Li, Y., Evans, C., Crasta, O. R., … Saxena, D. (2011). Microbial diversity in saliva of oral squamous cell carcinoma. *FEMS Immunology and Medical Microbiology*, *61*(3), 269–277. doi:10.1111/j.1574-695X.2010.00773.x

R Core Team. (2017). R: A Language and Environment for Statistical Computing. Vienna, Austria. Retrieved from https://www.r-project.org/

Ramirez-Garcia, A., Rementeria, A., Aguirre-Urizar, J. M., Moragues, M. D., Antoran, A., Pellon, A., … Hernando, F. L. (2016). *Candida albicans* and cancer: can this yeast induce cancer development or progression? *Critical Reviews in Microbiology*, *42*(2), 181–193. doi:10.3109/1040841X.2014.913004

Rathnayake, N., Åkerman, S., Klinge, B., Lundegren, N., Jansson, H., Tryselius, Y., … Gustafsson, A. (2013). Salivary biomarkers of oral health - a cross-sectional study. *Journal of Clinical Periodontology*, *40*(2), 140–147. doi:10.1111/jcpe.12038

Riccia, D. N. Della, Bizzini, F., Perilli, M. G., Polimeni, A., Trinchieri, V., Amicosante, G., & Cifone, M. G. (2007). Anti-inflammatory effects of *Lactobacillus brevis* (CD2) on periodontal disease. *Oral Diseases*, *13*(4), 376–385. doi:10.1111/j.1601-0825.2006.01291.x

Robertson, L. (2018). Can oral hygiene intervention reduce the severity of oral mucositis in adults about to undergo chemotherapy and radiotherapy for treatment of mouth cancer? *British Society of Dental Hygiene & Therapy: Dental Health*, *57*(6), 38–42.

Robledo-Sierra, J., Ben-Amy, D. P., Varoni, E., Bavarian, R., Simonsen, J. L., Paster, B. J., … Frandsen Lau, E. (2019). World workshop on oral medicine VII: targeting the oral microbiome part 2: current knowledge on malignant and potentially malignant oral

disorders. *Oral Diseases*, *25*(S1), 28–48. doi:10.1111/odi.13107

Rôças, I. N., Siqueira, J. F., Santos, K. R. N., & Coelho, A. M. A. (2001). "Red complex" (*Bacteroides forsythus*, *Porphyromonas gingivalis*, and *Treponema denticola*) in endodontic infections: a molecular approach. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*, *91*(4), 468–471. doi:10.1067/moe.2001.114379

Rodríguez-Caballero, A., Torres-Lagares, D., Robles-García, M., Pachón-Ibáñez, J., González-Padilla, D., & Gutiérrez-Pérez, J. L. (2012). Cancer treatment-induced oral mucositis: a critical review. *International Journal of Oral and Maxillofacial Surgery*, *41*(2), 225–238. doi:10.1016/j.ijom.2011.10.011

Rogers, S. N., Ahad, S. A., & Murphy, A. P. (2007). A structured review and theme analysis of papers published on "quality of life" in head and neck cancer: 2000-2005. *Oral Oncology*, *43*(9), 843–868. doi:10.1016/j.oraloncology.2007.02.006

Saito, H., Watanabe, Y., Sato, K., Ikawa, H., Yoshida, Y., Katakura, A., … Sato, M. (2014). Effects of professional oral health care on reducing the risk of chemotherapy-induced oral mucositis. *Supportive Care in Cancer*, *22*(11), 2935–2940. doi:10.1007/s00520-014-2282-4

Sakamoto, M., Umeda, M., Ishikawa, I., & Benno, Y. (2000). Comparison of the oral bacterial flora in saliva from a healthy subject and two periodontitis patients by sequence analysis of 16S rDNA libraries. *Microbiology and Immunology*, *44*(8), 643–652. doi:10.1111/j.1348-0421.2000.tb02545.x

Sampaio-Maia, B. (2014). Interindividual variability and intraindividual stability of oral fungal microbiota over time. *Medical Mycology*, *52*(5), 498–505. doi:10.1093/mmy/myu027

Segata, N., Haake, S., Mannon, P., Lemon, K. P., Waldron, L., Gevers, D., … Westcott, S. (2012). Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples. *Genome Biology*, *13*(6), R42. doi:10.1186/gb-2012-13-6-r42

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome Biology*, *12*(6), R60. doi:10.1186/gb-2011-12-6-r60

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., … Ideker, T. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Research*, *13*(11), 2498–2504. doi:10.1101/gr.1239303

Shaw, R., & Beasley, N. (2016). Aetiology and risk factors for head and neck cancer: United Kingdom National Multidisciplinary Guidelines. *The Journal of Laryngology & Otology*, *130*(S2), S9–S12. doi:10.1017/s0022215116000360

Shimauchi, H., Mayanagi, G., Nakaya, S., Minamibuchi, M., Ito, Y., Yamaki, K., & Hirata, H. (2008). Improvement of periodontal condition by probiotics with *Lactobacillus salivarius* WB21: a randomized, double-blind, placebo-controlled study. *Journal of Clinical Periodontology*, *35*(10), 897–905. doi:10.1111/j.1600-051X.2008.01306.x

Simón-Soro, A., & Mira, A. (2015). Solving the etiology of dental caries. *Trends in Microbiology*, *23*(2), 76–82. doi:0.1016/j.tim.2014.10.010

Socransky, S. S., Haffajee, D., Cugini, M., Smith, C., & Kent, R. L. (1998). Microbial complexes in subgingival plaque. *Journal of Clinical Periodontology*, *25*(2), 134–144. doi:10.1111/j.1600-051X.1998.tb02419.x

Sohrabi, M., Nair, R. G., Samaranayake, L. P., Zhang, L., Zulfiker, A. H. M., Ahmetagic, A.,

… Wei, M. Q. (2016). The yield and quality of cellular and bacterial DNA extracts from human oral rinse samples are variably affected by the cell lysis methodology. *Journal of Microbiological Methods*, *122*, 64–72. doi:10.1016/j.mimet.2016.01.013

Sonis, S.T. (2002). The biologic role for nuclear factor-κB in disease and its potential involvement in mucosal injury associated with anti-neoplastic therapy. *Critical Reviews in Oral Biology and Medicine*, *13*(5), 380–389. doi:10.1177/154411130201300502

Sonis, S.T. (2004). The pathobiology of mucositis. *Nature Reviews Cancer*, *4*, 277–284. doi:10.1038/nrc1318

Sonis, S.T. (2009). Mucositis: The impact, biology and therapeutic opportunities of oral mucositis. *Oral Oncology, 45*(12), 1015–1020. doi:10.1016/j.oraloncology.2009.08.006

Sonis, S.T. (2012). The pathobiology of oral mucositis. In: S.T. Sonis (Ed.), *Oral Mucositis* (pp. 7–13). Tarporley, England: Springer Healthcare. doi:10.1007/978-1-907673-46-7_2

Sonis, S. T., Elting, L. S., Keefe, D., Peterson, D. E., Schubert, M., Hauer-Jensen, M., … Rubenstein, E. B. (2004). Perspectives on cancer therapy-induced mucosal injury. *Cancer*, *100*(S9), 1995–2025. doi:10.1002/cncr.20162

Sroussi, H. Y., Epstein, J. B., Bensadoun, R. J., Saunders, D. P., Lalla, R. V., Migliorati, C. A., … Zumsteg, Z. S. (2017). Common oral complications of head and neck cancer radiation therapy: mucositis, infections, saliva change, fibrosis, sensory dysfunctions, dental caries, periodontal disease, and osteoradionecrosis. *Cancer Medicine*, *6*(12), 2918–2931. doi:10.1002/cam4.1221

Staab, B., Eick, S., Knöfler, G., & Jentsch, H. (2009). The influence of a probiotic milk drink on the development of gingivitis: a pilot study. *Journal of Clinical Periodontology*, *36*(10), 850–856. doi:10.1111/j.1600-051X.2009.01459.x

Stringer, A. M., & Logan, R. M. (2015). The role of oral flora in the development of chemotherapy-induced oral mucositis. *Journal of Oral Pathology and Medicine*, *44*(2), 81–87. doi:10.1111/jop.12152

Sturgis, E. M., Wei, Q., & Spitz, M. R. (2004). Descriptive epidemiology and risk factors for head and neck cancer. *Seminars in Oncology*, *31*(6), 726–733. doi:10.1053/j.seminoncol.2004.09.013

Sztukowska, M. N., Dutton, L. C., Delaney, C., Ramsdale, M., Ramage, G., Jenkinson, H. F., … Lamont, J. (2018). Community development between *Porphyromonas gingivalis* and *Candida albicans* mediated by InlJ and Als3, *9*(2), 1–16. doi:10.1128/mBio.00202-18

Takeshita, T., Kageyama, S., Furuta, M., Tsuboi, H., Takeuchi, K., Shibata, Y., … Yamashita, Y. (2016). Bacterial diversity in saliva and oral health-related conditions: the Hisayama Study. *Scientific Reports*, *6*, 22164. doi:10.1038/srep22164

Tamai, R., Sugamata, M., & Kiyoura, Y. (2011). *Candida albicans* enhances invasion of human gingival epithelial cells and gingival fibroblasts by *Porphyromonas gingivalis*. *Microbial Pathogenesis*, *51*(4), 250–254. doi:10.1016/j.micpath.2011.06.009

Tatakis, D. N., & Kumar, P. S. (2005). Etiology and pathogenesis of periodontal diseases. *Dental Clinics*, *49*(3), 491–516. doi:10.1016/j.cden.2005.03.001

Tateda, M., Shiga, K., Saijo, S., Sone, M., Hori, T., Yokoyama, J., … Miyagi, T. (2000). *Streptococcus anginosus* in head and neck squamous cell carcinoma: implication in carcinogenesis. *International Journal of Molecular Medicine*, *6*(6), 699–703. doi:10.3892/ijmm.6.6.699

Tekce, M., Ince, G., Gursoy, H., Dirikan Ipci, S., Cakar, G., Kadir, T., & Yilmaz, S. (2015). Clinical and microbiological effects of probiotic lozenges in the treatment of chronic

periodontitis: a 1-year follow-up study. *Journal of Clinical Periodontology*, *42*(4), 363–372. doi:10.1111/jcpe.12387

Teughels, W., Durukan, A., Ozcelik, O., Pauwels, M., Quirynen, M., & Haytac, M. C. (2013). Clinical and microbiological effects of *Lactobacillus reuteri* probiotics in the treatment of chronic periodontitis: a randomized placebo-controlled study. *Journal of Clinical Periodontology*, *40*(11), 1025–1035. doi:10.1111/jcpe.12155

Tezal, M., Sullivan, M. A., Hyland, A., Marshall, J. R., Stoler, D., Reid, M. E., … Scannapieco, F. A. (2009). Chronic periodontitis and the incidence of head and neck squamous cell carcinoma. *Cancer Epidemiology Biomarkers & Prevention*, *18*(9), 2406–2412. doi:10.1158/1055-9965.EPI-09-0334

Uehara, A., & Takada, H. (2007). Functional TLRs and NODs in human gingival fibroblasts. *Journal of Dental Research*, *86*(3), 249–254. doi:10.1177/154405910708600310

Vanhoecke, B., De Ryck, T., Stringer, A., Van de Wiele, T., & Keefe, D. (2015). Microbiota and their role in the pathogenesis of oral mucositis. *Oral Diseases*, *21*(1), 17–30. https://doi.org/10.1111/odi.12224

Vasconcelos, R. M., Sanfilippo, N., Paster, B. J., Kerr, A. R., Li, Y., Ramalho, L., … Corby, P. M. (2016). Host-microbiome cross-talk in oral mucositis. *Journal of Dental Research*, *95*(7), 725–733. doi:10.1177/0022034516641890

Vera-Llonch, M., Oster, G., Hagiwara, M., & Sonis, S. (2006). Oral mucositis in patients undergoing radiation treatment for head and neck carcinoma. *Cancer*, *106*(2), 329–336. doi:10.1002/cncr.21622

Vesty, A., Biswas, K., Taylor, M. W., Gear, K., & Douglas, R. G. (2017). Evaluating the impact of DNA extraction method on the representation of human oral bacterial and

fungal communities. *PLoS One*, *12*(1): e0169877. doi:10.1371/journal.pone.0169877

Vesty, A., Gear, K., Biswas, K., Radcliff, F. J., Taylor, M. W., & Douglas, R. G. (2018). Microbial and inflammatory-based salivary biomarkers of head and neck squamous cell carcinoma. *Clinical and Experimental Dental Research*, *4*(6), 255–262. doi:10.1002/cre2.139

Vissink, A., Jansma, J., Spijkervet, F. K. L., Burlage, F. R., & Coppes, R. P. (2003). Oral sequelae of head and neck radiotherapy. *Critical Reviews in Oral Biology & Medicine*, *14*(3), 199–212. doi:10.1177/154411130301400305

Vissink, A., Spijkervet, F. K. L., & Brennan, M. T. (2018). Xerostomia and dental problems in the head and neck radiation patient. In I. Olver (Ed.), *The MASCC Textbook of Cancer Supportive Care and Survivorship* (pp. 363–378). Cham: Springer International Publishing. doi:10.1007/978-3-319-90990-5_24

Vivekananda, M. R., Vandana, K. L., & Bhat, K. G. (2010). Effect of the probiotic *Lactobacilli reuteri* (Prodentis) in the management of periodontal disease: a preliminary randomized clinical trial. *Journal of Oral Microbiology*, *2*(1), 5344. doi:10.3402/jom.v2i0.5344

Vyshenska, D., Lam, K. C., Shulzhenko, N., & Morgun, A. (2017). Interplay between viruses and bacterial microbiota in cancer development. *Seminars in Immunology*, *32*, 14–24. doi:10.1016/j.smim.2017.05.003

Wade, W. G. (2013). The oral microbiome in health and disease. *Pharmacological Research*, *69*(1), 137–143. doi:10.1016/j.phrs.2012.11.006

Wang, M., Krauss, J. L., Domon, H., Hosur, K. B., Liang, S., Magotti, P., … Hajishengallis, G. (2010). Microbial hijacking of complement-toll-like receptor crosstalk. *Science*

Signaling, *3*(109), ra11. doi:10.1126/scisignal.2000697

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, *73*(16), 5261–5267. doi:10.1128/AEM.00062-07

Weerasekera, M. M., Sissons, C. H., Wong, L., Anderson, S., Holmes, A. R., & Cannon, R. D. (2013). Use of denaturing gradient gel electrophoresis for the identification of mixed oral yeasts in human saliva. *Journal of Medical Microbiology*, *62*(2), 319–330. doi:10.1099/jmm.0.050237-0

Wei, T., & Simko, V. (2016). Corrplot: visualization of a correlation matrix. Retrieved from https://cran.r-project.org/package=corrplot

Wescombe, P. A., Hale, J. D., Heng, N. C., & Tagg, J. R. (2012). Developing oral probiotics from *Streptococcus salivarius*. *Future Microbiology*, *7*(12), 1355–1371. doi:10.2217/fmb.12.113

White, T., Bruns, T., Lee, S., & Taylor, J. (1990). Analysis of phylogenetic relationships by amplification and direct sequencing of ribosomal RNA genes. In M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White (Eds.) *PCR Protocols: A Guide to Methods and Applications* (pp. 315–322). New York: Academic Press

Wolf, A., Moissl-Eichinger, C., Perras, A., Koskinen, K., & Peter, V. (2017). The salivary microbiome as an indicator of carcinogenesis in patients with oropharyngeal squamous cell carcinoma : a pilot study. *Scientific Reports, 7*(1), 5867. doi:10.1038/s41598-017-06361-2

World Health Organisation. (2012). Oral health. Retrieved from http://www.who.int/oral_health/publications/factsheet/en/

Xiao, C., Ran, S., Huang, Z., & Liang, J. (2016). Bacterial diversity and community structure of supragingival plaques in adults with dental health or caries revealed by 16S pyrosequencing. *Frontiers in Microbiology, 7*, 1145. doi:10.3389/fmicb.2016.01145

Xu, H., & Dongari-Bagtzoglou, A. (2015). Shaping the oral mycobiota: interactions of opportunistic fungi with oral bacteria and the host. *Current Opinion in Microbiology*, *26*, 65–70. doi:10.1016/j.mib.2015.06.002

Xu, X., He, J., Xue, J., Wang, Y., Li, K., Zhang, K., … Zhou, X. (2015). Oral cavity contains distinct niches with dynamic microbial communities. *Environmental Microbiology*, *17*(3), 699–710. doi:/10.1111/1462-2920.12502

Yadav, K., & Prakash, S. (2017). Dental caries: a microbiological approach. *Journal of Clinical Infectious Diseases & Practice*, *2*(1), 1–15. doi:10.4172/2476-213X.1000118

Yao, Q. W., Zhou, D. S., Peng, H. J., Ji, P., & Liu, D. S. (2014). Association of periodontal disease with oral cancer: a meta-analysis. *Tumor Biology*, *35*(7), 7073–7077. doi:10.1007/s13277-014-1951-8

Yost, S., Stashenko, P., Choi, Y., Kukuruzinska, M., Genco, C. A., Salama, A., … Frias-Lopez, J. (2018). Increased virulence of the oral microbiome in oral squamous cell carcinoma revealed by metatranscriptome analyses. *International Journal of Oral Science*, *10*(4), 32. doi:10.1038/s41368-018-0037-7

Zakrzewski, M., Proietti, C., Ellis, J. J., Hasan, S., Brion, M. J., Berger, B., & Krause, L. (2017). Calypso: a user-friendly web-server for mining and visualizing microbiome-environment interactions. *Bioinformatics*, *33*(5), 782–783. doi:10.1093/bioinformatics/btw725

Zhu, X. X., Yang, X. J., Chao, Y. L., Zheng, H. M., Sheng, H. F., Liu, H. Y., … Zhou, H. W.

(2017). The potential effect of oral microbiota in the prediction of mucositis during radiotherapy for nasopharyngeal carcinoma. *EBioMedicine*, *18*, 23–31. doi:10.1016/j.ebiom.2017.02.002

Zijnge, V., Van Leeuwen, M. B. M., Degener, J. E., Abbas, F., Thurnheer, T., Gmür, R., & Harmsen, H. J. M. (2010). Oral biofilm architecture on natural teeth. *PLoS One*, *5*(2), e9321. doi:10.1371/journal.pone.0009321