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The impact of denture related disease on the oral microbiome of denture wearers

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Abstract

Advances in healthcare over the last 100 years has resulted in an ever increasing elderly population. This presents greater challenges for adequate systemic and oral healthcare delivery. With increasing age there is a natural decline in oral health, leading to the loss of teeth and ultimately for some having to wear denture prosthesis. It is currently estimated that approximately one fifth of the UK and US populations have some form of removable prosthesis. The microbiology of denture induced mucosal inflammation is a pivotal factor to consider in denture care management, similar to many other oral diseases of microbial influence, such as caries, gingivitis and periodontitis.

Dentures support the growth of microbial biofilms, structures commonly known as denture plaque. Microbiologically, denture stomatitis (DS) is a disease primarily considered to be of yeast aetiology, with the literature disproportionately focussed on *Candida* spp. However, the denture surface is capable of carrying up to 10^{11} microbes per milligram, the majority of which are bacteria. Thus it is apparent that denture plaque is more diverse than we assume. There is a fundamental gap in our understanding of the bacterial composition of denture plaque and the role that they may play in denture related disease such as DS. This is categorised as inflammation of the oral mucosa, a disease affecting around half of all denture wearers. It has been proposed that bacteria and fungi interact on the denture surface and that these polymicrobial interactions lead to synergism and increased DS pathogenesis. Therefore, understanding the denture microbiome composition is the key step to beginning to understand disease pathogenesis, and ultimately help improve treatments and identify novel targets for therapeutic and preventative strategies.

A group of 131 patients were included within this study in which they provided samples from their dentures, palatal mucosa, saliva and dental plaque. Microbes residing on the denture surface were quantified using standard Miles and Misra culture technique which investigated the presence of *Candida*, aerobes and anaerobes. These clinical samples also underwent next generation sequencing using the Miseq Illumina platform to give a more global representation of the microbes present at each of these sites in the oral cavity of these denture wearers.

This data was then used to compare the composition and diversity of denture, mucosal and dental plaque between one another, as well as between healthy and diseased individuals. Additional comparisons included denture type and the presence or absence of natural teeth. Furthermore, microbiome data was used to assess differences between patients with varying levels of oral hygiene. The host response to the denture microbiome was investigated by screening the patients saliva for the presence and quantification of a range of antimicrobial peptides that are associated with the oral cavity. Based on the microbiome data an *in vitro* biofilm model was developed that reflected the composition of denture plaque. These biofilms were then used to assess quantitative and compositional changes over time and in response to denture cleansing treatments. Finally, the systemic implications of denture plaque were assessed by screening denture plaque samples for the presence of nine well known respiratory pathogens using quantitative PCR.

The results from this study have shown that the bacterial microbiome composition of denture wearers is not consistent throughout the mouth and varies depending on sample site. Moreover, the presence of natural dentition has a significant impact on the microbiome composition. As for healthy and diseased patients the data suggests that compositional changes responsible for disease progression are occurring at the mucosa, and that dentures may in fact be a reservoir for these microbes. In terms of denture hygiene practices, sleeping with a denture *in situ* was found to be a common occurrence. Furthermore, significant shifts in denture microbiome composition were found in these individuals when compared to the denture microbiome of those that removed their denture at night. As for the host response, some antimicrobial peptides were found to be significantly reduced in the absence of natural dentition, indicating that the oral immune response is gradually impaired with the loss of teeth. This study also identified potentially serious systemic implications in terms of respiratory infection, as 64.6% of patients carried respiratory pathogens on their denture.

In conclusion, this is the first study to provide a detailed understanding of the oral microbiome of denture wearers, and has provided evidence that DS development is more complex than simply a candidal infection. Both fungal and bacterial kingdoms clearly play a role in defining the progression of DS. The biofilm model created in this study demonstrated its potential as a platform to test novel actives.

Future use of this model will aid in greater understanding of host: biofilm interactions. Such findings are applicable to oral health and beyond, and may help to identify novel therapeutic targets for the treatment of DS and other biofilm associated diseases.

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List of publications based on thesis

O'Donnell LE, Robertson D, Nile CJ, Cross LJ, Riggio M, Sherriff A, et al. The Oral Microbiome of Denture Wearers Is Influenced by Levels of Natural Dentition. PloS one. 2015;10(9):e0137717.

O'Donnell LE, Smith K, Williams C, Nile CJ, Lappin DF, Bradshaw D, et al. Dentures are a Reservoir for Respiratory Pathogens. Journal of prosthodontics : official journal of the American College of Prosthodontists. 2015.

O'Donnell LE, Millhouse E, Sherry L, Kean R, Malcolm J, Nile CJ, et al. Polymicrobial Candida biofilms: friends and foe in the oral cavity. FEMS yeast research. 2015;15(7
E. Rosa (ed.), *Oral Candidosis: Physiopathology, Decision Making, and Therapeutics*, Candida Virulence Factors**. Lindsay E. O'Donnell**, Douglas Robertson and Gordon Ramage © Springer-Verlag Berlin Heidelberg 2015

Related Publications

Smith, K., A. Collier, E. M. Townsend, **L. E. O'Donnell**, A. M. Bal, J. Butcher, W. G. Mackay, G. Ramage and C. Williams (2016). "One step closer to understanding the role of bacteria in diabetic foot ulcers: characterising the microbiome of ulcers." BMC Microbiol **16**(1): 54.

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Author's Declaration

I declare that I have carried out the work described in this thesis unless otherwise acknowledged or cited, under the supervision Professor Gordon Ramage, Dr Douglas Robertson and Dr Christopher Nile. I further declare that this thesis has not in whole or in part, been submitted for any other degree.

Lindsay O'Donnell
June 2016

Abbreviations

ABTS:	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
ALS:	Agglutinin-like sequences
AMP:	Antimicrobial peptide
ATP:	Adenosine triphosphate
AP:	Aspiration pneumonia
AS:	Artificial saliva
BD1:	Beta defensin 1
BSA:	Bovine albumin serum
CAP:	Community acquired pneumonia
CCA:	Cannonical correspondance analysis
CFE:	Colony forming equivelant
CFU:	Colony forming unit
COPD:	Chronic obstructive ulmonary disease
CV:	Crystal violet
DC:	Denture cleanser
DNA:	Deoxyribonucleic acid
DS:	Denture stomatitis
DsDNA:	double stranded DNA
ECM:	Extracellular matrix
eDNA:	Extracellular DNA
ELISA:	Enzyme-linked immunosorbant assay
EU:	European union
HAP:	Hospital acquired pneumonia
HBD:	human beta defensin
HCL:	Hydrochloric acid
HMDS:	Hexamethyldisilazane
HNP:	human neutrophil peptide
HOMD:	Human oral microbiome database
HW:	Hard water
Hwp1:	Hyphal wall protein-1
KO:	Knock out
LB:	Lysogeny broth

LPS:	Lipopolysaccaride
LTCF:	Long term care facility
MGS:	Mitis group streptococci
MRSA:	Methicillin-resstant Staphylococcus aureus
NGS:	Next generation sequencing
NHS:	National health service
OHIP-14:	Oral health impact profile 14
OHRQoL:	Oral health related quality of life
OTU:	Operational taxonomic unit
PAMP	Pattern associated molecular pattern
PBS:	Phosphate buffered saline
PCA:	Prncipal component analysis
PCR:	Polymerase chain reaction
PD:	Periodontal disease
PMMA:	Polymethylmethacrylate
PRR:	Pattern recognition receptors
qPCR:	Quantatative PCR
QS:	Quorom sensing
RHOE:	reonstituted human oral epithelium
RNA:	ribonucleic acid
rRNA:	Ribosomal RNA
SEM:	Scanning electron microscopy
TLR:	Toll-like receptors
TMB:	3,3',5,5'-tetramethylbenzide
TSB:	Tryptic soy broth
VAP:	Ventilator associated pneumonia
XTT:	2,3 bis(2-methoxy-4-nitro-5sulfo-phenyl)-2H-tetrazolium-5-carboxanilide

1 Introduction

1.1 Introduction

Edentulousness is categorised as the loss of all natural teeth, which is an irreversible clinical condition that can be described as the ultimate marker of oral disease burden (Cunha-Cruz et al., 2007). It is also associated with socioeconomic factors, with higher prevalence reported in the poor and women (Bedos et al., 2003). With the sheer numbers of individuals routinely wearing and using a denture then there are profound implications for care and management. Many ageing individuals will experience a general decline in oral health, with current estimates reporting the international prevalence of edentulism varies from 7 to 69% of the adult population (Felton et al., 2011). Within the US and UK around 20% of the population already wear some form of removable denture (Coulthwaite and Verran, 2007, Shulman et al., 2004), with 70% of UK adults aged 75 years and above falling into this category (Iinuma et al., 2014). Nonetheless, dentures provide much-needed assistance to the patients, through improved nutritional intake and aesthetic appearance, which generally enhance the patients' quality of life. Though, as with any indwelling foreign body there are consequences, and these need to be managed accordingly to minimise denture-induced disease.

This chapter provides a review of the current literature relating to denture research, including denture microbiology, immunology, and emphasises the local and systemic implications of wearing dentures. Furthermore, this will highlight the importance of utilising advanced molecular technology OMICs approaches, such as next generation sequencing (NGS), when investigating microbial associated disease. Some aspects of this chapter have been published in:

O'Donnell, L. E., E. Millhouse, L. Sherry, R. Kean, J. Malcolm, C. J. Nile and G. Ramage (2015). "Polymicrobial *Candida* biofilms: friends and foe in the oral cavity." FEMS Yeast Res **15**(7).

E. Rosa (ed.), *Oral Candidosis: Physiopathology, Decision Making, and Therapeutics*, *Candida* Virulence Factors**. Lindsay E. O'Donnell**, Douglas Robertson and Gordon Ramage © Springer-Verlag Berlin Heidelberg 2015.

1.2 Denture Stomatitis

1.2.1 Introduction

Denture stomatitis (DS) refers to inflammation of the oral mucosa and pathological changes associated with denture surfaces adjacent to tissue (Jeganathan and Lin, 1992). Approximately two thirds of individuals who wear removable complete dentures suffer from DS, though most individuals are asymptomatic (Gendreau and Loewy, 2011). With 15 million dentures wearers in the UK this is not an inconsequential disease (Coulthwaite and Verran, 2007). DS sufferers can experience pain, discomfort, a burning or itching sensation and sometimes disturbance of taste. DS is associated with microbial factors, primarily infection with *Candida albicans* (Budtz-Jorgensen E, 1975). Other terminologies are used to refer to DS including denture-related candidiasis or *Candida*-associated denture-related stomatitis.

1.2.2 Classification

DS is diagnosed by clinical examination of inflammation or swelling of the mucosal tissues covered by the denture. The severity of DS is classified by the colour, texture, and overall appearance of the upper palate. The most frequently utilised scale of severity in the literature is known as Newton's Types (NT), and is described by three variables (Fig 1.1) (Newton, 1962a):

- **0-** No inflammation (Healthy)
- **1-** Localised inflammation (reddened areas within the tissue)
- **2-** Diffuse inflammation (diffuse hyperaemia of tissues with denture)
- **3-** Granular inflammation (increased papillary regions and swelling)

Although microbial factors play a major role in the inflammation of the palatal mucosa of DS patients; severity cannot always be attributed to the number of organisms present. Therefore, other factors such as poor fitting dentures and poor oral hygiene appear to play a crucial role (Coco et al., 2008b).

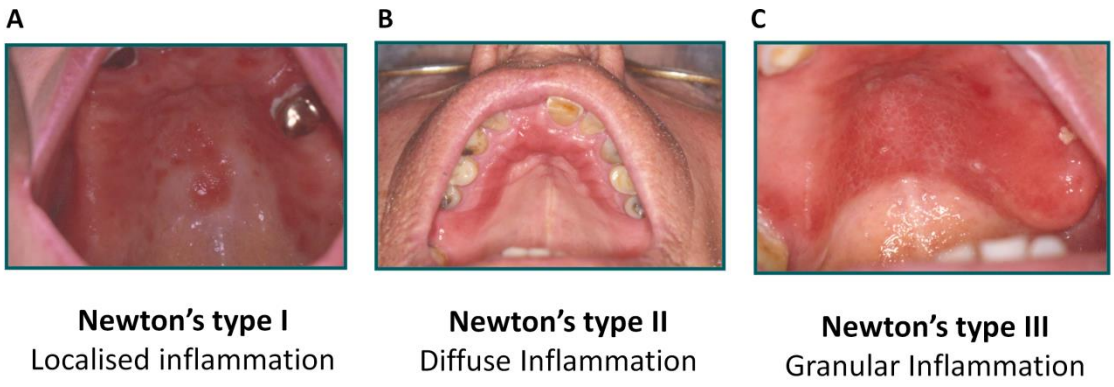


Figure 1.1:Newton's Type scale for the classification of inflammation present in DS.

A) Localised inflammation, **B)** Diffuse inflammation and **C)** Granular inflammation (Gendreau and Loewy, 2011).

1.2.3 Prevalence

Prevalence rates of DS vary considerably amongst the studies conducted; these variations can be attributed to differences within the study populations assessed. Table 1 provides the prevalence rates of numerous DS studies conducted worldwide. A large proportion of these studies were undertaken in treatment clinics designed specifically for denture patients, thus this may bias results as these patients already had pre-existing problems with their dentures. Furthermore, this may explain the high prevalence rates found in the majority of the prosthetic clinic based studies, with 67% of studies indicating a prevalence rate >45% (Gendreau and Loewy, 2011). Other studies obtained their prevalence rates by analysing representative population samples, for example, the NHANES III survey looked at a representative US population. They found that of 33,994 individuals, 3,450 wore removable dentures, 28% of which had DS (Shulman et al., 2004). The remaining population based studies from Spain, Slovenia and Turkey reported DS occurrence between 14.7-19.6% (Garcia-Pola Vallejo et al., 2002, Kovac-Kovacic and Skaleric, 2000, Mumcu et al., 2005). Yet, one representative study based in Denmark was somewhat of an outlier as it revealed a prevalence rate of 65%. However, this was later attributed to poor denture hygiene and a high occurrence of *Candida* infection within this population (Budtz-Jorgensen E, 1975). Furthermore, two studies undertaken in Finland, one from a national sample (Mikkonen et al., 1984) and the other from a group of home living elderly residents (Nevalainen et al., 1997), found a DS prevalence rate of 48% and 35%, respectively.

Interestingly, the study based solely on the elderly population had a lower prevalence rate. However, this could perhaps be explained by the fact that according to the national sample, 44% of adults in Finland wear dentures, which is an unusually high number in comparison to 20% of the US population (Shulman et al., 2004). Highlighting the fact that variation in dental and denture practices across different cultures makes defining DS prevalence particularly complex.

Table 1.1: Prevalence of Denture Stomatitis from studies conducted worldwide.
(Gendreau and Loewy, 2011).

Study Population /Country	Year of sampling	Age (years)	# Denture wearers	# with DS	Prevalence Rate %	Diagnosis method
Random *USA*	1988-94	59.2±0.5	3,450	963	28	Newton's
Random *Denmark*	1975	74.5	463	291	65	NR
Age-stratified, Random Finland	1984	81	260	91	35	NR
Age-stratified Random *Germany*	1997	Y-39 O- 69.5	655	NR	Y- 2.5 O- 18.3	WHO guidelines
Representative *Finland*	1989-91	NR	3,875	1860	48	NR
Representative *Turkey*	2005	NR	178	3	18.5	WHO guidelines
Age-stratified *Spain*	2002	54.3±13.5	102	20	19.6	NR
Random Age-stratified *Slovenia*	2000	25-75	163	24	14.7	NR
Stratified *Chile*	2003	65 to >75	574	198	34.5	WHO
LTCF residents *Denmark*	1987	64≥85	582	197	33.9	NR
Elderly patients attending clinic *Turkey*	2002	NR	70	31	44	Budtz-Jorgensen

Study Population /Country	Year of sampling	Age (years)	# Denture wearers	# with DS	Prevalence Rate %	Diagnosis method
LTCF *Finland*	2004	83.3±8.1	106	26	25	NR
Randomley selected LTCF *UK*	2000	84.5±8.3	331	110	33.2	Budtz-Jorgensen
All LTCF in Taubate *Brazil*	2006	74.9±12.5	201	108	54	NR
LTCF patients *Belgium*	1996	85.6±6.9	146	104	71	Budtz-Jorgensen
LTCF *Canada*	1999	83.4±17.6	38	13	34.2	Newton
Population –based *Greece*	2005	65-99	222	33	14.9	Newton
LTCF Brazil	2008	66.7±10.2	59	26	44.1	Newton
Population-based Rural *Brazil*	2008	NR	146	85	58.2	NR
Dental clinic Endentulous referral *Canada*	1976-1983	24-90	200	34	17	NR
Prothetic clinic *Mexico*	2005	Mean 67	105	50	47.6	NR
Volunteer Clinic *Brazil*	2004	62±12.8	236	NR	42.4	NR
Volunteer Clinic *Turkey*	2006	45-81	234	130	55.5	NR
Pros Clinic *Jordan*	2002	18-100 Mean 65±10.1	321	94	29	NR

Study Population /Country	Year of sampling	Age (years)	# Denture wearers	# with DS	Prevalence Rate %	Diagnosis method
Pros Clinic *Turkey*	2009	60-85	310	111	35.8	Newton
Pros Clinic *UK*	2008	49-89 Mean 73	37	26	70.3	Newton
Pros Clinic *Turkey*	2008	36-82	70	49	70	Budtz-Jorgesen
Pros Clinic *Canada*	2007	Mean 64.5	40	31	77.5	Newton
Folllow-up Clinic 1 year after denture	2008	72.1±4.4	173	110	63.6	Newton
Pros clinic Spain	2009	40-87	100	45	45	Newton
Pros Clinic *Jordan*	2007	39-100	300	157	52	Newton

LTCF: Long term care facility, Y:Youngest, O:Oldest, NR: Not reported, WHO: World health organisation

1.2.4 Aetiology

DS aetiology can be described as multi-factorial, (Fig 1.2) (Wilson, 1998). These factors include poor fitting dentures which can lead to mucosal trauma, poor denture hygiene, continual wearing of dentures and microbial infection. Therefore, treating DS is complex given the many factors contributing towards the disease, yet hygiene and microbial burden are most predominantly associated with causing disease and thus are the major targets for treatment.

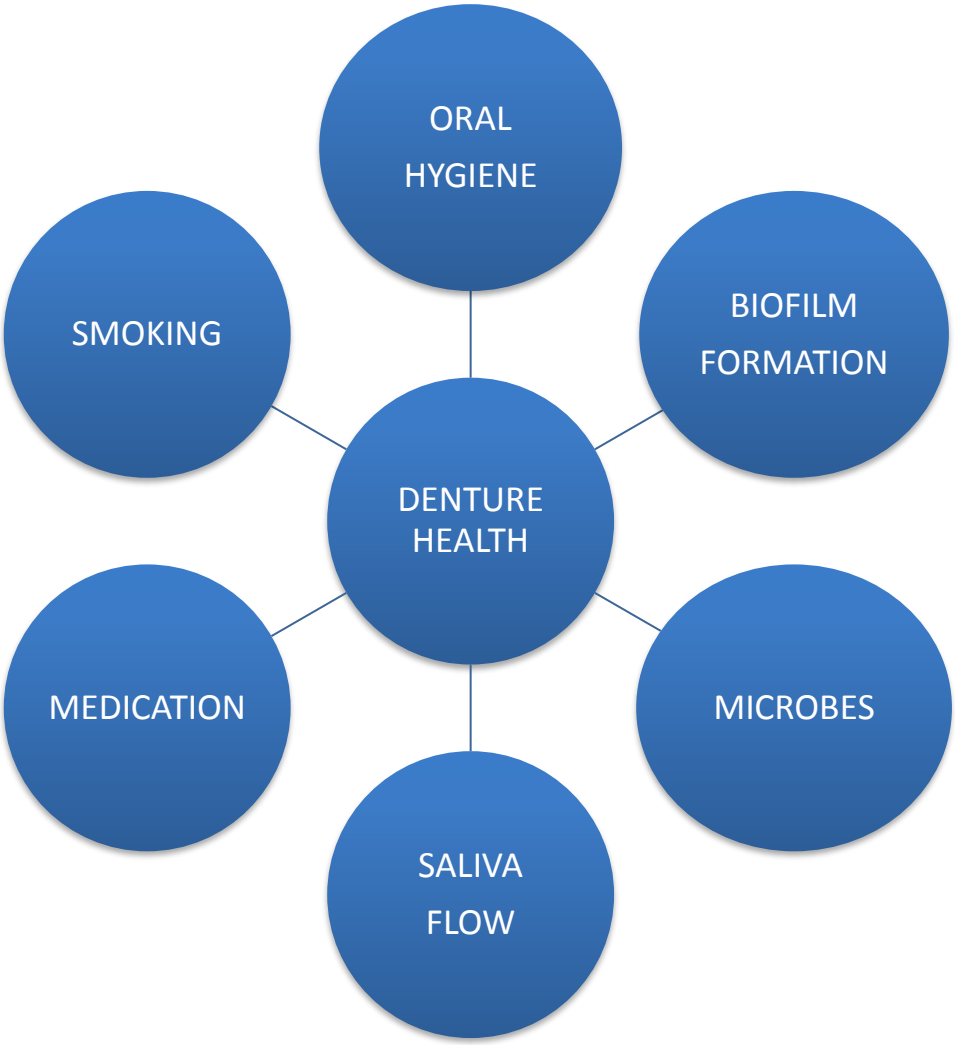


Figure 1.2: Aetiological factors associated with denture health.

1.2.4.1 Denture trauma

Denture-induced trauma, primarily associated with poor fitting dentures, has been identified as a high risk factor for developing DS. Approximately one half of denture wearers with poor fitting dentures are thought to experience localised Newton's type I inflammation (Mumcu et al., 2005, Frenkel et al., 2000, Kuc et al., 1999, Coco et al., 2008a). A study by Enammi *et al* (2008) compared DS incidence between elderly patients given new conventional dentures and those given two-implant overdentures. The overdentures reduced occlusal pressure on the mucosa and improved denture fit and stability. They concluded that the improved stability of overdentures caused less mucosal trauma and significantly reduced the likelihood of developing DS (Emami et al., 2008). Other studies imply that both trauma and *Candida* infection are responsible for disease pathogenesis, as it has been suggested that mucosal trauma induced by the denture increases the permeability of the epithelium, enhancing the ability of *Candida* to penetrate the mucosa (Cawson, 1965).

1.2.4.2 Oral hygiene

More severe forms of DS have been associated with poor hygiene in numerous studies. These studies reported that the majority of denture wearers rely on mechanical cleaning with a toothbrush as the sole denture cleaning method (Gendreau and Loewy, 2011). Poorly cleaned dentures allows for the colonisation of yeasts and bacteria, which form complex microbial communities known as biofilms, and if left unchecked may lead to inflammation of the oral mucosa (Kulak-Ozkan et al., 2002). Dikbas et al (2006) found that of 234 patients, only 11.9% had clean dentures and showed a significant correlation between unclean dentures and occurrence of DS (Dikbas et al., 2006). Furthermore, Budtz-Jorgensen and Bertram (1970) associated poor hygiene with increased *Candida* infection, mucosal trauma and inflammation (Budtz-Jorgensen and Bertram, 1970). While in contrast, Cross et al (2004) demonstrated that despite maintenance of excellent denture hygiene over three years, 18% of subjects relapsed, indicating factors other than hygiene are involved (Cross et al., 2004). For example, dentures set up a barrier to natural mechanical cleaning by saliva flow; therefore it is important to give time for this natural mechanism to have an effect by removing the denture whilst sleeping. Nonetheless, sleeping with

dentures *in situ* is still a common occurrence. This can lead to the development of an anaerobic environment between the denture and mucosa, favouring the growth of more pathogenic microbes and has been related to increased risk of developing DS (Gendreau and Loewy, 2011).

1.2.4.3 Salivary flow, medication and diet

Other factors that can impact denture health include diet and medication, which can consequently have a significant impact on the patients' salivary flow. Saliva plays an important role in maintaining physiological homeostasis within the oral cavity (Anil et al., 2016). It is involved in lubricating oral tissues, swallowing and speaking and humidifying the oral environment. It is estimated the normal salivary flow rate is approximately 0.3 mL/min at rest, which can increase up to more than 3 mL/min when stimulated (Watanabe and Dawes, 1988, Watanabe et al., 1995). Xerostomia is the subjective feeling of a dry mouth which is a symptom associated with alterations to the quality and quantity of saliva and is often a consequence of poor health, certain medications or radiation therapy (Cassolato and Turnbull, 2003). The implications of xerostomia include problems eating, diminished taste, mucosal infections and denture intolerance. Xerostomia is predominantly associated with the elderly population and can lead to major complications in their general health and well being (Anil et al., 2016). Xerostomia can cause alterations to the oral microbiome, which often results in the growth of acidogenic bacteria, as well as favouring growth of *Candida* spp (Samaranayake, 2011). The moisture created by saliva in the oral cavity, plays a role in retention of dentures by contributing towards the suction mechanism required to maintain stability. Thus without this stability dentures are likely to rub against palatial tissues causing sore spots and trauma to the mucosa (Edgerton et al.). Moreover, numerous widely used medications can cause xerostomia, such as simvastatin, lisinopril, omeprazole, Amlodipine etc. Thus, medications which induce xerostomia are a contributor towards denture associated disease and trauma.

Diet is a factor which can be associated with DS and can be implicated as both a cause and effect of the disease. According to the literature the primary microbiological cause of DS is candidal infection. Candida spp (Pereira-Cenci et al., 2008). requires nutrients for survival, including a rich source of glucose. Therefore, a diet high in carbohydrates has been linked to increase candidal

growth and survival (Brown et al., 2014). Thus, in patients plagued by oral candidiasis a decreased intake of food high in sugars is recommended. Furthermore, diet can also be affected by DS in that those with pre-existing disease and sore inflamed mouths may have difficulty eating. If this continues without treatment, the patients' nutritional intake could be compromised which could have more serious consequences on their general health and wellbeing.

1.2.4.4 Smoking

Many factors, as previously mentioned, can contribute towards the development of DS, and whether smoking can be included as one of these factors has been considered for many years. Several studies have shown that *Candida* oral carriage rate is significantly elevated in smokers when compared to non-smokers (Abu-Elteen and Abu-Alteen, 1998, Willis et al., 1999, Crockett et al., 1992). The mechanism by which smoking increases *Candida* growth is not fully understood, however a proposed mechanism is that nicotine triggers the liver into releasing glycogen into the blood stream. This then elevates the blood sugar level, providing a source of nutrients for *Candida*, however, the blood sugar level is now too high so the liver releases insulin to bring it back down. This causes blood sugar to drop, which causes cravings for another cigarette, thus creating a vicious cycle (Soysa and Ellepola, 2005). Inhibition of the immune response in the oral cavity as one study demonstrated that chronic exposure of rats to nicotine inhibits the antibody-forming response and this immunosuppression is due to impaired antigen mediated T cell signalling (Lee et al., 2012). Furthermore, nicotine is said to have a vasoconstrictve effect on blood vessels by stimulating the α1-adrenergic receptors was suggested as an explanation for the reduced recruitment of inflammatory cells to the oral cavity seen in smokers (Johnson et al., 1991). Therefore, specific mechanisms by which smoking affect host microbiology and immunology needs to be identified as it is a vital step toward finding therapeutic approaches required to manage oral diseases in smokers.

1.3 Denture Microbiology

The oral cavity is one of the main portals of entry into the human host, and is resident to a rich and diverse microbial flora. It is an advantageous habitat for both prokaryotes and eukaryotes, despite being bathed in saliva, an important innate defence mechanism that is abundant in antimicrobial molecules. Moreover, it is thought that up to 10^8 microbes per millilitre of saliva are present (Guo and Shi, 2013).

The microbiology of denture induced mucosal inflammation is a pivotal factor to consider in denture care management, similar to many other oral diseases of microbial influence, such as caries, gingivitis and periodontitis. The overall smooth appearance of dentures, both visually and to the touch, can be misleading as on closer inspection microscopically the denture may contain tiny cracks and fissures, which provide excellent habitats for microorganisms. Dentures support the growth of microbial biofilms, structures also known as denture plaque. These biofilm communities dominate the denture surface, with up to 10^{11} microbes per milligram of denture plaque (Nikawa et al., 1998), benefiting from the varied topography associated with denture acrylics and resins (Ramage et al., 2004a). Complex polymicrobial consortia of bacteria and yeasts reside within the cracks, crevices and fissures, and within surface pores, which give the appearance of a lunar landscape at high magnification. The nature of the surface of the denture provide optimal conditions for oral biofilm formation given that the acrylic surface is non-shedding and the reduced salivary flow, which means less exposure to immune factors (Barbeau et al., 2003). The acrylic surface of PMMA allows the salivary pellicle to form readily on the surface of the denture. The salivary pellicle coats the oral surfaces, dentures included, with a layer of proteins, carbohydrates and lipids to which the primary colonisers, *Streptococci*, *Actinomyces* etc. can adhere (Cavalcanti et al., 2016). The pellicle components on dentures vary per individual, yet the general level of immune factors such as IgG, IgA, lysozyme etc, bound were reduced when compared to the oral mucosa, thus, it is suggested that this may influence the microbial colonisation of the denture base (Gocke et al., 2002). Thus, this architecturally heterogeneous panorama provides structural stability and an environment protected from key innate immune defences, as well as chemotherapeutic agents and mechanical disruption methods (Verran et al., 2014).

1.3.1 *Candida*

The prevalence of invasive fungal infections has risen significantly worldwide, and although over 600 fungal species are reported as human pathogens, *Candida* species are arguably the most frequently isolated and are the most important fungal cause of morbidity and mortality in humans. In fact, *Candida* species are considered the fourth most common cause of hospital acquired bloodstream infections in the US (Lass-Florl, 2009). *Candida albicans* is generally attributed as being the main causative agent of DS, and this form of oral candidiasis affects approximately 30-70% of denture wearers. In most instances *C. albicans* exists as a commensal in the oral cavity; however, if the host defences become compromised and if the conditions within the oral cavity become favourable for the growth and adhesion of the yeast, then the fungus can become pathogenic.

Microbiologically, DS is a disease primarily considered to be of yeast aetiology, with the literature disproportionately focussed on *C. albicans* (Bagg et al., 2003, Coleman et al., 1997, Li et al., 2007, Redding et al., 2004). It is able to exist as a commensal in the oral cavity of 25-50% of the healthy population but can become pathogenic under optimal conditions (Dagistan et al., 2009a). Two morphological forms of *Candida* exist, yeast and hyphal. *Candida* species take on the circular yeast morphology, however, hyphae are formed as a germ-tube projections from the yeast cell, forming branches which are divided by septa into separate fungal units (Fig.1.3). The hyphal form is referred to as the more invasive form of the fungus, with an enhanced ability to adhere to and colonise the prosthesis surface and is frequently isolated in DS sufferers (Gendreau and Loewy, 2011).

A **B**

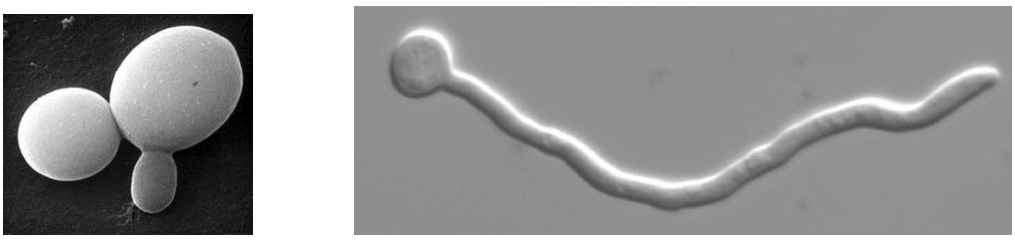


Figure 1.3: The different morphological forms of *C. albicans*.
C. albicans yeast morphology **(A),** *C. albicans* hyphal morphology **(B).**

1.3.1.1 *Candida* virulence factors

The success of *Candida* species, and in particular *C. albicans*, as a human pathogenic yeast can almost solely be attributed to their extensive arsenal of virulence factors. These include phenotypic plasticity, the ability to adhere to host or biomaterial surfaces, biofilm formation and subsequent dispersion of yeast cells from these structures (Mayer et al., 2013). Collectively, these make up the principal pathogenic mechanism assisting in the survival of these pathogenic yeasts.

1.3.1.1.1 Adhesion

Before becoming symptomatic to an infection *Candida* species must first adhere to and colonise host cells or an abiotic substrate. The adhesion proteins of *C. albicans* have been intensely studied and the agglutinin-like sequence (ALS) proteins have been identified as the central players (Hoyer et al., 2008). There are eight known ALS proteins (ALS 1-7 and ALS9) and of these ALS3 has been identified as the most significant due to its vast up-regulation during infection and ability to bind cadherins on host cells and induce endocytosis of the pathogen (Murciano et al., 2012, Phan et al., 2007). Hyphal-associated GPI-linked protein (Hwp1) has been identified as another key candidal adhesion protein. Knock out (KO) mouse model studies have shown reduced infection in models of systemic candidiasis with these proteins (Sundstrom et al., 2002, Phan et al., 2007). *Candida glabrata,* has a lower adherence capacity to gingival cells when compared to *C. albicans* and *Candida tropicalis*, however it adheres to dentures at a two-fold greater rate than *C. albicans*; indicating that it has a stronger affinity for binding to prosthetics materials. (Li et al., 2007). A caveat to this finding would be that the yeast morphology of *C. glabrata* would be associated with greater viable cell numbers due to the homogenous nature of the yeast growth, whereas intertwined hyphal elements that are more commonly associated with *C. albicans* may be quantitatively greater, yet conventional microbial culture leads to a profound underestimation.

1.3.1.1.2 Hyphal formation

Pressure generated by the hyphae is thought to be the reason for its effective infiltration of epithelial cells (Gow et al., 2002). The mechanics behind hyphal tip pressure remain little understood (Brand, 2012). It has been proposed *Candida's* penetrative nature is intrinsic and that they have been programmed to infiltrate any surface they contact. Hyphae have been shown to penetrate into silicone material despite a lack of any biological interaction with the surface (Leonhard et al., 2010). Directional hyphal growth, known as thigmotropism, can occur as a result of contact sensing with the cell surface and can lead the hyphae to weakened areas of the cell wall (Hube and Naglik, 2001, Gow et al., 1994). Characteristic thigmotropic behaviour has been demonstrated in *C. albicans* and *Candida dubliniensis* (Watts et al., 1998, Chen et al., 2011).

1.3.1.1.3 Biofilm formation

The attachment of fungal cells is closely followed by cell division, proliferation and the development of a biofilm (Fig. 1.4) (Kumamoto, 2002). The capacity of some *Candida* species to form biofilms is classed as a virulence factor; a biofilm is characterised as a structured microbial community attached to both a surface and one another surrounded by a protective extracellular matrix (ECM) (Costerton et al., 1995), and it is now believed that the majority of microorganisms utilise this form of growth. The encased structure of the mature biofilm provides protection by preventing the penetration of host immune factors and antifungals into the ECM when compared to planktonic cells (Ramage et al., 2009). *C. albicans, Candida parapsilosis and Candida tropicalis* all have the ability to form biofilms and have been associated with higher levels of morbidity than that of non-biofilm forming *Candida* species (Kumamoto, 2002).

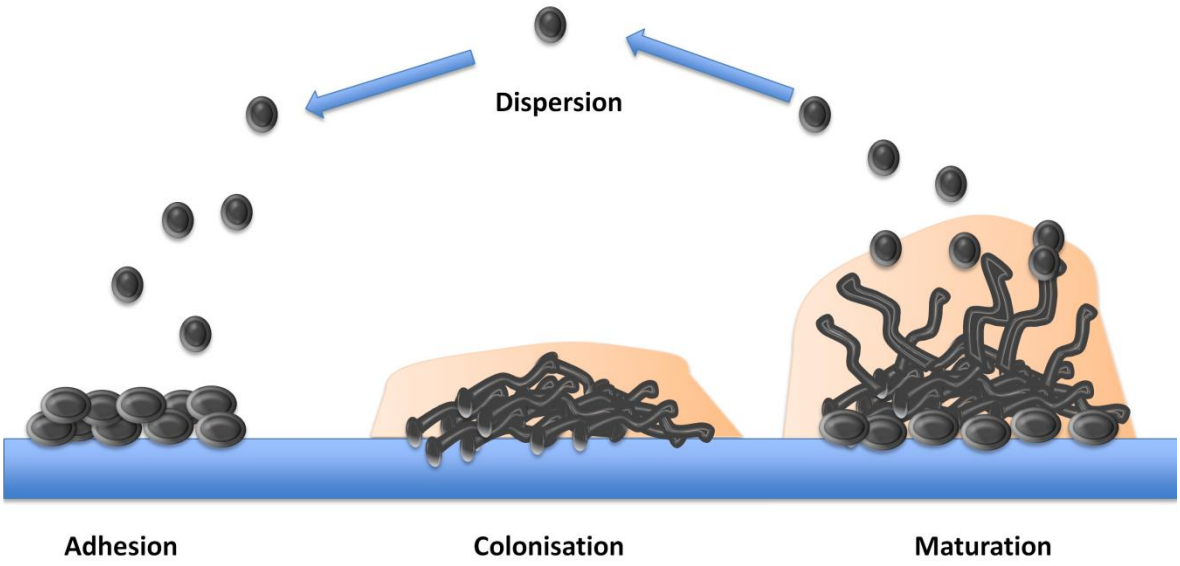


Figure 1.4: Developmental stages of *C. albicans* biofilm formation.
The attachment of fungal cells is closely followed by cell division and proliferation, thus establishing colonisation. The production of hyphal growth and ECM leads to mature stable biofilm architecture. The mature biofilm then disperses yeast cells, subsequently leading to the formation of new colonies and further biofilm development.

Hyphal production is a requisite for the formation of stable 3-D architecture within mature biofilms. The biofilm structure is stabilised by filamentous growth, however, the matrix is not impenetrable as there are water channels that deliver essential nutrients to the cells, and the cells can also escape the matrix and seed to other areas initiating the spread of infection (Douglas, 2003). The ECM composition of *C. albicans* biofilms consists of carbohydrates, mainly ß-1,3 glucan, proteins, phosphorus and hexoamines. Positive regulators of ß-1,3 glucan such as glucoamylases (Gca1 and Gca2), glucan transferases (Bgl2 and Phr1) and exo-glucanase Xog1, seem to play an integral role in protecting the fungal cells as the biofilm becomes more susceptible to antifungals when they are absent (Taff et al., 2012).

Once a mature biofilm is fully established yeast cells can then disseminate out to other areas subsequently leading to the spread of infection. Therefore, the formation of a biofilm is a fundamental mechanism exerted by *Candida* species, which aids their success as a pathogen by providing a protective niche for these fungi to grow, proliferate and subsequently disperse whilst defending against potentially devastating assaults from the immune system. Furthermore, increasing evidence indicates that *Candida* does not act alone when colonising dentures and

infecting host mucosa as bacteria have been found to be incorporated into the denture biofilms of DS sufferers (Tournu and Van Dijck, 2012).

1.3.1.2 Mixed *Candida* infection

C. glabrata, despite being unable to form hyphae or form successful biofilms, remains the second most commonly isolated *Candida* species in most reported cases of candidiasis (Li et al., 2007). Moreover, it is hypothesised that *Candida spp.*, in particular *C. glabrata* benefit from *C. albicans*. There have been suggestions that DS pathology may be promoted by the synergistic interaction between these species within denture biofilms. Coco and colleagues (2008) first reported that *C. glabrata* and *C. albicans* were often co-isolated from patients, particularly those with severe inflammation (Coco et al., 2008a). *C. glabrata*, unable to form hyphae, forms structurally poor and unstable biofilms, yet is linked with disease. Consequently, it was hypothesised that C. *glabrata* uses *C. albicans* as a structural scaffold to gain entry into the host. Further studies have confirmed this, where *C. albicans* appeared to assist the invasive capacity of *C. glabrata* within an *in vitro* reconstituted epithelial biofilm model (Silva et al., 2011). The mechanism of this interaction remains unknown. This group has shown similar data with work in a reconstituted human vaginal epithelial model, where *C. glabrata* on its own caused minimal tissue damage, yet there was a significant increase in *C. glabrata* colonisation and invasiveness in combination with *C. albicans* (Alves et al., 2014). Thus, further studies using *in vivo* models are required to investigate the pathogenesis of DS where *C. albicans* and *C. glabrata* are co-isolated.

1.3.2 Bacterial denture plaque

It could be inferred from a rudimentary scan of the literature that there is a candidal bias in DS, with little else of microbial importance in the context of dentures. Though in both healthy and diseased individuals it is apparent that denture plaque is more diverse than assumed. The denture surface capable of carrying up to 10^{11} microbes per milligram (Nikawa et al., 1998). The majority of studies investigating the bacteria colonising dentures typically use culture based methods and therefore only study a limited number of culturable bacteria (Sumi et al., 2003, Ribeiro et al., 2012, Baena-Monroy et al., 2005, Theilade and Budtz-Jorgensen, 1988). *Streptococcus* species are amongst the most predominant

microflora detected on dentures, with species including *Streptococcus oralis, Streptococcus salivarius, Streptococcus sanguinus, Streptococcus mutans* and *Streptococcus mitis* (Coulthwaite and Verran, 2007). *Staphylococcus* spp. are also commonly isolated from dentures using selective media, most frequently *Staphylococcus aureus* (Ribeiro et al., 2012, Sumi et al., 2003). However, with the advent of molecular technologies, recent studies have utilised methods such as polymerase chain reaction (PCR) and DNA-DNA hybridization to gain a more detailed insight into the microbial composition of denture plaque. Using PCR, Campos et al (2008) were able to identify bacterial phylotypes found on healthy dentures and how they differed to those identified on those with DS (Campos et al., 2008). Healthy dentures were predominantly represented by bacteria within the *Streptococcus* and *Veillonella* genera, whereas phylotypes unique to DS were mainly of *Streptococcus*, *Atopobium* and *Prevotella* genera. Moreover, DNA-DNA hybridization of denture plaque revealed the presence of *Actinomyces* spp., *Porphyromonas gingivalis*, *Veillonella parvula, Neisseria mucosa* and *Eikenella corrodens* (Teles et al., 2012, Sachdeo et al., 2008). Of note, numerous studies have identified the presence of periodontal pathogens on dentures, or in the oral cavity of denture wearers, including, *P. gingivalis*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans*, which were previously thought to disappear from the oral cavity with the complete removal of natural teeth (Fernandes et al., 2010, Yasui et al., 2012).

Studies have shown that there is indeed a diverse denture microbiome consisting of various orally important bacterial species, many of which have been shown to co-aggregate with *Candida* species. Some of these bacteria include putative periodontal pathogens, including *F. nucleatum, A. actinomycetemcomitans* and *P. gingivalis* (Sachdeo et al., 2008, Yasui et al., 2012), though caries-associated species such as *Streptococcus* and *Lactobacillus* species appear to be more prominent (Teles et al., 2012), possibly through their ability to coaggregate with *C. albicans* hyphae (Bilhan et al., 2009, Ribeiro et al., 2012). Indeed, it has been reported that polymicrobial interactions between *Candida* and bacteria may lead to synergism and increased DS pathogenesis (Stacy et al., 2014). Indeed, oral microbiologists have devoted significant time and effort to distinguishing the importance of specific bacterial-bacterial interactions, while investigations into polymicrobial interactions have not received the same level of attention or rigour.

This has led to a gap in the literature regarding candidal-bacterial interactions within the oral environment. Some of these key interactions will now be discussed.

1.3.3 Polymicrobial interkingdom interactions

The vast majority of the studies looking at fungal-bacterial interactions only look at a few species *in vitro* in oversimplified conditions. This highlights the importance of contemporary sequencing techniques to gauge a comprehensive understanding of the complete oral microbiome in order to fully understand fungal-bacterial relationships (Kraneveld et al., 2012, Shirtliff et al., 2009). The importance of *Candida* within these complex communities is now slowly being discovered as a result of advances in genome sequencing (Nobbs and Jenkinson, 2015). For example, microbiome analysis of the saliva from elderly Dutch patients showed that an increased candidal load led to dysbiosis in bacterial flora, excluding pathogenic anaerobic bacteria and favouring co-existence with oral streptococci (Kraneveld et al., 2012). *Candida* species have been isolated from various sites within the oral cavity which include both soft and hard tissues of biological and non-biological origin including, periodontal pockets, root canals, orthodontic appliances, enamel, dentures and mucosal surfaces (Arslan et al., 2008, de Carvalho et al., 2006, Dongari-Bagtzoglou et al., 2009, Freitas et al., 2014, Ramage et al., 2004b, Sardi et al., 2010). A moisture rich environment, sufficient nutrients, hyphal growth and the presence of commensal bacteria, are all involved in influencing successful *C. albicans* biofilm architecture and virulence (Bertolini et al., 2015).

1.3.3.1 Candida and *Streptococcus*

A large proportion of the primary colonisers of the oral cavity are comprised of streptococcal species (Moore et al., 1982, Syed and Loesche, 1978). Oral streptococci are classed as mitis group streptococci (MGS), which include *S. mitis, S. oralis, S. gordonii, S. sanguinis and S. parasanguinis* species (Kawamura et al., 1995). MGS streptococci are conventionally associated with the early colonisation of dental surfaces, comprising between 60-80% of the flora (Diaz et al., 2012a). Moreover, studies using high throughput gene sequencing technology has revealed

them to also be principal colonisers of oral mucosal surfaces (Diaz et al., 2012a). Recent metagenomic studies looking at the complete oral microbiome found that as *Candida* load increased the bacterial genus bacilli increased, with streptococci contributing to 34% of this group. Increasing *Candida* load has been shown to decrease the pH, this correlates with streptococci spp. due to it being extremely acid-tolerant (Kraneveld et al., 2012).

The relationship between streptococci and *Candida* is synergistic in nature, as advanced microscopy has shown streptococci interacting with *Candida* in both yeast and hyphal forms (Fig. 1.5) (Dutton et al., 2014). *C. albicans* has evolved a mechanism allowing it to bind directly to MGS species, including *S. oralis, S. mitis* and *S. gordonii* (Jenkinson et al., 1990). Interactions via *C. albicans* hyphal cell wall protein Als3, and the streptococcal cell surface adhesins SspA and SspB (Holmes et al., 1996), proteins that belong to the antigen I/II polypeptide family are involved in adherence between these two species (Bamford et al., 2009). Direct binding of SspB and Als3 is required for bacterial-fungal adherence.

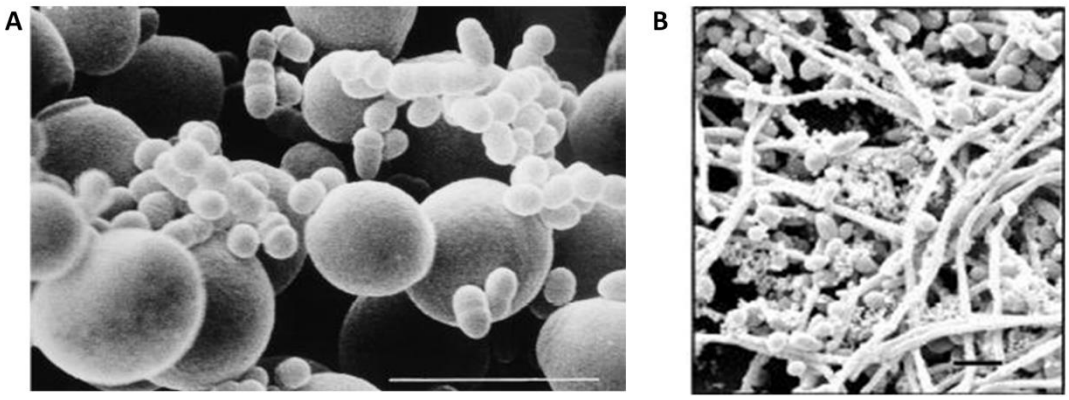


Figure 1.5: *Candida* and *Streptococcus* species interactions.
Streptococcus species are able to interact with *Candida* species in both the yeast morphology **(A)** and hyphal morphology **(B) (Jenkinson and Douglas, 2002).**

C. albicans can support the survival of streptococci by reducing oxygen tension levels to that more suitable for streptococcal growth, as well as providing nutrients required for bacterial survival (Douglas, 2002). This interaction is bidirectional, as streptococci supply *Candida* with nutrients from the salivary pellicle, such as glucose and lactate, which the yeast exploits as a source of carbon (Holmes et al., 2006). Moreover, streptococci are aciduric bacteria and therefore

create an acidic environment via the fermentation of carbohydrates (Takahashi and Nyvad, 2011). Yet, *Candida* can grow and survive at a lower pH (<4.5) when co-colonised with streptoccoci, and the H_2O_2 produced by streptococci can induce oxidative stress, which encourages hyphal growth (Jenkinson et al., 1990, Nasution et al., 2008). Though, there are instances when this interaction can be detrimental to the host. Studies have shown that streptococci augment the persistence of *Candida* spp. Xu and colleagues (2014) established that co-infection with *C. albicans* and *S. oralis* led to a more severe inflammatory response when compared with the monospecies infection, which is due to an exaggerated up-regulation of TLR2 dependant inflammatory genes (Xu et al., 2014, Dutton et al., 2014).

A significant component of a biofilm is the ECM, which provides protection against antimicrobials (Xu et al., 2014). The ECM of *Candida* biofilms is composed of ß-glucans (Gregoire et al., 2011), whereas the streptococcal biofilm ECM is chiefly comprised of α -glucans (Al-Fattani and Douglas, 2006, Taff et al., 2012). *S. mutans* utilises its ECM components by depositing α-glucans on the surface of hyphae to enhance adhesion to fungal cells (Gregoire et al., 2011). Furthermore, interaction between *S. mutans* and *C. albicans* is promoted by glucosyltransferase-derived ECM and expression of the *S. mutans* virulence gene gtfB (Falsetta et al., 2014). This study further demonstrated that *Candida*-derived ß1,3-glucans contribute towards the ECM structure, at the same time fungal ß-glucan and mannan present sites for gtfB binding. Additionally, ß-glucans are found on the surface of hyphae as well as inside the matrix (Dongari-Bagtzoglou et al., 2009), therefore indicating that streptococci use these proteins to adhere to hyphal filaments. Together, this suggests the biofilm ECM may affect this synergistic behavior, favoring their co-existence in the oral environment to the detriment of the host.

Quorum sensing (QS) plays a significant role in the association between *Candida* and streptococci. The key QS molecule associated with *C. albicans* is farnesol, a tetraprenoid alcohol and a key intermediate in the sterol biosynthetic pathway in eukaryotic cells, its primary role is involved in the repression of hyphal growth and biofilm formation (Ramage et al., 2002). Yet, a study by Bamford et al (2009) has showed that *S. gordonii* can suppress farnesol induced inhibition of biofilm formation, via autoinducer 2 (AI-2). AI-2 is associated with the *luxS* gene, and

mutants of this gene were less effective at permitting hyphal formation, however, the mode of action has yet to be elucidated (Bamford et al., 2009). Farnesol also inhibits *S. mutans* biofilm formation and polysaccharide production (Koo et al., 2003). From this and other studies it has been suggested that it may be used to control its competitiveness in mixed species biofilms and could be targeted as a chemotherapeutic strategy (Jeon et al., 2011). AI-2 is the chief QS molecule released by bacteria that allows inter-species communication (Vendeville et al., 2005). *luxS* streptococcal mutants are able to form monospecies biofilms, however, when co-colonised with *C. albicans,* biofilm formation becomes abrogated, suggesting this molecule is plays a role in cellular communication (McNab et al., 2003, Bamford et al., 2009).

1.3.3.2 Candida and *Lactobacillus*

The general consensus is that lactobacilli antagonise candidal species colonisation and growth, and is one of the main reasons they are included in probiotics (Young et al., 1956). Probiotics have proven to reduce candidal levels at various sites, including the oral cavity, bloodstream and urinary tract (Kumar et al., 2013, Mendonca et al., 2012). Initial studies suggested that lactobacilli decreased *C. albicans* levels via provision of nutrients for lactobacilli resulting in lactic acid production, thus hampering candidal growth through pH dependant inhibition. Evidence indicates that there is a close association between the both organisms, but to date the majority of data has come from studies into vaginal infection. The role played by lactobacilli in maintaining homeostasis at the vaginal mucosa originally came to light due to the occurrence of vaginal candidiasis during treatment with systemic antibiotics. The mechanistics behind *Lactobacillus* induced inhibition of *Candida* spp. growth and virulence are not yet fully understood, but perhaps the production of hydrogen peroxide contributes as it has been associated with anti-candidal activity, albeit in some strains of lactobacilli (Strus et al., 2005). Furthermore, one of the key virulence factors associated with Lactobacillus is its ability to produce antimicrobial proteins known as bacteriocins (Yang et al., 2014). Bateriocins have broad spectrum activity which can kill other closely related or non-related microbes but not the organism itself and are generally harmless to the human body. They are increasingly being used as a food preservative due to their antimicrobial effect against many food spoilage and pathogenic bacteria (Garsa et al., 2014). Their mechanism of action includes

binding to the main transporter of peptidoglycan subunits and preventing correct cell wall synthesis, as well as membrane insertion which leads to pore formation and ultimately cell death (Cotter et al., 2005). Therefore, it is possible that the release of bacteriocins by *lactobacilli* may be responsible for the antagonistic effect on *Candida* spp, however to date bacteriocins have not been directly linked to candidal inhibition. Thus, other interactions also contribute towards disease development, such as modulating the host response, in that lactobacilli cells have been shown induce the production of inflammatory cytokines when co-cultured with *C. albicans* (Martinez et al., 2009), potentially explaining the clearance of candidal infection.

Yet, in spite of the overwhelming evidence that their relationship is antagonistic, there is some studies indicate that they may mutually benefit one another. Modulating the environmental pH to that of a more acidic environment is a common trait of lactobacilli as a result of producing lactic acid (Messaoudi et al., 2013). This is generally considered a pathogenic mechanism due to the inability of other bacteria to tolerate a low pH. However, a low pH induces a stress response in *Candida*, leading to hyphal formation in certain species of oral *Lactobacillus*, specifically *L. casei*, which has been shown to stimulate *C. albicans* hyphal growth (Orsi et al., 2014). Furthermore, *Candida* hyphae can co-aggregate and maintain lactobacilli levels in patients with more severe of oral disease (Bilhan et al., 2009). Thus, the induction of hyphae may be beneficial to lactobacilli as it can provide a stable scaffold to which the lactobacilli can adhere. Nonetheless, further investigation is essential to understand interactions in detail to establish the true extent of the dynamic relationship; given that the conceived antagonism may be specific to *C. albicans*. For example, Jiang et al (2015) demonstrated that only one of six probiotic *Lactobacillus* species had an inhibitory effect on *C. glabrata* growth (Jiang et al., 2015). This suggests that the interactions between *Candida* and lactobacilli may be dependent on the particular species they interact with.

1.3.3.3 Anaerobic Gram-negative interactions

The environment of subgingival plaque is highly anaerobic, favouring many obligate periodontal disease (PD) pathogens such as *P. gingivalis, F. nucleatum* and *Prevotella intermedia*. However, given the indeterminate association

between *Candida spp* and PD, then this remains a reasonably ignored area of research. Studies investigating interactions between *C. albicans* and *P. gingivalis* have produced contradictory results. *P. gingivalis* was shown to suppress *Candida* biofilm formation by reducing the number of viable yeast cells coincidental with an increasing *P. gingivalis* concentration (Thein et al., 2006). On the other hand, it was demonstrated that *P. gingivalis* induces germ-tube formation in *C. albicans,* leading to a more invasive phenotype, thus increasing the risk of infection (Nair et al., 2001). Additionally, both microbes appear to demonstrate a mutually antagonistic effect on one another in relation to host cell adhesion, as *P. gingivalis* blocked the adhesion of *C. albicans* to buccal epithelial cells (Nair and Samaranayake, 1996), whilst the presence of *C. albicans* inhibited adhesion to gingival epithelial cells or gingival fibroblasts by *P. gingivalis* (Tamai et al., 2011). However, the same study also showed that pre-exposure of gingival epithelial cells and fibroblasts to *C. albicans* enhanced cell invasion by *P. gingivalis*. What is clear is that further studies are necessary to understand the interactions between these two microorganisms.

Co-aggregation studies have shown that *F. nucleatum* has the ability to adhere to *C. albicans* species (Grimaudo and Nesbitt, 1997), as well as *C. dubliniensis* (Jabra-Rizk et al., 1999). Although, the co-aggregation with *C. albicans* may be temperature dependant as when *C. albicans* was grown at 37°C, it did not adhere to *F. nucleatum*, yet the two species did co-aggregate when grown at 25°C and 45°C (Jabra-Rizk et al., 1999). The mechanisms responsible for these interactions are unknown, however these observations suggest *C. albicans and F. nucleatum* interactions may play an important role in oral colonisation by yeasts.

Thus, understanding the composition of denture biology is key to beginning to understand the complex microbial interactions. These interactions, whether synergistic or antagonistic in nature can result in enhanced pathogenicity, which can lead to local infection, yet on a more serious note, may contribute towards the development of a systemic infection.

1.4 Systemic implications of wearing dentures

In recent years the systemic effect of oral bacteria has become increasingly apparent, as it is now implicated in numerous systemic diseases including rheumatoid arthritis, cardiovascular disease, kidney disease and respiratory infection (Pizzo et al., 2010, Farquharson et al., 2012). However, the majority of this evidence to date has primarily focused on the link between PD and systemic infections. Whereas, given that the composition of denture plaque is considerably understudied, there is little evidence on the systemic impact of denture plaque.

1.4.1 Respiratory infection

The 2010 Global Burden of Disease Study reported that lower respiratory tract infections; including pneumonia are the fourth leading cause of death worldwide (Lozano et al., 2012). Approximately 90% of deaths caused by pneumonia occur in those aged 65 and over, with one in 20 people over the age of 85 presenting with a new case of pneumonia every year, and with the increasingly ageing population more cases are likely to present (Torres et al., 2013). Poor oral hygiene has been connected to respiratory infection with common respiratory pathogens being identified within dental plaque, convincingly linking this with the development of pneumonia in intensive care patients and the dependant elderly (Sumi et al., 2007, Russell et al., 1999).

Aspiration pneumonia (AP) is a clinical phenotype associated with the aspiration of oropharyngeal secretions into the lower respiratory tract (Raghavendran et al., 2011). With advances in medicine people are living longer and thus an increasing number of these individuals are at risk of developing AP. As the majority of AP cases occur in individuals >65 years old, it means a large proportion of these people will have dentures (Falcone et al., 2012, Centre, 2011). While the constituents of dental plaque have been well established, the composition of denture plaque has been understudied and thus the extent to which respiratory pathogens may colonise the prosthesis is not yet fully understood. The few studies which have investigated the presence of respiratory pathogens within the oral cavity have prominently relied on standard microbial culturing methods for identification of the microorganisms (Sumi et al., 2007, Sumi et al., 2002). This

therefore calls for the opportunity to make use of advanced molecular techniques to give more accurate insight into the potential systemic consequences.

1.4.1.1 Categories of Pneumonia

Pneumonia is classified into either community acquired pneumonia (CAP) or hospital acquired pneumonia (HAP). Other than the place of infection, these two categories differ as the microorganisms involved in each case are distinct (Falcone et al., 2012).

During the past decade hospitalisation rates due to pneumonia has increased by 34% (Trotter et al., 2008). Currently CAP is the leading cause of death due to infection within Europe, and while the reported incidence of CAP varies from country to country, they all conclusively report an increased progression rate with increasing age (Torres et al., 2013). HAP is defined as occurring more than 48 hours after admission to the hospital and can be divided into ventilator associated pneumonia (VAP) and non-ventilator associated pneumonia (Niederman, 2010). VAP develops 48 hours or more after intubation and has a mortality rate between 17-50% and the cost of treating ventilated patients with pneumonia is significantly higher than those without (Lahoorpour et al., 2013).

It is estimated within the next 30 years, 40% of adults will spend some time in a long term care facility (LTCF) before dying (El-Solh, 2011b). Institutionalised elders are a group at great risk of developing CAP, currently the incidence of pneumonia in nursing homes or LTCF's is 0.3-2.3 occurrences every 1,000 resident care days (Muder, 1998). These individuals often have co-morbidities such as COPD, cardiovascular disease, diabetes and neurological disorders such as dementia, all of which increase their risk of developing pneumonia (Falcone et al., 2012). Thus this highlights the importance of gaining an improved understanding of the pathophysiology of this disease for prevention purposes which will markedly improve health care costs and clinical outcomes.

1.4.1.2 Microbiology of pneumonia

The microorganisms most commonly associated with CAP are *Streptococcus pneumoniae, Haemophilus influenzae, Mycoplasma pneumoniae, Chlamydia*

pneumoniae, Moraxella catarrhalis, and Legionella spp. However, multi-drug resistant bacteria such as *Pseudomonas aeruginosa*, *Enterobacteriaceae spp.* or *methicillin-resistant S. aureus* (MRSA) are more frequently responsible for HAP (Falcone et al., 2012). Although the prevalence rates of these organisms vary amongst studies of elderly individuals with aspiration pneumonia, gram-negative bacilli are the predominant organisms (49 %), followed by anaerobic bacteria (16 %) and *S. aureus* (12 %) (Mandell, 2009, Falcone et al., 2009, El-Solh et al., 2003). *S. pneumoniae* is the primary aetiological pathogen associated with CAP, with approximately 92 strains existing, all varying in their carriage rates, prevalence and pathogenicity (Blasi et al., 2012). The most commonly encountered strains are 3 (16.9%), 19 (10.7%) and 14 (7.5%). Strain 2 frequently causes invasive disease, Strains 3, 4, 6A, 6B, 7, 9N, 9V, 11, 12, 14, 15A, 15F, 16, 18C, 22, 23A, 23B, 31, 33 and 35 are associated with fatality (Blasi et al., 2012).

PD is suggested to cause increased levels of oral pathogens in the saliva which are subsequently aspirated into the lungs. In cases which involved anaerobic pulmonary infection, there was a significantly elevated level of periodontal pathogens, such as *P. gingivalis*, isolated from these patients when compared with age-matched controls, thus supporting a role between periodontal infection and anaerobic pulmonary infection (Paju and Scannapieco, 2007). Furthermore, although clinically infections caused by *Fusobacteria* are rare, these bacteria are associated with simple diseases such as aspiration pneumonia to more severe infections such as necrotizing pneumonia (Brook, 2004). *Fusobacteria nucleatum* is associated with causing the majority of these infections (Nohrstrom et al., 2011). *F. nucleatum* is normally found as a commensal of the oropharyngeal, gastrointestinal and respiratory tract, yet has been shown to become pathogenic in those that frequently aspirate oropharyngeal contents (Bartlett, 2012). Aspiration of oropharyngeal contents is increased in those with impaired cough reflexes, difficulty swallowing and those that sleep whilst wearing their dentures overnight.

1.4.1.3 Dentures and pneumonia

As over 70% of individuals >75 years old either wear a partial or complete denture, and as men are now expected to live to the age of 78 and women to 82, there are

a significant number of denture wearers in the population who could be at risk of AP (Centre, 2011, Kyte and Gordon, 2009).

Edentulous individuals are unlikely to suffer from pneumonia caused by periodontal pathogens; nonetheless these bacteria can persist in the mouths of edentulous subjects for extended periods of time after the extraction of all teeth (Fernandes et al., 2010, Danser et al., 1995). Adequate oral and denture care procedures within nursing homes are rare, mainly due to the individual's inability to perform sufficient oral care themselves, a lack of personnel designated to oral hygiene care, inadequate staff training and patient noncompliance (El-Solh, 2011a). Nonetheless, several studies conducted into the effectiveness of oral and denture care in nursing home patients saw a reduction of respiratory infections in patients that began intense oral hygiene regimens. Yoneyama et al (2002) conducted a study with 417 patients in nursing homes; patients were selected randomly for the control or intervention groups (Yoneyama et al., 2002). The intervention group had their mouths cleaned after every meal and dental hygienists provided professional health care once a week, the control group were responsible for their own oral health care. Both groups had their dentures cleaned once a day by their care providers. The results found that the control group had significantly higher incidences of pneumonia than the intervention group. Similar studies also found that when professional oral care was provided the risk of AP was significantly lowered. Studies were carried out looking at patients with dysphagia and impaired cough reflex sensitivity by Yoshino et al (2006) and Watando et al (2004), respectively. Both studies saw a significant improvement in cough reflexes and swallowing reflexes in the intervention groups, reducing the risk of AP (Yoshida et al., 2006, Watando et al., 2004). Therefore, this evidence highlights the need to implement an oral hygiene programme within nursing homes and LTCF's. A programme of oral health care consisting of tooth brushing after each meal, cleaning dentures once a day, and professional oral health care once a week, seems the best intervention to reduce the incidence of AP, and subsequently reducing the burden of the health care system.

As with all diseases of microbial origin, particularly those of the mucosa where a diverse microbial habitat exists, this leads to difficulties in determining the causative organism. Currently, no complete assessment using NGS has been

performed for dentures and the associated mucosa, thus understanding the interkingdom interactions and systemic risk factors involved cannot take place without first knowing what is there in the first place. Therefore, improved techniques to dissect and characterise the microbial ecology are welcomed, such as the advent of NGS.

1.5 Next generation sequencing

1.5.1 Introduction

Dr Fredrick Sanger began research into DNA sequencing in the early 1970's after successfully publishing a methods describing RNA sequencing (Sanger et al., 1965, Barrell and Sanger, 1969, Brownlee et al., 1967). In 1977 Sanger published a paper demonstrating a successful method for sequencing DNA, now commonly known as the Sanger method (Sanger et al., 1977). The method involved a complex process by which DNA incorporated nucleotides with a slight modification, the addition of a 3' hydroxyl group which blocks the addition of further nucleotides. Mixtures of DNA polymerase and the four unaltered nucleotides were mixed with a modified nucleotide either A, C, G or T. The fragments produced from these reactions were separated by size on polyacrylamide gels, the reactions containing the modified A, C G and T were run on separate adjacent lanes. The gels were dried and exposed to x-ray films and the fragments were then visually separated based from top to bottom (shortest to longest fragments) to generate the DNA sequence, thus was laborious and an extremely time consuming process (Sanger et al., 1977, Mardis, 2013). However, various improvements were introduced since its invention including the introduction of DNA synthesis chemistry, the advent of oligonucleotide primers and the use of longer and thinner polyacrylamide gels (Mardis, 2013). Nonetheless, despite these improvements none were sufficient to make this method scalable for high throughput sequencing needs.

Throughout the years the process of DNA sequencing has become more sophisticated and advanced, and with that came the advent of NGS (Mardis, 2008). NGS allows the sequencing of large amounts of DNA rapidly and at substantially lower costs than previous methods, as previously it took over a decade to sequence the entire human genome, yet now with NGS technology it takes hours (Mardis, 2013). These NGS platforms are opening doors for research allowing for the

investigation of genomes, microbiomes, ecological diversity and identification of unknown aetiological agents. Today there are several NGS platforms in use, the more predominantly used is the 454 sequencing platform and the Illumina sequencing platform (Li et al., 2014).

1.5.2 Next generation sequencing methods

1.5.2.1 454 sequencing

The 454 platform was the first next generation sequencer to be introduced commercially in 2004 using a method known as pyrosequencing. This method generates DNA fragments from the original DNA strand. These DNA fragments are used to generate fragment libraries to which adapters (short DNA sequences) are added to the DNA blunt ends. The library fragments are then mixed with agarose beads with bound oligonucleotides sequences which are complimentary to the 454-specific adapter sequences, thus each bead is associated with a single fragment. Beads are isolated into individual oil:water micelles containing PCR reactants for amplification of the fragments, producing approximately one million copies of each fragment. The beads are separated into individual wells on a picotitre plate, providing a set location were each sequencing reaction can be observed. Each fragment is then sequenced en masse. Enzyme mixtures which initiate the downstream pyrosequencing reaction are then added to the wells. The picotitre plate functions as a flow cell by which each specific nucleotide solution is added in a stepwise manner. In pyrosequencing the incorporation of a nucleotide initiates the release of a pyrophosphate molecule, which leads to a series of reactions, ultimately producing light by the enzyme luciferase. The amount of light emitted is proportional to the number of nucleotides incorporated, and thus the DNA sequence is generated (Mardis, 2008).

454 sequencing was for a long time the standard method used for NGS sequencing due to its longer sequence reads and ability to assign taxonomy down to species level, and thus was the main competitor for other sequencing platforms such as Illumina (Mardis, 2013). However, in 2013 Rosche announced the withdrawal of the 454 platform due to its inability to compete with the ever evolving NGS platforms (Goodwin et al., 2016). The lengthy read length that comes with the 454 platform leads to increased insertion and deletion errors. Homopolymer

regions are problematic with this platform as read accuracy is lost when more than 6 homopolymer bp are present (Loman et al., 2012, Forgetta et al., 2013). Whilst other platforms which use a similar method such as the Ion torrent have kept up with other rapidly evolving NGS platforms, the 454 has failed to keep up with yield or cost. The platform would not be fully discontinued until 2016 and thus current literature is still being published using this method, and thus it is still important to consider studies using the 454 method for comparison.

1.5.2.2 Illumina sequencing

As with the 454 platform a fragment library must be generated before sequencing can begin. The Illumina platform uses a chip-based bridge amplification procedure followed by sequencing synthesis using reverse terminator dye nucleotides, which takes place and occurs on the surface of an 8-channel oligo-derived flow cell. Adapters are annealed to the DNA fragments before they are introduced to the flow cell, which has oligonucliotides, complimentary to the adapters, already attached. Fragments attached to the oligonucleotides on the flow cell then undergo brigde amplification using DNA polymerase to generate clusters. Each cluster represents the same fragment sequence and produces approximately one million copies of each, which provides a fluorescent signal strong enough to be detected during nucleotide incorporation. Unlike the 454 platform, Ilumina introduces all four nucleotides simultaneously, as each base has a unique fluorescent label attached. A laser is then used to activate the fluorescent labels and the colour emitted is read. Each base contains a 3'-OH group to prevent incorporation of more than one nucleotide. After each cycle the 3'-OH group is chemically removed and the process is repeated, eventually generating the DNA sequence (Mardis, 2008).

1.5.3 Sequencing the 16S gene

Research into understanding microbial communities has been revolutionised by NGS, as we are now able to gain an in depth insight into the microbiome composition of various microbial communities. The majority of microbiome studies sequence the 16S rRNA gene, however the region within the gene that is sequenced varies from study to study. Bacterial rRNA 16S genes contain nine hypervariable regions (V1-V9), which express extensive sequence diversity across

bacteria (Van de Peer et al., 1996). Sections within these regions that are known to be species specific are often targeted as it allows for identification down to the species level. None of the hypervariable regions are able to differentiate between all bacterial species; therefore this led to numerous studies all comparing different variable regions. Chakravorty et al (2007) sequenced the different variable regions to compare the advantages of each region for the specific diagnostic goals required (Chakravorty et al., 2007). There results indicated that different variable regions were better than others for differentiating between certain genera. In terms of oral microbiome studies the most popular regions sequenced include the V1-V3 and V3-V4 regions (Liu et al., 2016, Kennedy et al., 2016, Macovei et al., 2015, Yun et al., 2016, Harris et al., 2015, Johansson et al., 2016). The V1-V3 region remains popular because it has been shown to produce an overall similar OUT profile as the V3-V4 when tested on the same samples, however the V1-V3 provides a greater phylotype richness and evenness which is thought to support a more representative assessment of the community (Zheng et al., 2015b). However, Kozich et al (2013) in a recent study set out to address problems associated with the MiSeq platform and attempt to improve the quality of data produced (Kozich et al., 2013). Comparison of three different sequencing regions V34, V4 and V45 found that surprisingly the shorter V45 region had a higher error rate than the V34 region. Interestingly however, the V4 region alone had a lower error rate than both the V34 and V45. Thus, they deduced that the fraction and length of sequences retained could be organised so that it is comparable to platforms with longer sequence reads (Schloss et al., 2011). Furthermore, the shorter V4 region is approximately 250 bp in length, therefore using the Miseq platform with the 500 bp read, gives almost complete overlap of the sequences and produces a lower error rate (Kozich et al., 2013). Therefore, the sequencing region of choice is ultimately dependant on whether a lower error rate or increased species richness is desired.

1.5.3.1 Advantages and disadvantages of using the Illumina Miseq platform and alternatives

Advances in the technology of these next generation sequencers has raised questions on which is the most suitable platform to use for in depth analysis of microbial communities. Traditionally he most commonly used platform was the 454, however its inability to keep up with new advancing NGS platforms led to its

discontinuation. However, interest in other platforms such as Illumina continues to grow as the technology becomes more sophisticated and advanced (Gloor et al., 2010), yet it is essential that with these advancements comes improvement in sequencing quality as this cannot be sacrificed for an increased sequence output. Other factors which do need to be taken into account when selecting an NGS platform include cost, how many reads can you get per run and per pound, and also the length of the sequence because the longer the sequence the more accurate the assignment to a taxonomic group.

The Illumina platform is able to generate the largest amount of sequencing data at the cheapest price (Caporaso et al., 2012). Currently up to 300 and 500 nucleotide reads of sequence are obtainable from the HiSeq and MiSeq platforms respectively, which are split into two reads. The HiSeq platform has a substantially greater read depth and is more costly to run than the MiSeq, and is more commonly used for shotgun metagenomics. However, Miseq Illumina is more appropriate for 16S rRNA gene sequencing due to its longer sequence reads (Caporaso et al., 2012). Traditionally 454 was favoured over the Illumina platform because Illumina had trouble sequencing samples with low genetic diversity, however, the introduction of the phage PhiX into the DNA library, to increase the diversity corrected this problem, so with improved technology as little as 5% of the DNA library is comprised of PhiX (Kozich et al., 2013). Therefore, taking into account the shorter sequencing time, the increased read depth and considerably cheaper cost the MiSeq Illumina platform is able to cost-effectively produce high quality sequencing data and is currently considered the clear favourite NGS platform for large scale sampling.

However, the Illumina platform is not without its limitations. One of the main drawbacks of this technology is the short sequence read lengths, as the majority of samples are unable to be assigned to the species level as most stop at the genus level. However, databases including the human microbiome project and the human oral microbiome project etc, these can be used alongside sequencing data to build an understanding of the microbial environment. Furthermore, another limitation of NGS sequencing, is contamination of samples from the DNA extraction kits used before sequencing, known as the ‘kit-ome’, however this is a problem which affects all NGS platforms and not specifically the Illumina. Studies have been conducted in which blank control that have been passed through the

extraction kits were sequenced and detected microbial contamination, all be it at a low biomass (Salter et al., 2014). Contaminating genera include Proteobacteria, Actinobacteria, Firmicutes, Bacteriodetes and Acidobacteria. Therefore, kit contamination can significantly influence the results of microbiome studies, and particular care should be taken for samples with a low biomass. The inclusion of negative controls for sequencing may help distinguish contaminating microbes from the samples and help limit false conclusions.

As NGS continues to evolve, more and more sequencing platforms begin to emerge, other alternatives include the ion torrent, PacBio and Minlon platforms. The Ion torrent system, like the 454 involves sequencing by synthesis and is able to generate superior read lengths of approximately 400 bp (Goodwin et al., 2016). This platform involves works by detecting the H^+ ions released as each nucleotide is incorporated and the change in pH is detected by a sensor. The change in pH is directly proportional to the number nucleotides integrated (Loman et al., 2012). Although facing similar drawbacks to the 454 such as insertion and deletion errors, the ion torrent has been able to keep pace with the rapidly changing NGS field. The PacBio system is the most commonly used long-read platform (Eid et al., 2009). It involves fixing DNA polymerase to the bottom of the flow cell and allows the DNA strand to progress through zero mode waveguides (Levene et al., 2003). A laser records the colour of the emitted light as the labelled nucleotide is incorporated, the polymerase then cleaves the fluorophore before the next nucleotide is added. The PacBio system can generate read lengths of 10-15 kb, however it is not without its limitations as the long read length means a higher error rate, yet, PacBio provide a circular template which provides a level for error correction (Goodwin et al., 2016). Moreover, the MinION platform is a nanopore sequencer, which directly detects the DNA composition of ssDNA without the hybridisation or incorporation of nucleotides along a template DNA strand. Instead, DNA is passed through a protein pore which results in a shift in voltage through the pore, these voltage shifts are characteristic of a particular DNA sequence (Clarke et al., 2009). The advantages of MinION are that it can be run from a personal computer, is easily portable and is ideal for use in clinics when rapid responses are required. However, although there are few limitations as of the size of fragment that can be sequenced, the MinION has a large error rate. Nonetheless, improvements in the Chemistry involved are improving error rates

and read accuracy (Goodwin et al., 2015, Jain et al., 2015). Therefore, although of late the hugely successful Illumina instruments have dominated the NGS field and some technologies that could not keep up with the ever evolving technology have become casualties, new technologies continue to be developed, challenging Illumina for its place at the top.

Moreover, whilst it cannot be argued that the advent of NGS has revolutionised these types of studies, whether it is investigating the microbiome, genome, metabolome etc, simply knowing what is there is not sufficient in terms of understanding the disease pathogenesis. The microbes are not always the sole players involved in disease; as the host can also play a role. Take PD for example, the over activation of the immune system is strongly associated with hard and soft tissue destruction. Thus, understanding the role of the host is crucial for investigating disease pathogenesis and the intricate interplay between the host and microbiome.

1.6 Host response

The host response at the oral mucosa has been thoroughly investigated in terms of dental related disease including gingivitis and PD (Millhouse et al., 2014), however there is a sufficient lack in understanding regarding the role of the oral mucosal immune response in terms of DS. As we have previously stated the literature is heavily biased towards *Candida* in terms of DS (Coco et al., 2008a, Davenport and Hamada, 1979, Iacopino and Wathen, 1992), therefore, studies in this area have focused on the protection during infection by *Candida* (Wei et al., 2011, Yanez et al., 2011, Carvalho et al., 2012). The key factors involved are primarily associated with cell-mediated immunity. Microbial componants such as lipopolysaccharide and peptidoglycan are primarily recognised by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) expressed on the host epithelium (Tietze et al., 2006). The binding of a PRR to its cognate Pathogen associated molecular pattern (PAMP) triggers a signalling cascade which can lead to the activation and modulation of the innate and adaptive immune responses, and production of antimicrobial mediators such as antimicrobial peptides (AMPs) including defensins, cathelicidins and defensins (Akira and Takeda, 2004). In terms of *Candida,* it is recognised by PRRs including Dectin-1,CR3, TLR2, TLR3 and TLR4 on the host oral epithelial cells (Yanez et al., 2011, Gasparoto et al., 2010,

Takahara et al., 2012). Dectin-1 and CR3 recognise ß-glucans on *C. albicans* cell wall in both yeast and hyphal forms (Zheng et al., 2015a). The role of TLR2 and TLR4 is controversial as some studies have shown an upregulation during Candidal infection, yet others showed no differences in the epithelial cell cytokine profile when these receptors were blocked by antibodies (Weindl et al., 2007). However, intracellular TLR3 is activated in response to host cell invasion by *C. albicans (Muller et al., 2007)*.In terms of DS, Gasparoto et al (2012) demonstrated that the expression of TLR2 on neutrophils is significantly reduced in elderly individuals with DS in comparison to younger controls with DS, suggesting that with the natural ageing of the immune system, PRRs gradually become deficient (Gasparoto et al., 2012).

The immune cells normally involved in innate immunity of the oral mucosa include dendritic cells, macrophages and neutrophils, all of which drive adaptive immune responses involving T cells and B cells (Wei et al., 2011). In general it is accepted that CD4+ T cells of Th1/Th17 lineage responses are protective against candidal infection (Schaller et al., 2004, Pandiyan et al., 2011), whilst a Th2 response is detrimental (Clemons and Stevens, 2001). These CD4+ cells orchestrate the immune response by the release of inflammatory and anti-inflammatory cytokines. A study recently conducted by Rogers et al demonstrated that cytokines associated with a Th1/Th17 response were elevated within DS patients in comparison to healthy denture wearing controls, thus the appropriate response is being elicited (Rogers et al., 2013). However, there is a delicate balance between host immunity and pathogen growth as excessive Th1 and Th17 responses could also contribute to inflammation and tissue damage. Nonetheless, in spite of the evidence concerning host immune responses during *Candida* infection, limited studies have been undertaken regarding the local inflammatory response associated with DS, particularly in terms of the role of bacteria in this disease.

Moreover, in addition to the cellular immune response and their related cytokines, there are other immune factors involved in maintaining healthy oral mucosa. A range of AMPs have been isolated within the oral cavity. They are classed as a major innate arsenal against microbes and have demonstrated effective protection against oral disease, including DS.

1.6.1 Antimicrobial peptides

Cationic AMPs are small peptides that, by definition, exhibit some, often broad-spectrum, antimicrobial activity. AMPs can protect against a wide variety of bacterial, viral, fungal and protozoan infections and as a result are often referred to as host defence peptides. They also play an important role in the innate immune system, promoting actions that protect against microbial infection (Andersson et al., 2016). AMPs are synthesised within granules of phagocytic cells or are secreted by epithelia (Gudmundsson and Agerberth, 1999). In humans and other mammals, there are sites within the body that are generally free of microorganisms (e.g. lung), yet other sites such as the oral cavity, are heavily colonised by microorganisms. The oral cavity and respiratory tract express a similar pattern of AMPs, and there has been an increased interest in the roles that AMPs have at these sites against conditions such as PD and cystic fibrosis (Devine, 2003). An increasing resistance of microbes to anti-microbial drugs has lead researchers to look for new approaches, with the outcome being an increase in studies into using antimicrobial peptides (AMP's) as a potential new therapy (da Silva et al., 2012).

1.6.1.1 Histatin 5

Histatins in particular have shown strong antifungal properties predominantly against *Candida* biofilms and promisingly has a potent effect on fungal species, which show strong resistance to classic antifungals (Edgerton and Koshlukova, 2000). There are three major histatins (Hst1, Hst3 and Hst5), yet histatin 5 has the most potent antifungal activity, with the ability to kill both yeast and hyphal forms of *C. albicans* (Xu et al., 1991). Histatin 5 gains entry into the cell by binding to the *C. albicans* cell wall proteins Ssa1/2 and glucans, it is then transferred into the cytosol via fungal polyamine transporters in an energy dependant manner (Puri and Edgerton, 2014). It is thought that histatins function by exerting action at microbial membranes leading to the loss of ions, primarily adenosine triphosphate (ATP) (Vylkova et al., 2007). Furthermore, histatin 5 induces the formation of reactive oxygen species (ROS) inside *C. albicans* cells and in the mitochondria, with high ROS levels correlating with increased cell death (Helmerhorst et al., 2001).

The efficacy of histatin 5 in preventing adherence of *Candida* species to denture acrylic has been successfully demonstrated, as well as its ability to inhibit the adhesion of *C. albicans* to reconstituted human oral epithelium (RHOE) (Yoshinari et al., 2006, Edgerton et al., 1995, Moffa et al., 2015). Whilst *C. albicans* biofilms are highly susceptible to histatin 5, *C. glabrata* biofilms on denture surfaces are less susceptible, whilst planktonic *C. glabrata* cells are completely insensitive (Konopka et al., 2010). Therefore, similar to antifungal agents currently used clinically, histatin 5 is vulnerable to the resistant abilities of *C. glabrata*.

1.6.1.2 LL-37

Several studies have demonstrated that LL-37 assists in the initiation of the immune response by its ability to induce migration, chemotaxis and activation of innate immune cells (Diamond et al., 2009, Gordon et al., 2005). LL-37 is the only cathelicidin found in humans and becomes active when proteinase 3 cleaves the C-terminus to create an active 37 residue antimicrobial peptide (Sorensen et al., 2001). This peptide was initially found within neutrophil granules but was later discovered in monocytes, T cells and the respiratory mucosa (Diamond et al., 2008b). Like histatin 5, LL-37 has broad spectrum activity targeting *Candida*, Gram-positive and Gram-negative bacteria (Overhage et al., 2008, Dean et al., 2011, Amer et al., 2010, Murakami et al., 2004, Wong et al., 2011). LL-37 contains several hydrophobic residues and is positively charged, these factors allow it to interact with negatively charged microbial membranes (Tsai et al., 2011a). These electrostatic interactions can then either lead to the peptide being taken into the cell or the accumulation of enough peptides leading to the formation of a pore and the loss of cellular homeostasis (Brogden, 2005, Lee et al., 2011, Henzler Wildman et al., 2003).

In terms of fungal interactions, LL-37 has been shown to block *C. albicans* adhesion to mucosal membranes by interacting with surface carbohydrates on the mucosal membrane. ß-1-3 glucan is a major component of fungal cell membranes, and an interaction between related exo ß-1-3 glucan, Xog1p, significantly decreases *C. albicans* adhesion to epithelium (Tsai et al., 2011b). Thus LL-37 could be utilised for detection of other cell wall components involved in *Candida* adhesion. As for bacterial interactions, LL-37 has been shown to prevent biofilm formation at low concentrations and at higher concentrations is able to destroy preformed *P.*

aeruginosa biofilms. Studies investigating LL-37-treated *P. aeruginosa* biofilms saw a down-regulation of quorum-sensing (*LasI*, *rhlR*)-controlled genes and an up-regulation of type IV pili, which led to twitching of the bacteria and prevented adhesion to surfaces (Overhage et al., 2008, Dean et al., 2011). LL-37 also demonstrated an anti-biofilm effect against *Francisella novicida* (Amer et al., 2010). Nonetheless, some bacteria have developed a resistance mechanism against LL-37, which involves the release of proteolytic enzymes to cleave the active form of the peptide (Sieprawska-Lupa et al., 2004).

1.6.1.3 Alpha defensins

Alpha defensins, or as they are more commonly known, human neutrophil peptides (HNP), are named so because they were first isolated from neutrophil granules (Yang et al., 2004). HNP 1-3 are abundant in the oral cavity, found in tissue and secretions including oral and salivary gland tissues as well as saliva and gingival crevicular fluid (Gorr and Abdolhosseini, 2011). The HNP's all are similar in size and structure containing of between 29-35 amino acids, with only slight changes in composition distinguishing them (Kohlgraf et al., 2010).

Increases in the level of HNP's found in the oral cavity are seen in a number of oral diseases such as PD, lichen planus, Behcets disease and aphthous stomatitis (Kucukkolbasi et al., 2011). α defensins exert their antimicrobial effects by direct antimicrobial activity, by disrupting the cellular membrane or by inducing non-oxidative killing in phagocytes (Dale and Krisanaprakornkit, 2001). HNP-1-3 have been shown to directly target oral bacteria including *S. mutans, A. actinomycetemcomitans* and *P. gingivalis (Gorr and Abdolhosseini, 2011)*. One study investigating *Escherichia coli* found that HNP1-3 permeabilised the outer then the inner membrane, and upon breach of the inner membrane cellular homeostasis was compromised and DNA, RNA and protein synthesis ceased (Lehrer et al., 1989). However, the antimicrobial activity of HNP's are not as potent against oral bacteria as beta defensins (Dale and Fredericks, 2005).

1.6.1.4 Beta Defensins

Beta defensins were originally found in the respiratory epithelium but were subsequently discovered to be expressed at most epithelial surfaces, suggesting

that they play an important role in protecting the epithelium against invading pathogens. Three defensins are expressed by human oral epithelial cells (hBD 1-3). Hbd-1 is expressed at low levels constitutively, however, hBD-2 and 3 have low expression but can be up-regulated upon stimulation by microbes or the immune system (Diamond and Ryan, 2011). Their mechanism of action is thought to directly target the microbial membrane (Hans and Madaan Hans, 2014). As ß-defensins are positively charged, they target negatively charged areas of the microbial membrane, which for gram positive bacteria is lipoteichoic acid and for gram negative bacteria; lipopolysaccharide (LPS) (Weinberg et al., 1998, Hans and Madaan Hans, 2014). It is proposed that interaction with the membrane leads to peptide deposition and pore formation (Agawa et al., 1991).

ß- defensins have a broad spectrum of activity, as they possess antibacterial, antifungal and antiviral properties. In terms of the oral cavity, these peptides have been shown to be active against periodontal pathogens including, *A. actinomycetemcomitans*, *F. nucleatum* and *S. mutans* (Ji et al., 2010, Joly et al., 2004, Song et al., 2009). Nonetheless, some oral pathogens have developed resistance to ß- defensins, as *P. gingivalis* does not induce the expression of defensins (Krisanaprakornkit et al., 2000) and *Treponema denticola* suppresses the induction by interacting with the signal transduction pathway (Brissette et al., 2008, Shin and Choi, 2010). As for *Candida*, studies of oral candidiasis showed an increased level of expression of ß- defensins in the oral epithelium and that hBD2 and hBD3 were more effective at killing *C. albicans* than hBD1 (Sawaki et al., 2002, Feng et al., 2005). However, hBD-1 has been shown to play an important role in preventing early infection by *C. albicans,* (Tomalka et al., 2015) suggesting that hBD1 is required during early infection and hBD2 and hBD3 become involved later.

1.6.1.5 Calprotectin

Calprotectin, also known as calgranulin, is found to be constitutively expressed in several immune cells including neutrophils, monocytes, macrophages as well as epithelial cells (Nacken et al., 2003, Dale et al., 1985, Odink et al., 1987, Brandtzaeg et al., 1987). Its levels are elevated in saliva, plasma and synovial fluid during inflammation as a result of diseases such as PD (Nisapakultorn et al., 2001, Kido et al., 1999). Calprotectin exerts its antimicrobial activity by sequestering

metal ions, namely zinc. When released into an inflammatory environment, it will bind these ions, which are essential for microbial function, thus provides host defence by inhibiting microbial growth (Damo et al., 2013).

In whole unstimulated saliva, calprotectin is found at levels around 22 mg/L (Cuida et al., 1995). It has been shown to confer protection against *S. aureus*, *Staphylococcus epidermidis*, and *E. coli (Steinbakk et al., 1990)*. Moreover, in the oral cavity, calprotectin was shown to contribute towards the resistance of gingival epithelial cells to invasion with *P. gingivalis* (Nisapakultorn et al., 2001). In terms of its antifungal properties, higher levels of calprotectin were found in the saliva of patients with oral candidiasis than those without (Kleinegger et al., 2001). Furthermore, several studies have demonstrated its ability to inhibit *C. albicans* growth (Sohnle et al., 1996, Okutomi et al., 1998, Murthy et al., 1993). The recent phenomenon of neutrophil extracellular traps (NETs) has been an area of great interest in medical research and calprotectin has been shown to be one on the primary constituents released by these NETs (Urban et al., 2009). Urban et al (2009) demonstrated that calprotectin KO's used in *C. albicans* mouse infection models completely lost their antifungal activity, demonstrating the importance of this peptide against fungal infection.

1.6.1.6 Lactoferrin

Lactoferrin is one of the few proteins which are expressed at all mucosal sites throughout the body and is found in saliva, nasal secretions, gastric secretions, tears, breast milk and amniotic fluid. It has been shown to demonstrate anti-bacterial, anti-fungal and anti-viral properties (Farnaud and Evans, 2003). Traditionally lactoferrin was thought to play an indirect role in terms of its antimicrobial activity by sequestering iron which was essential for bacterial survival. However, it was then discovered that lactoferrin demonstrated bactericidal activity that was independent of binding iron. Interactions with bacteria are thought to happen through binding to the lipid A portion of LPS or lipoteichoic acid for Gram-negative and Gram-positive bacteria, respectively. This interaction occurs when positively charged lysine or arginine residues interact with negatively charged regions of the lipid A or lipoteichoic acid, resulting in disruption of the outer membrane and disturbs the membrane stability. In terms

of oral bacteria, the binding *of P. gingivalis* and *A. actinomycetemcomitans* to epithelial cells is blocked by iron saturated lactoferrin (Alugupalli and Kalfas, 1995). Elevated levels of oral lactoferrin have been suggested as a marker of PD (Friedman et al., 1983, Adonogianaki et al., 1993). Several studies have demonstrated the effective anti-fungal activity of lactoferrin against *Candida* spp. (Lupetti et al., 2007, Andres et al., 2008, Al-Sheikh, 2009, Kondori et al., 2011, Velliyagounder et al., 2015). Moreover, Kobayashi et al (2011) saw interesting effects of synergism when using lactoferrin in combination with traditional antifungals fluconazole and itraconazole, on certain strains of *C. albicans* (Kobayashi et al., 2011). Thus, providing evidence of a new potential therapeutic for the treatment of candidiasis.

Consequently, the importance of the immune system in controlling microbial growth around the denture environment cannot be understated. Given the relationship between the elderly and denture wear, then a depleted immune system in this patient group creates an increased risk of infection. Thus, given the increasing elderly population, due to medical advances, this emphasises the importance of understanding the host response of a disease, which affects a large proportion of the population, and thus alleviating the burden on the National Health Service (NHS).

1.7 Conclusions

It is clear by reviewing the literature that there is an extreme lack of investigation regarding the microbial composition of denture plaque. At present, those studies that have looked at denture microbiology have used less complex techniques such as culturing and standard PCR. Currently, to our knowledge, no group has made use of NGS technology in this context. In addition to this, understanding the innate arsenal of innate immune factors, such as AMPs, that are present within the oral environment of a denture wearer will further contribute towards understanding the pathogenesis of DS.

1.8 Aims and hypothesis

Denture biofilms are able to form on all surfaces of the denture, however it is those biofilms which form on the upper denture surface in direct contact with

palatal mucosa that are predominantly associated with denture related disease. Currently there is limited understanding of the role of bacteria in this disease.

The aims of this study were therefore to:

1) Investigate the microbiome of dentures and other tissues within the mouths of denture wearers using NGS technology

2) Compare the microbiome of healthy and diseased individuals suffering from DS of varying severity

3) Identify the potential systemic implications of the denture microbiome, in terms of respiratory pathogens colonising dentures

4) To use the knowledge gained from the NGS to develop an accurate denture biofilm model the can be used for various applications including the testing of chemotherapeutics.

5) To investigate the host response in relation to denture plaque by studying salivary AMPs in order to understand DS pathogenesis

2 Patient Demographics

2.1 Introduction

Healthcare improvements in the last century have led to an increasingly elderly population. Worldwide, 810 million people are aged 60 years or over, which is predicted to increase to at least two billion by 2050 (22% of the entire global population) (Guzmán et al., 2012). In the EU alone the proportion of the population who are 65 years and older is predicted to reach 53% by the year 2025 (Muenz, 2007). This demographic change will result in significant challenges for oral healthcare delivery to an increasingly aged population with declining oral health. As the population ages oral diseases become more relevant with respect to their local and systemic impact, which can have profound implications for healthcare provision (Griffin et al., 2012, Meurman and Hamalainen, 2006).

The oral cavity is a complex environment that is continually exposed to numerous opportunistic microbial pathogens. These are kept in check by a robust arsenal of immune factors that maintain a healthy oral environment and prevent the development of disease. This arena has gradually become a key area of biomedical research, which has led to a greater understanding of the causes, pathogenesis and host response against oral disease, with the majority of research focussing on diseases affecting dentate individuals, such as gingivitis, periodontitis and caries. Conversely, there is relatively less research regarding denture related disease. Despite major improvements in oral health worldwide, recent estimates report that the rate of edentulouness still varies from 7 to 69% of the worlds adult population (Felton et al., 2011, Petersen, 2003), and in the US and UK populations around one fifth wear some form of removable denture (Coulthwaite and Verran, 2007, Shulman et al., 2004). This continued high prevalence should convince researchers that there is a requirement to develop an understanding of the implications of dentures on oral health. Moreover, according to the literature, the key to understanding the role of dentures in oral health lies with the denture microbiology. As the majority of studies regarding denture related disease indicate a microbial cause, yet an in depth analysis of denture plaque microbiology has yet to be carried out.

Furthermore, focusing solely on clinical and microbiological indicators of dental disease may not fully reflect the problems people have with their dentures (Guyatt et al., 1989). Fiske and co-workers (1998) indicated that tooth loss and wearing dentures has broader psychosocial consequences than the obvious functional limitations (Fiske et al., 1998). Patient centred outcomes are increasingly being used to measure the impact of health and disease on quality of life (Wilson and Cleary, 1995), including the Oral Health Impact Profile–14 (OHIP-14) questionnaire, which was developed and validated based on Locker's conceptual framework for assessing oral health (Locker, 1988, Locker et al., 2004).

2.2 Aims

The aim of this chapter was to report patient demographic data in order to gain a clearer understanding of the patient cohort that will be included in this observational cross sectional study. Furthermore, to understand how patient demographics such as gender, health, denture type and hygiene affect both the basic denture microbiology and oral health related quality of life measures. The following key questions were investigated:

- How are the patient demographics, including gender, health status, denture type and dentate status distributed proportionally?
- Does denture stomatitis affect the qualitative microbiology on the denture surface?
- Does the denture type alter the qualitative microbiology on the denture surface?
- Does the presence of natural teeth alter the qualitative microbiology on the denture surface?
- Do oral hygiene habits and practices alter the qualitative microbiology on the denture surface?
- How does wearing dentures impact the oral health related quality of life of our participants?
- Do certain oral hygiene habits and practices have an impact on oral health related quality of life?

2.3 Materials and Methods

2.3.1 Patient recruitment

131 denture wearing patients attending the University of Glasgow Dental School and Hospital were enrolled in the study. Patients that were attending the hospital had appointments to have new dentures made, which included healthy patients and those having trouble with their dentures. This therefore avoided the sample bias of only recruiting patients with denture associated problems. Convenience sampling was used based on the patient availability on recruitment days. Patients were recruited by a designated PhD student (self) or a research nurse. Patients were given a study information sheet (Appendix I), written informed consent (Appendix II) was obtained from all participants. Ethical approval for the study was granted by the West of Scotland Research Ethics Service (12/WS/0121). All patients wore full or partial removable dentures. A team of qualified dental clinicians were responsible for the collection of samples and the recording of clinical features (Appendix III). There was no age related exclusion criteria for this study. Newton's classification method for DS was used to score the appearance of the patient's palatal mucosa (Newton, 1962b). The following scores were applied; 0= healthy mucosa, 1= pin-point hyperaemic lesions (localized erythema), 2= diffuse erythema (generalized simple inflammation), and 3= hyperplastic granular surface (inflammatory papillary hyperplasia). For standardisation, all clinicians received training to calibrate scoring the extent of erythema. Patients were excluded from the study if they were pregnant, had previous radiotherapy for the treatment of head and neck malignancy, had been receiving antimicrobial/antifungal treatment, using prescription mouthwashes or had received immunosuppressant therapy within six months previous to sampling.

2.3.2 Clinical sample collection

Ethylene oxide sterilised swabs (Fisher Scientific, Loughborough, UK) were used to collect samples from the denture surface in contact with the palatal mucosa and the palatal mucosal surface covered by the denture. If any natural teeth were present the clinician took a plaque sample using a sterile dental probe, which was immediately placed into a 2 mL collection tube (Fisher Scientific) containing

RNAlater® (QIAgen, Manchester, UK). Dentures were removed from the patients' mouth and placed in sterile bags (Fisher Scientific) filled with 50 mL PBS (Sigma-Aldrich, Dorset, UK), then placed in a sonic bath (Ultrawave, Cardiff, UK) and sonicated for 5 min at at 35 kHz to remove adherent denture plaque. The denture sonicate was then transferred to a 50 mL tube and centrifuged for 10 min at 3700 x *g*, and the plaque pellet re-suspended in 2 mL of RNAlater® (QIAgen). Swab tips were removed and stored in RNAlater®. Denture plaque, dental plaque and swab samples were all stored at -80ºC (Triple Red Lab Technology, Long Credon). In total, samples from 131 patients were collected, which included 131 denture swabs, 131 mucosal swabs and 79 dental plaque samples.

2.3.3 Collection of clinical data and questionnaire

Data on patient age, gender, smoking status and any history of recent antimicrobial medication were recorded on a clinical information sheet (Appendix IV). Patients were then asked to complete a questionnaire covering aspects of their routine oral hygiene regimens. Included within the questionnaire were questions from the OHIP – 14, which assesses seven dimensions of impact of oral conditions on people's oral health related quality of life (OHRQoL), including functional limitation, physical pain, psychological discomfort, physical disability, psychological disability, social disability and handicap (Allen and McMillan, 1999). A 5-point Likert scale anchors each of the OHIP-14 questions. Each response was provided a score as follows; Never = 0, Hardly ever =1, occasionally = 2, Fairly often = 3, Very often = 4. The sum of each patient's questionnaire was calculated by adding the response for each question. The maximum possible score is 56. The greater the score the greater the impact on the patients oral health related quality of life. All patients were given a unique study code, which was used for subsequent analysis throughout the study to ensure the anonymity of the patients.

2.3.4 Microbial quantification of denture sonicate

Prior to centrifugation, 1 mL of the denture sonicate was used to prepare serial ten-fold dilutions ranging from 10^0 – 10^{-5} in phosphate buffered saline (PBS) [Sigma-Aldrich, Dorset, UK]. Serial dilutions were then used to perform colony forming unit (CFU) counts standard using colorex *Candida* (E & O labs, Bonnybridge, UK),

colorex *S. aureus* (E and O labs), MacConkey (E & O labs) and BHI blood (E & O labs) agar plates. 100 µl of each serial dilution was spread across each of the plates. Colorex® *Candida* plates were incubated at 30ºC for 72 hours and MacConkey, colorex *S. aureus* and BHI blood plates were incubated at 37ºC for 24 hours. Additional blood agar plates were maintained under anaerobic conditions (85% N_2, 10% CO_2 and 5% H_2 [Don Whitley Scientific Limited, Shipley, UK]) at 37ºC for 24 hours. Bacteria and *Candida* were quantified by counting colonies formed for each dilution and the average number of bacteria or *Candida* cells/mL was calculated. The quantity (cells/mL) for both bacteria and *Candida* were then calculated based on initial dilutions (multiplied 50 x in order to represent the original 50 mL of PBS in which the denture was sonicated).

2.3.5 *Candida* species strain collection

Candida isolates from dentures grown on Colorex® *Candida* plates were assigned a species based on the colour of the colonies, green for *C. albicans*, pink for *C. glabrata* and blue for *C. tropicalis*. Purity plates were made for each isolate on Sabouraud dextrose agar (SAB [Sigma-Aldrich, Dorset, UK]). All isolates were stored in Microbank® vials (Pro-Lab Diagnostics, Cheshire, UK) at -80ºC until further use (Triple Red Lab Technology).

2.3.6 Statistical Analysis

Statistical analysis and graph production were performed using GraphPad Prism (version 4; La Jolla, CA, USA) or IBM SPSS statistics (version 21; Chicago, Il, USA). All data was normalised by log transformation and statistical analysis carried out on these values, as indicated by the mean and median values throughout the results section. The Mann-Whitney U test was used to test for differences between microbial culture data across patient demographic groups and hygiene categories as the data were not normally distributed. The Mann Whitney U test was also used to test for differences in OHIP scores in relation to oral hygiene, cleaning frequency and sleeping with or without a denture *in situ*. A Kruskal-Wallis test with Dunns post-hoc test was employed to compare differences when the groups were further split into healthy and DS groups to account for multiple pair-wise comparisons.

2.4 Results

2.4.1 Patient Demographics

Samples from 131 patients were included in this study, of which the primary demographics of these patients are shown in Table 2.1. The average patient age was 71.6 years (min: 33, max: 95), the patient age distribution is shown in Figure 2.1, with an average denture age of 4.5 years (min: 0.2 max: 40). Females represented the majority of the population at 65%, with males contributing only 35% (Fig 2.2A). 63% of participants were found to have healthy oral mucosa and the remaining 37% were diagnosed with DS of varying degrees of severity (Fig 2.2B). Overall, 18.3% of the patients were current smokers, and when split into healthy and DS groups, smokers represented 13.4% and 26.5%, respectively. The majority of participants wore a complete denture (67.9%), with the remaining 32.1% wearing a partial denture (Fig 2.2C). The average number of teeth remaining was 5.6 for the population as a whole; the distribution of the number of remaining natural teeth is shown in Figure 2.3.

Table 2.1: Patient demographics.

Patient Demographics		Denture induced stomatitis			
	Healthy	grade 1	grade 2	grade 3	Total
N	82 (62.6%)	25 (19.2%)	14 (10.7%)	10 (7.5%)	49 (37.4%)
Male	26 (31.7%)	11 (44.0%)	6 (42.9%)	3 (30.0%)	20 (40.8%)
Female	56 (68.3%)	14 (66.0%)	8 (57.1%)	7 (70.0%)	29 (59.2%)
Mean Age Median Age	72 72	69.0 72	69.6 70	64.4 65	68.2 70
Median Denture Age	3	4.4	3.6	6.2	4.5
Complete dentures	61 (74.4%)	16 (64.0%)	8 (57.1%)	4 (40.0%)	28 (57.1%)
Partial dentures	21 (25.6%)	9 (36.0%)	6 (42.9%)	6 (60.0%)	21 (42.9%)
Mean natural teeth remaining	5.2	5.2	6.1	8.5	6.1
Current smoker	11 (13.4%)	5 (20.0%)	3 (21.4%)	5 (50.0%)	13 (26.5%)

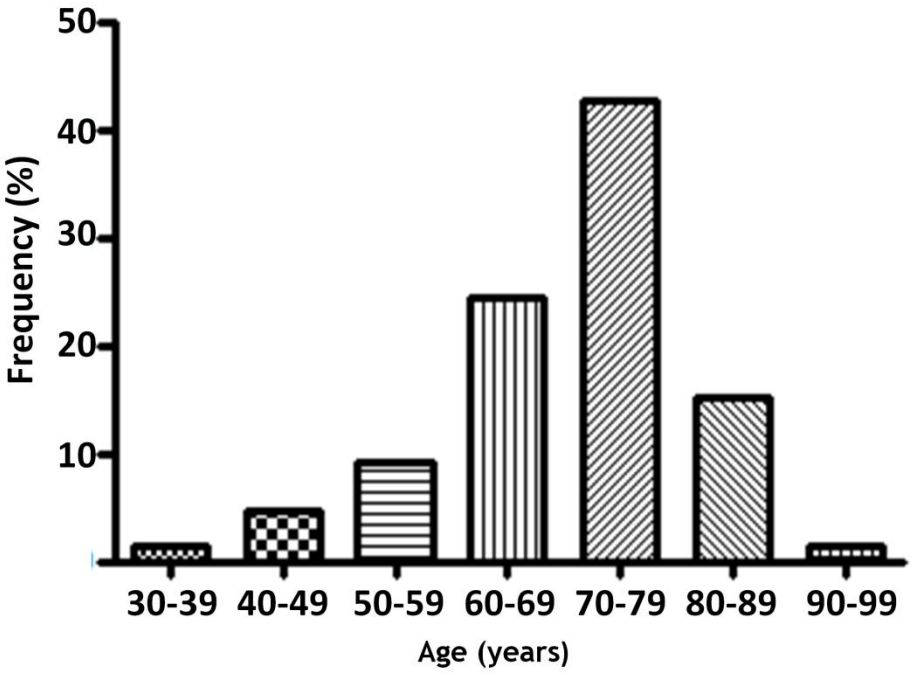


Figure 2.1: Patient age distribution of denture wearers.
The proportion of denture wearers in the study representing each age group.

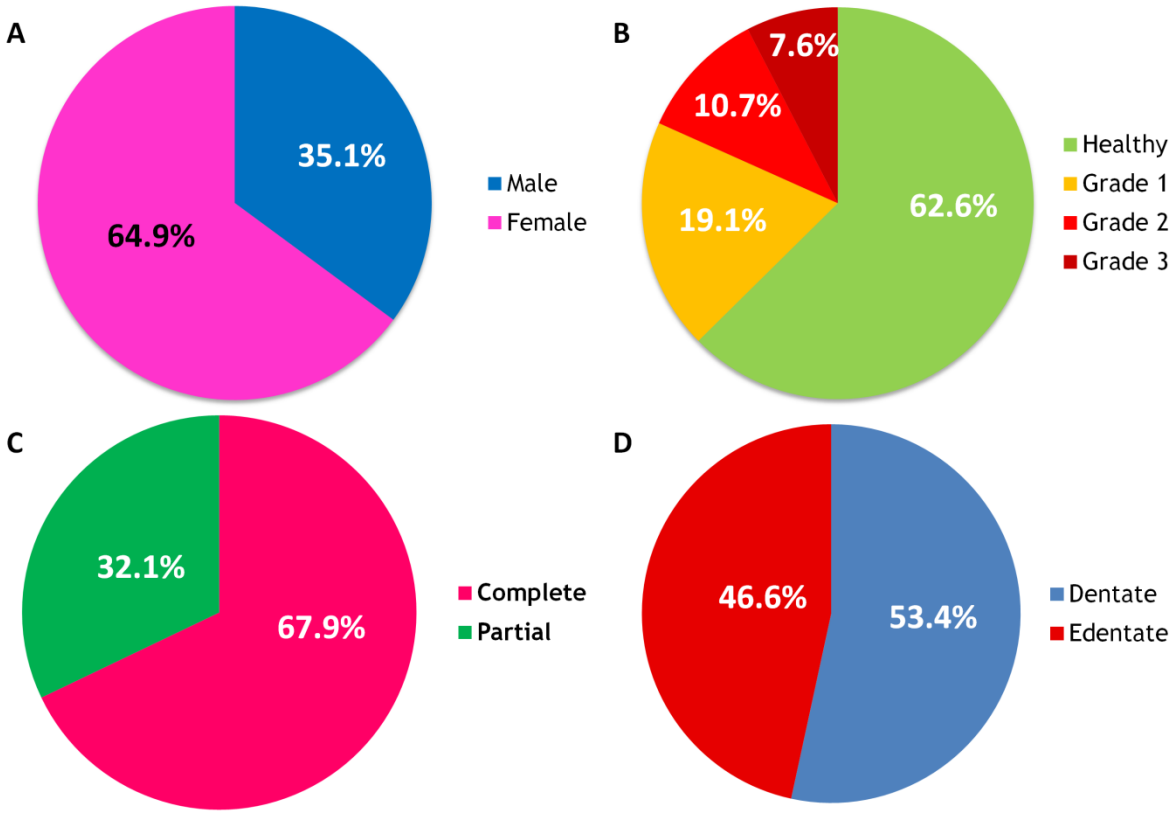


Figure 2.2: Patient demographics of the study cohort.
Pie charts representing the key patient demographics proportionally for **(A)** gender, **(B)** health & disease, **(C)** denture type and **(D)** dentate status.

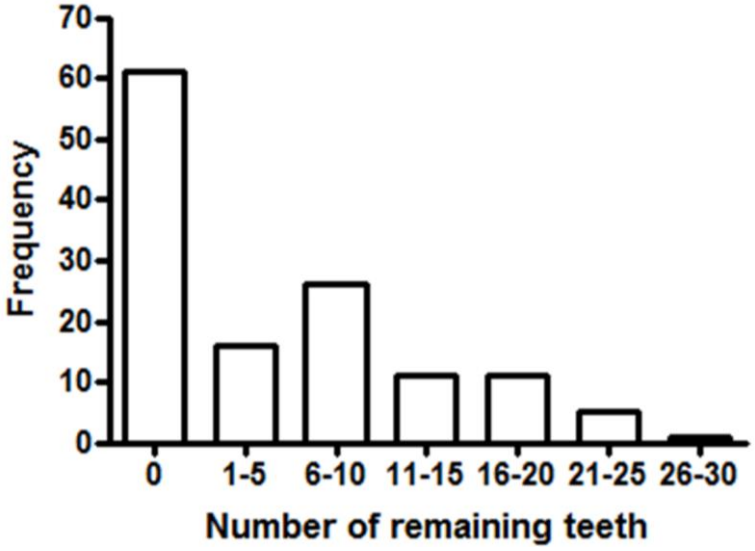


Figure 2.3: Frequency of natural remaining teeth in patient cohort.
The participants were separated into groups based on the number of natural teeth they had remaining in their oral cavity.

2.4.2 Oral hygiene practices

The clinical and questionnaire data collected also gave an insight into patient oral hygiene habits and practices (Table 2.2). Clinicians classed participants as having excellent (16%), good (56.5%) or poor (27.5%) oral hygiene. However, when split into healthy and diseased, only 20.7% of healthy patients were classed as having poor oral hygiene, in comparison to 38.8% of DS sufferers. This difference is even more apparent within the most severe inflammation group, grade 3, where 50% of patients have poor hygiene. Denture cleaning habits indicate that 32.1% of participants clean their denture at least once a day, 45.8% clean twice per day and the remaining 19.8% more than twice per day. Yet when split into health and disease, no noticeable differences were observed. Sleeping with a denture in is commonplace amongst denture wearers, as for this study it was found that 44.3% slept with their denture *in situ*, 44.3% did not sleep with their denture *in situ*, and 11.4 % sometimes slept with their denture *in situ*. When separated into health and disease, however, 57.2% were found to sleep with their dentures *in situ* compared to the 36.6% of those with a healthy palatial mucosa.

Table 2.2: Oral hygiene practices and DS.

Denture Hygiene	Healthy	Denture induced stomatitis			
		grade 1	grade 2	grade 3	Total
Excellent	16 (19.5%)	1 (4.0%)	2 (14.3%)	2 (20.0%)	5 (10.2%)
Good	49 (59.8%)	13 (52.0%)	9 (64.3%)	3 (30.0%)	25 (51.0%)
Poor	17 (20.7%)	11 (44.0%)	3 (21.4%)	5 (50.0%)	19 (38.8%)
Denture cleaning	**Healthy**	**Denture induced stomatitis**			
		grade 1	**grade 2**	**grade 3**	**Total**
Never	1 (1.2%)	0	0	0	0
<once/day	1 (1.2%)	0	1 (7.1%)	0	1 (2.1%)
once/day	24 (29.3%)	9 (36.0%)	6 (42.9%)	3 (30.0%)	18 (36.7%)
twice/day	40 (48.8%)	10 (40.0%)	6 (42.9%)	4 (40.0%)	20 (40.8%)
>twice/day	16 (19.5%)	6 (24.0%)	1 (7.1%)	3 (30.0%)	10 (20.4%)
Sleeping with denture	**Healthy**	**Denture induced stomatitis**			
		grade 1	**grade 2**	**grade 3**	**Total**
No	43 (52.4%)	9 (37.5%)	4 (28.6%)	2 (20.0%)	15 (30.6%)
Yes	30 (36.6%)	13 (54.2%)	9 (64.3%)	6 (60.0%)	28 (57.2%)
Sometimes	9 (11.0%)	3 (8.3%)	1 (7.1%)	2 (20.0%)	6 (12.2%)

2.4.3 Frequency and impact of oral pain and mouth dryness of denture wearers

Adjusting to wearing dentures is often a difficult experience, with many suffering pain and discomfort. Of the 131 patients assessed, 82 (62.6%) were currently experiencing some form of denture related pain (Table 2.3). Using a scale from 1 (no pain) to 10 (Intolerable pain), the average pain score was 5.29, with the most common pain described as a dull ache. Separation into health and DS groups found 50 (61%) and 32 (65.3%) participants experiencing pain, respectively. Moreover, the mean pain score reported was lower for DS group, 4.56, than the healthy group, 5.8.

Table 2.3: Participants experience of pain caused by dentures.

	Experiencing Pain	mean pain score	Most common type of pain
Total	82 (62.59%)	5.29	Dull ache
Healthy (n=82)	50 (60.97%)	5.80	Dull ache
Denture induced stomatitis (n=49)	32 (65.30%)	4.56	Throbbing and dull ache

The vast majority of study participants (66.5%) found that they were unaware of any dryness of the mouth. Only 19.8% felt that they had too little saliva, with 13.7% feeling like they have too much saliva. Suffering from DS appeared to affect mouth dryness (Table 2.4).

Table 2.4: Mouth dryness.

	Too little saliva	Too much saliva	Unaware
Healthy (n=82)	13 (15.8%)	14 (17.1%)	55 (67.1%)
Denture induced stomatitis (n=49)	13 (26.5%)	4 (8.2%)	32 (65.3%)

2.4.4 Prevalence of *Candida* on dentures

72% of patient's dentures were colonised by *Candida*. The prevalence of *Candida* species isolated from dentures of healthy and DS patients is shown in Table 2.5. The overall prevalence of *Candida* was higher on dentures from DS sufferers (78%) when compared with their healthy counterparts (64%). At the species level *C. albicans* was more predominant on DS individuals' dentures (76%), whereas for *C. glabrata* there were little differences in prevalence between health (41%) and disease (40%). Furthermore, the number of patients in which *C. albicans* and *C. glabrata* were co-isolated was also more common in DS sufferers (36%).

Table 2.5: Prevalence of *Candida* species isolated from dentures of healthy and diseased patients.

	Candida spp. **+ve**	***C. albicans*** **+ve**	***C. glabrata*** **+ve**	**Mixed *C. albicans* / *C. glabrata*** **+ve**
Healthy n (%) n= 78	50 (64)	32 (41)	32 (41)	14 (18)
DS n(%) n= 45	35 (78)	34 (76)	18(40)	16 (36)

2.4.5 Quantitative analysis of denture microbiology

The bacterial and fungal load colonising the dentures of our participants was quantified. The quantity of aerobic and anaerobic bacteria and *Candida* residing on the denture surface was enumerated by counting CFUs from the denture sonicate. Figure 2.4 compares the average number of CFUs detected in healthy patients against those with DS. In terms of total bacteria (Fig 2.4A), participants with DS had significantly higher levels of bacteria on their dentures [Median: 8.5 (min: 5.3, max: 9.65) v 9.17 (min: 6.52, max: 10.19), $p<0.05$]. No significant differences in the number of aerobic bacteria were determined (Fig 2.4B) [Median: 8.11 (min: 4.11, max: 9.61) v 8.53 (min: 4.38, max: 10.13), $p>0.05$], but DS participants appeared to have significantly more anaerobic bacteria than their healthy counterparts (Fig 2.4C) [Median: 8.05 (min: 5, max: 9.31) v 8.91 (min: 6.04, max: 9.64), $p<0.05$]. As for *Candida*, patients that carried *Candida* on their dentures had significantly elevated levels if they suffered from DS (Fig 2.4D) [Median: 5.27 (min: 2.7, max: 7.1) v 6.2 (min: 3.7, max: 8.11), $p<0.01$]. When stratified into species (*C. albicans* [Fig 2.4E] and *C. glabrata* [Fig 2.4F]), only *C. albicans* showed a significant difference between the groups [Median: 5.14 (min: 2.7, max: 6.85) v 5.75 (min: 3.65, max: 7.19), $p<0.01$], [Median: 5.22 (min: 2.7, max: 7.1) v 5.43 (min: 2.7, max: 8.1), $p>0.05$] respectively.

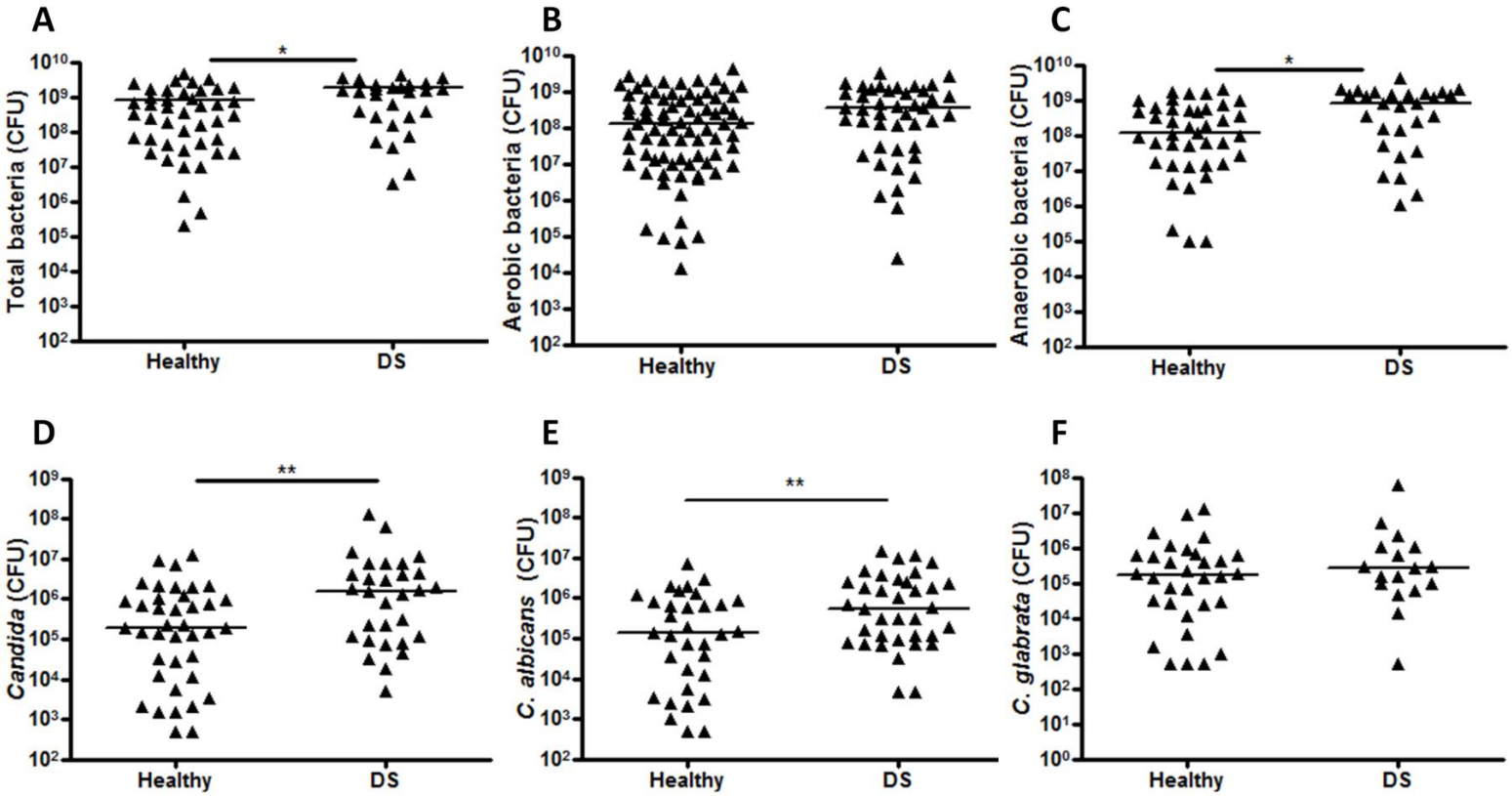


Figure 2.4: CFU analysis of denture microbiology of healthy and DS patients.
Denture sonicate was used to quantify bacteria and yeasts by plating out on blood agar and colorex *Candida* plates respectively. Blood agar plates were stored under aerobic and anaerobic conditions. Colorex *Candida* plates allowed for differentiation between *C. albicans and C. glabrata* species. Colonies formed were quantified and total bacteria **(A)**, total aerobes **(B)**, total anaerobes **(C)**, total Candida **(D)**, total *C. albicans* **(E)** and total *C. glabrata* **(F)** were separated into healthy and DS groups and compared. Data represents median (* $p<0.05$, ** $p<0.01$). Statistical analysis was performed using a Mann Whitney test as data did not conform to a normal distribution.

Participants were then split into groups depending on denture type (complete or partial), and also on whether they had some or no natural teeth remaining (dentate or edentate). Total bacteria CFU and total *Candida* CFU was compared between denture types and no differences were found for bacteria (Fig 2.5A) [Median: 8.69 (min: 5.3, max: 10.19) v 9.12 (min: 6.15, max: 9.65), $p>0.05$]. In contrast, *Candida* levels were significantly elevated on complete dentures (Fig 2.5B) [Median: 6.01 (min: 3.18, max: 8.11) v 5.18 (min: 2.7, max: 6.89), $p<0.01$]. As for dentate status, as with the denture type, bacteria levels showed no differences (Fig 2.5C) [Median: 8.77 (min: 5.30, max: 9.53) v 8.75 (min: 6.78, max: 10.19), $p>0.05$], but *Candida* levels were significantly elevated in edentulous individuals (Fig 2.5D) [Median: 5.43 (min: 2.7, max: 7.1) v 5.89 (min: 3.18, max: 8.11), $p<0.05$].

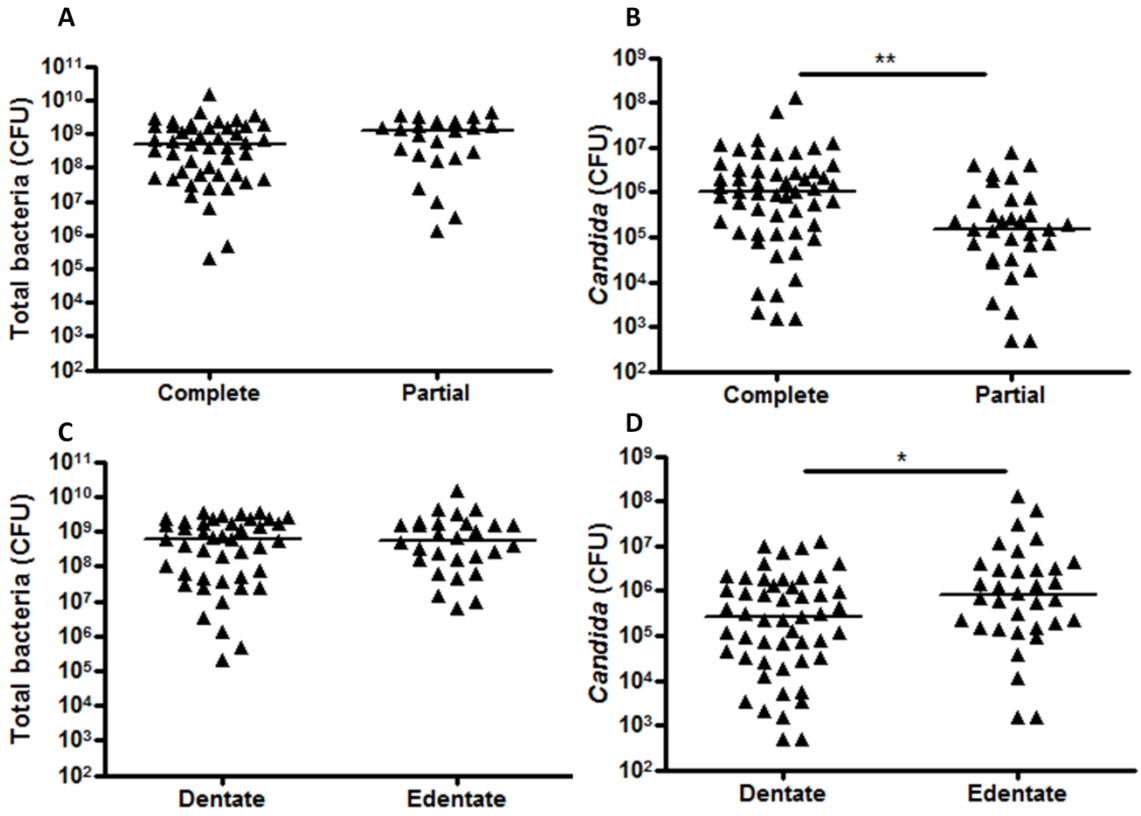


Figure 2.5: CFU analysis of denture microbiology based on denture type and presence of natural teeth.
Denture sonicate was used to quantify bacteria and yeasts by plating out on blood agar and colorex *Candida* plates respectively. The average CFU counts were then compared between complete and partial dentures for bacteria **(A)** and *Candida* **(B)** and between dentate and edentate patients for bacteria **(C)** and *Candida* **(D)**. Data represents median (* $p<0.05$, ** $p<0.01$). Statistical analysis was performed using a Mann Whitney test as data did not conform to a normal distribution.

The oral hygiene data shown in Table 2.2 was used to assess the impact of common oral hygiene habits and practices on denture microbiology. For ease of comparison the excellent and good oral hygiene participants were grouped together and compared against the poor oral hygiene group. The quantity of total bacteria (Fig 2.6A) [Median: 8.69 (min: 5.3, max: 10.19) v 9 (min: 6.15, max: 9.65), $p>0.05$] and total *Candida* spp. (Fig 2.6B) [Median: 5.49 (min: 2.7, max: 7.19) v 6.01 (min: 2.7, max: 8.11), $p>0.05$] was compared between good and poor oral hygiene groups, however, no differences were observed for either.

As for cleaning frequency, as previous, in order to make comparison easier those that selected that they never clean their denture or cleaned it less than once per day were excluded (3 participants). Furthermore, those that cleaned their denture twice a day were combined with those that clean more than twice per day. However, comparison of total bacteria (Fig 2.6C) [Median: 9.34 (min: 7.87, max: 9.85) v 9.61 (min: 7.24, max: 10.08), $p>0.05$] and *Candida spp*. (Fig 2.6D) [Median: 6.23 (min: 2.7, max: 7.1) v 6.78 (min: 3.18,max: 8.11), $p>0.05$] indicated no differences between the groups.

Finally, for the sleeping with or without a denture *in situ* category, those that said 'sometimes', were grouped with those that said 'yes'. Comparison of total bacteria (Fig 2.6E) [Median: 8.76 (min: 6.78, max: 9.65) v 8.81 (min: 5.3,max: 10.19), $p>0.05$] and *Candida* (Fig 2.6F) [Median: 5.83 (min: 2.7, max: 8.11) v 5.63 (min: 3.18,max: 7.1), $p>0.05$] also revealed no significant changes for either.

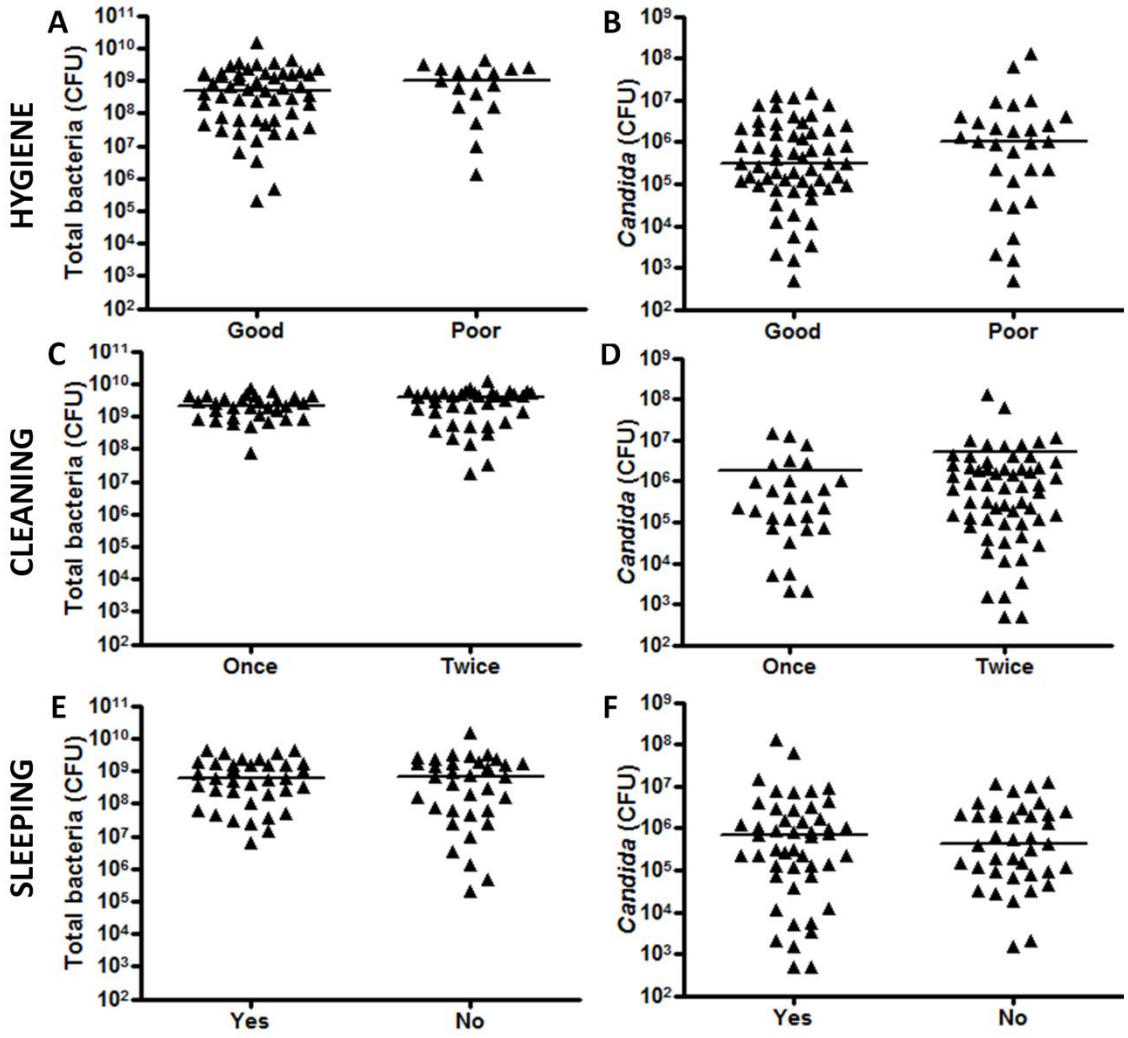


Figure 2.6: Comparison of changes in denture microbiology as a result of oral hygiene practices.
The denture sonicate was assessed by CFU analysis for differences in the quantity of bacteria and *Candida*, respectively for, hygiene status **A)** and **B)**, denture cleaning frequency **D)** and **E)** and sleeping with a denture *in situ* **G)** and **H)**. Data represents median. Statistical analysis was performed using a Mann Whitney test as data did not conform to a normal distribution.

2.4.6 OHIP and patient demographics

The median OHIP sum for the study cohort was 17, ranging between 0 and 56. The healthy group's median OHIP sum was 17.5, while the DS group score 16.2, which was not statistically significant (Kruskal-Wallis test 0.94). The stacked data of each patient's response to the individual questions for the OHIP-14 questionnaire are shown in Figure 2.7. Of the 7 domains this cohort of patients most frequently (very often) experience physical pain, and psychological discomfort. Social disability and handicap was least (never) experienced out of the 7 domains.

The overall OHIP score was calculated for each patient based on their answers from the OHIP-14 questionnaire. These scores were then used to compare between different patient demographic groups including gender, health and disease, denture type and the presence of teeth. Females had significantly higher OHIP scores than males, indicating that dentures have a bigger impact on their oral health related quality of life (Fig 2.8A) [Median: 13 (min: 0, max: 38) v 20 (min: 0, max: 51), $p<0.05$]. The OHIP score for individuals with healthy mucosa was significantly higher than those with DS (Fig 2.8B) [Median: 17.5 (min: 0, max: 56) v 17 (min: 0, max: 51), $p<0.01$]. Participants wearing complete dentures had a significantly higher OHIP scores when compared with those with partial dentures (Fig 2.8C) [Median: 19 (min: 0, max: 56) v 10 (min: 0, max: 41), $p<0.01$]. Edentulous individuals also scored higher than dentate individuals (Fig 2.8D) [Median: 13 (min: 0, max: 51) v 21 (min: 0, max: 56), $p<0.01$].

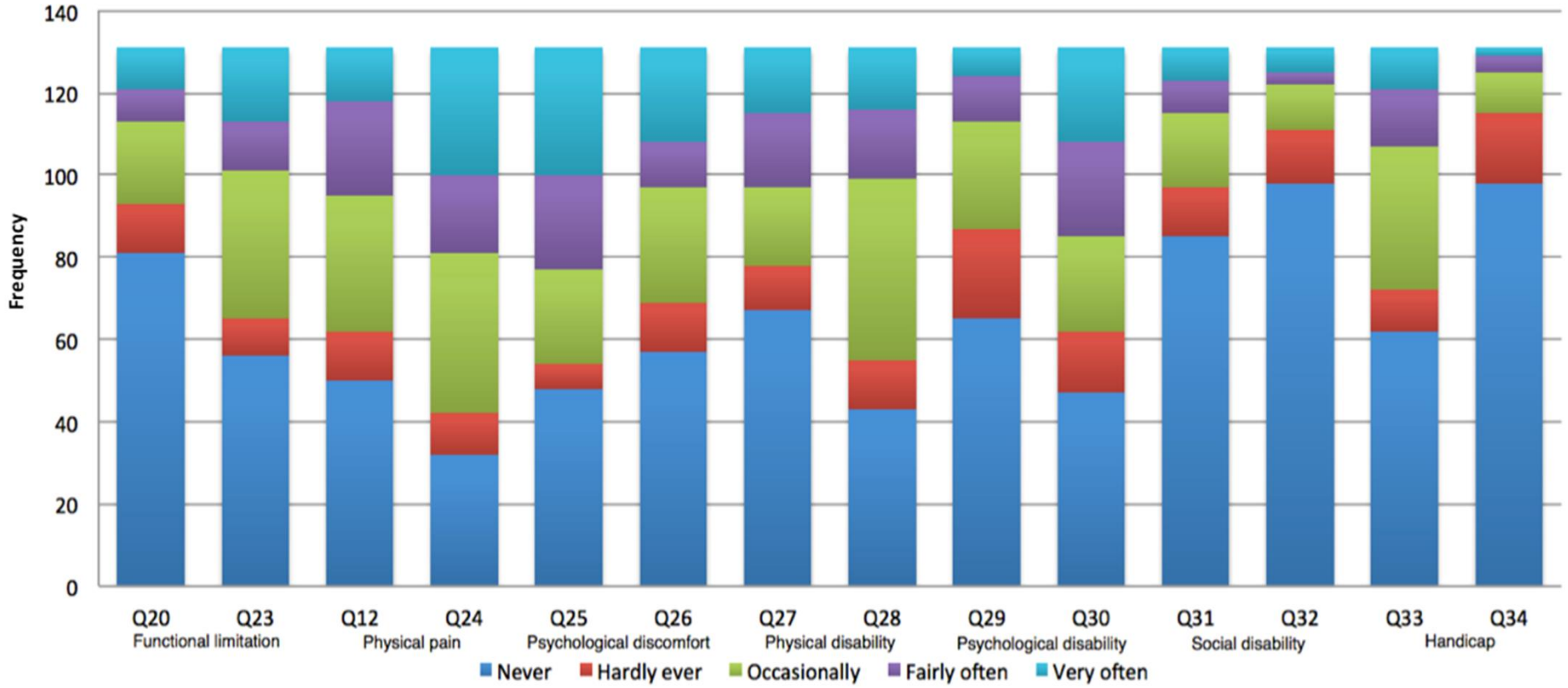


Figure 2.7: Frequency data stacked for each patient's answer of the OHIP-14 questionnaire.
Two questions each representing 7 domains of functional limitation, physical pain, psychological discomfort, physical disability, psychological disability, social disability and handicap were included in the OHIP-14 questionnaire. A Likert scale was used for each question, and the frequency by which patients gave a particular answer is shown for each question in this figure.

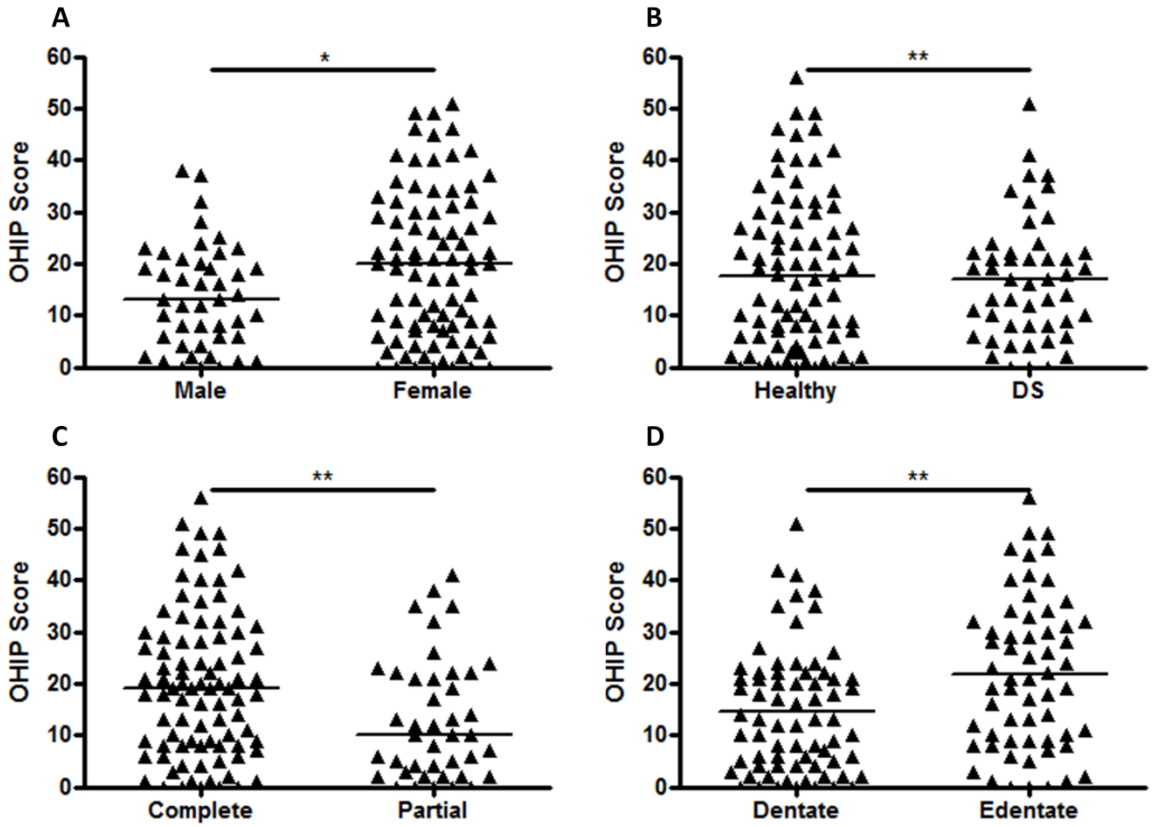


Figure 2.8: Patient demographics and the impact on oral health.
Based on their answers to the OHIP-14 questionnaire, each patient was given an OHIP score. Scores ranged from 0-56. Overall OHIP score was compared across a range of different patient demographics including, gender **(A)**, health & DS **(B),** denture type **(C)** and dentate status **(D)**. Data represents median (* $p<0.05$, ** $p<0.01$). Statistical analysis was performed using a Mann Whitney test as data did not conform to a normal distribution.

2.4.7 Patient and denture hygiene habits and the OHIP

The average overall OHIP score was measured and assessed to see if oral hygiene practices such as hygiene status, denture cleaning frequency and sleeping with a denture *in situ* had a significant impact on general oral health related quality of life. No significant differences in OHIP score were noted between those with good or poor oral hygiene (Fig 2.9A) [Median: 19 (min: 0, max: 51) v 12 (min: 0, max: 56), $p>0.05$] Frequency of cleaning dentures (Fig 2.9B) [Median: 17 (min: 0, max: 56) v 17 (min: 0, max: 51), $p>0.05$] or sleeping with denture *in situ*, (Fig 2.9C) [Median: 19 (min: 0,max: 51) v 10 (min: 0, max: 56), $p>0.05$] did not appear to have a significant impact on oral health related quality of life.

Each of the groups looking at patient and denture hygiene habits were further separated into healthy and DS groups, to investigate if disease status impacted oral health related quality of life when combined with poor oral hygiene habits. The presence and severity of DS appeared to have no effect on OHIP score when comparing hygiene status as no differences were detected between any of the groups (Fig 2.10A) Frequency of which dentures were cleaned each day did not appear to affect whether the patients were healthy or diseased (Fig 2.10B). Moreover, the findings indicated that the patients oral health related quality of life was unaffected by disease status when sleeping with a denture *in situ* (Fig 2.10C).

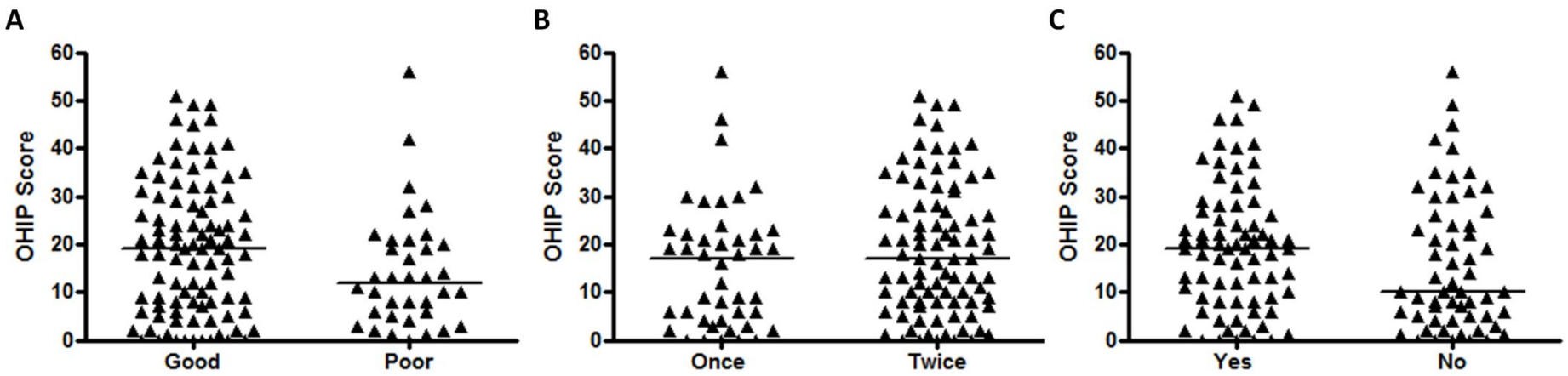


Figure 2.9: The oral health impact of common oral hygiene practices.
Overall OHIP score was assessed for differences between those with good or poor oral hygiene (**A**), those that clean their denture once or twice per day (**B**) and those that sleep with their denture *in situ* and those that do not (**C**). Data represents median. Statistical analysis was performed using a Mann Whitney test as data did not conform to a normal distribution.

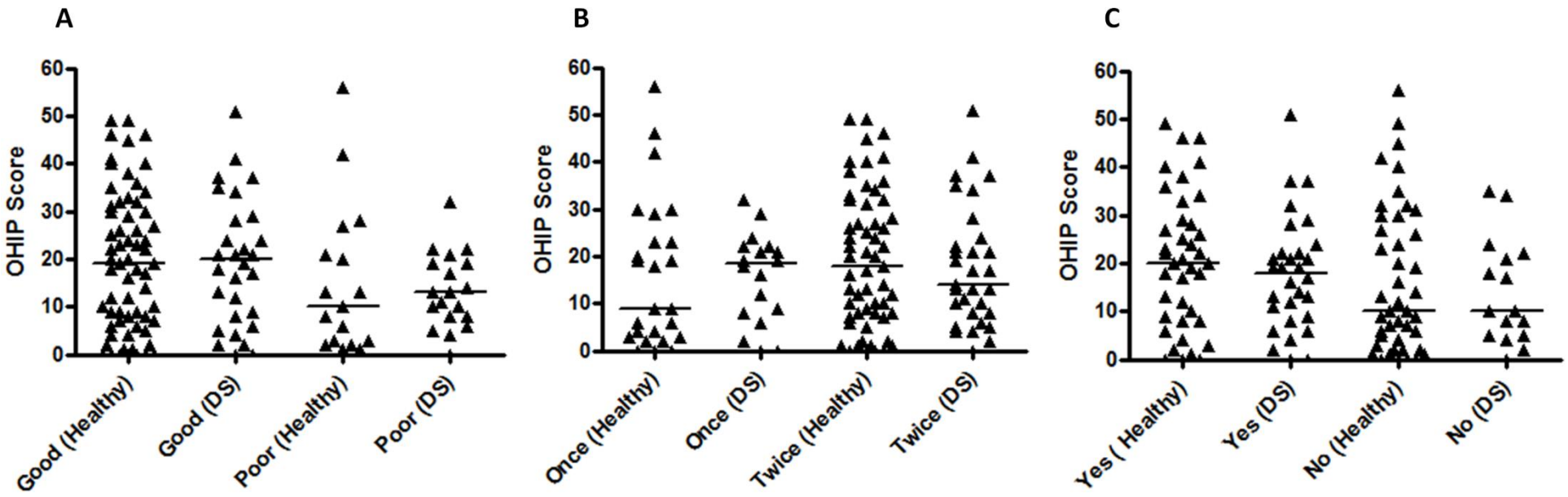


Figure 2.10: The oral health impact of common oral hygiene practices on healthy and DS paients.
Overall OHIP score was assessed for differences between healthy patients and those with DS. Healthy and diseased patient groups were further split into those with good or poor oral hygiene (**A),** those that clean their denture once or twice per day (**B)** and those that sleep with their denture *in situ* and those that do not (**C)** and assessed for differences that may be accounted for by disease status. Data represents median. Statistical analysis was performed using a Kruskal-Wallis test with Dunn's post-test to compare all groups to each other.

2.5 Discussion

Population growth and an increasingly elderly population is leading to a rising demand to deliver sufficient healthcare to the population. Those most often in need of healthcare are the elderly population, and they are most frequently associated with having declining oral health. The identification of associations between oral health and systemic diseases such as cardiovascular disease and pneumonia has led recently to an increase in research interest in this area (Inaba and Amano, 2010, Farquharson et al., 2012). Moreover, the population group most at risk of developing these diseases are the elderly population, i.e. those ≥70 years old. As greater than 70% of the population older than 75 years wear some form of removable prosthesis, it is possible that the associations being made between oral health and systemic disease may somehow implicate denture related hygiene and disease (Iinuma et al., 2015).

Our patient cohort was comprised of predominantly females, which supports previous studies that found a higher prevalence of denture wearers are female (Bertakis et al., 2000). Furthermore, epidemiological studies have found an association with women and increased risk of DS (Kovac-Kovacic and Skaleric, 2000, Nevalainen et al., 1997, Mikkonen et al., 1984). However, the reason why women seem to be more prone to developing DS is unknown.

The prevalence rates of DS, varies considerably, with studies reporting rates of between 15% and 78% (Gendreau and Loewy, 2011). This large variation can be explained by the multifactorial aetiology of DS. Factors such as poor denture fit, poor hygiene, lack of access to dental care and smoking are just some examples that may contribute towards DS development (Gendreau and Loewy, 2011). The DS prevalence rate of 37.4%, reported in this study, falls somewhere in the middle of these two extremes and is similar to other studies conducted within the UK, which report rates of 33.2% and 27% (Zissis et al., 2006, Frenkel et al., 2000). Nonetheless, of the studies reporting DS prevalence rates, there are concerns that not all of them are of a representative population and thus their outcomes may not be a true reflection of the population. Furthermore, amongst these studies different scales are used to categorise DS, whilst the majority used either

Newton's or Budtz-Jorgensen, which are similar and are therefore comparable, other studies do not report using any scales to grade DS. Consequently, inconsistencies across these studies most likely explains the large variation in reported prevalence rates (Gendreau and Loewy, 2011).

This study reports both the proportion of complete denture wearers in comparison to partial denture wearers as well as dentate individuals compared to edentate individuals. The reasoning of this is because not all of the complete denture wearers were fully edentulous, as some had mandibular teeth remaining. This categorisation allows us to understand how denture type and the presence of natural teeth may differentially affect oral health and microbiology of denture wearers. Furthermore, this study reported that 67.9% of participants wore a complete dentures, which is a similar to the rate of 64.6% reported (Bilhan et al., 2012). However, it is difficult to know if these figures are comparative to other denture studies as unfortunately the majority of studies failed to provide the information of whether dentures were complete or partial. As for natural dentition, this study demonstrated that 53.4% of participants were partially edentulous, with a range of 1- 28 natural teeth remaining. The loss of natural teeth has been shown to impact the denture wearer in several aspects of their lives such as, impairing their immune response (Davidopoulou et al., 2013), experiencing physical pain, social and psychological discomfort (Davis et al., 2000) and even compromising their nutritional status (Chauncey et al., 1984).

The significance of understanding as much about our patients as possible cannot be underestimated, given the influence they can for example, have on denture microbiology. Microbial colonisation of the denture is considered the primary cause of denture related disease, yet there is a large gap in understanding its pathogenesis. This is because it is difficult to pinpoint exactly what a healthy or diseased denture microbiome should look like given the vast number of biological and environmental variables that can affect the microbial composition. Of the literature that exists, what is apparent is that there is need of a more in depth understanding of the bacterial microbiology, as traditionally the literature is disproportionally biased towards fungal microbiology (Altarawneh et al., 2013, Dagistan et al., 2009b, Iacopino and Wathen, 1992).

In this study, one of the key questions is to investigate how the denture microbiology varies between healthy individuals and those suffering from DS. The initial CFU analysis revealed that the total bacteria numbers were significantly higher in the DS group, yet in terms of aerobes and anaerobes only anaerobes showed a difference. This suggests that the development of disease is accompanied by a conversion to a more anaerobic microbial composition, yet whether this is a cause or effect of disease requires further investigation. The microbiological approach used here is limiting and therefore the use of selective media may have been more appropriate. However, in terms of identifying the specific species responsible for these changes, a more sophisticated technique such as high throughput gene sequencing may be more appropriate. Furthermore, these findings are reflective of disease progression in periodontal disease (PD). As PD progresses from health to disease, the microbial composition becomes increasingly comprised of anaerobic bacteria (Haffajee and Socransky, 2006).

Our study identified a 72% prevalence of *Candida* species on dentures, of which *C. albicans* was the most predominant, and was the only species in which we saw a significantly higher CFU count in DS individuals. However, *C. glabrata* was also isolated in a high number of patients (40%), which is in line with studies showing the increasing emergence of this species, as it is currently responsible for approximately 13% of invasive candidosis cases (Pfaller et al., 2012, Lewis et al., 2013, Klingspor et al., 2015, Coco et al., 2008a). In addition, the frequency of *C. glabrata* oral carriage rate has been shown to increase with age. Of particular interest was the number of patients in which both *C. albicans* and *C. glabrata* were co-isolated, this was high particularly amongst DS sufferers (35%). Recent studies have suggested that when co-cultured *C. albicans* and *C. glabrata* form a more pathogenic and invasive biofilm than either species alone, this may contribute to more severe cases of DS (Alves et al., 2014). However, despite showing that DS is more common in dentate individuals, we found significantly higher *Candida* CFU counts on edentate patients and complete dentures when compared to dentate and partial dentures, respectively. This may be due to the less diverse microbiome of the edentulous patients, as with fewer microorganisms, this opens up a niche for *Candida* spp. to colonise.

Maintenance of oral hygiene is important to keep the number of microbes residing on the denture and in the mouth low in order to prevent disease development. Clinicians and dentists stress the importance of maintaining good denture hygiene, yet there is not currently a gold standard technique of what is the best method. Poor hygiene practices and habits are commonplace amongst denture wearers (Gendreau and Loewy, 2011). Yet, interestingly this study found no differences between groups for bacteria or *Candida* levels in the hygiene, cleaning frequency, or the sleeping categories. However, this may reflect limitations in the sensitivity of CFU analysis for quantifying microbes. Nonetheless, although there appears to be little change in the abundance of microbes, this is not to say that the microbiome composition had not altered.

Additionally, focusing solely on the microbiology of dentures does not completely represent all the problems people experience with them. The use of the OHIP-14 questionnaire has been shown to be extremely useful within this study in helping to identify key factors which may impact on oral health related quality of life (Allen and McMillan, 1999, Locker et al., 2001). Identifying these issues will help communication between patient and clinician during treatment as some of the patient related factors cannot be corrected by new dentures or hygiene regimens. The results of the OHIP-14 questionnaire show patients wearing dentures have a wide range of disorders impacting their daily lives. In particular physical pain and psychological discomfort was frequently reported. This is unlikely to be apparent from just a clinical examination, and is supported elsewhere (Jagger et al., 2006, Mericske-Stern, 1990).

The OHIP data suggests that dentures have a greater impact on females oral health related quality of life than males. This maybe a true difference between genders, however, as females are reported to access health care and report problems more frequently than males, this may explain our findings particularly given that 69% of the study cohort was female (Bertakis et al., 2000). Interestingly, individuals with DS did not report to having a more difficult experience with their dentures than their healthy counterparts, thus emphasising the point that factors other than disease must be taken into account when assessing oral health. Furthermore, fully edentulous individuals with complete dentures have a worse experience with dentures than partially dentate individuals. This could be because participants

feel that their partial dentures are more stable as they are supported by the remaining natural teeth, and are therefore more likely to stay in place. Unstable dentures may cause discomfort, and the increased risk that they may fall out could lead to both social and psychological anxiety.

In summary, understanding as much information regarding the study cohort is essential when undertaking such a comprehensive study. Looking at basic denture microbiology, we have shown that DS sufferers appear to have a more anaerobic microbiome, yet whether this indicates increased pathogenicity requires further study. Moreover, in terms of *Candida* species, the elevated levels of *C. albicans* in DS patients is unsurprising, however the increasing isolation of *C. glabrata* is interesting particularly given its association with antifungal resistance. Furthermore, using the OHIP-14 data we have demonstrated that other dentures can have an impact on several aspects of the patients' life, and is not solely disease related. Therefore, we have highlighted the importance for future denture related studies not to solely focus on the biology, but also to take into account the physical and psychological factors which may be affecting the patients' general oral health, in order to gain a more rounded understanding of the patient's condition.

CHAPTER FINDINGS

Anaerobic bacteria are significantly more abundant on the denture of DS sufferers.

Candida is significantly more abundant on the dentures of DS sufferers, and at the species level this could be attributed to *C. albicans*.

Edentulous individuals and complete denture wearers have significantly more *Candida* on their dentures.

Hygiene status and common hygiene habits and practices did not alter the CFU levels of bacteria or *Candida* on dentures.

Being fully edentulous and having a complete denture have a more negative impact on the individuals oral health related quality of life.

3 Defining the role of the oral microbiome of denture wearers

3.1 Introduction

Within the oral cavity, approximately 700 bacterial species have been identified using the human oral microbiome database (HOMD) (Chen et al., 2010). Given such a vast number of microbes, defining the composition of a 'typical healthy' oral microbiome is problematic as it is likely to be affected by a combination of both biological and environmental factors, each with the ability to alter the microbial composition. Furthermore, the addition of a prosthetic denture to the oral cavity will only further add to the complexity of understanding this environment. Ultimately, this means that each individual have their own unique oral microbiome.

The primary disease condition that denture wearers suffer from is denture stomatitis (DS). Given that around one fifth of adults wear some form of removable denture prosthesis, this represents a large proportion of the population, and thus DS should be considered a disease of greater importance (Shulman et al., 2004). DS refers to inflammation of the oral mucosa and pathological changes associated with the wearing of dentures (Gendreau and Loewy, 2011), and can be classified according to the severity of inflammation using a scale first described by Newton (Jeganathan and Lin, 1992, Newton, 1962b). The aetiology of DS is related to a variety of factors including poorly fitting dentures causing trauma and biological factors such as poor salivary flow, smoking or antibiotic treatment, as well as microbial infection (Salerno et al., 2011).

Despite the individuality of the oral microbiome certain oral diseases have a 'pattern' of particular bacteria associated with the disease. In periodontal disease there appears to be a well defined ecological shift in the microbiome, affected by different environmental stressors (Socransky et al., 1998). However in terms of denture related disease, there is very little evidence of the microbial changes that occur when going from a healthy to diseased phenotype. Nonetheless, without firm evidence base DS is regarded as a disease of yeast origin, primarily *Candida albicans*. It affects approximately 30-70% of denture wearers (Gendreau and Loewy, 2011), with *Candida spp*. colonising the denture surface to form co-aggregates with bacteria and other yeasts to build complex microbial communities

known as biofilms. The majority of literature in this area focuses solely on *Candida* spp. as the primary cause of infection, however, there is increasing evidence to suggest that this is very much a polymicrobial disease in which bacterial and yeast interactions play a role in disease pathogenesis (Morales and Hogan, 2010, Shirtliff et al., 2009). Several studies have isolated bacteria directly from the surface of dentures using standard microbial culturing techniques, primarily streptococci and staphylococci species (Sumi et al., 2002, Ealla et al., 2013, Daniluk et al., 2006, Sumi et al., 2003). However, culture based methods do not always give a comprehensive representation of the polymicrobial population, which can contain up to 10^{11} microbes per milligram of denture plaque (Nikawa et al., 1998). The advent of high throughput sequencing, however, has revolutionised our understanding of microbial ecosystems, and thus using this superior method we can for the first time gain an insight into the oral microbiome of denture wearers.

Understanding the microbiome may not be so straightforward, as many factors can alter the composition, with denture hygiene thought to have a strong impact. Moreover, good denture hygiene practice is not always commonplace amongst denture wearers. This could be attributed to the fact that the majority of individuals are elderly, many of whom are institutionalised and are unable to implement or are simply unaware of the importance of maintaining good denture hygiene (Petersen and Yamamoto, 2005). Failure to clean the denture and oral cavity sufficiently can lead to an accumulation of bacterial and fungal plaque on the denture surface and is thought to be one of the main causes of denture-related disease. Therefore, understanding how key factors such as hygiene habits and practices can impact the composition, is crucial to gaining a more comprehensive picture of the microbiome.

In addition to an incomplete understanding of the composition of denture plaque, we also have limited knowledge and understanding of the local host immune response. It has been established that the immune response is gradually impaired with increasing age (Muller and Pawelec, 2014, Castelo-Branco and Soveral, 2014), but with the addition of loss of natural teeth an even greater rapid decline in host protective responses in the oral cavity is reported (Davidopoulou et al., 2013). Antimicrobial peptides (AMP) including cathelicidin LL-37, histatins and defensins, which exhibit antimicrobial and immunoregulatory properties and protect mucosal

surfaces against pathogens, have been found to be present in saliva. However, no studies have yet investigated salivary AMPs in denture wearers and the potential role they may play in the protection against denture plaque. Thus, a more in depth investigation is required into the host-microbiome relationship in denture wearing individuals, which may help towards understanding the pathogenesis of DS.

3.2 Aims

It is hypothesised that the oral ecology of natural and non-natural surfaces of denture wearers is affected by host related factors, which in turn affects disease outcomes. Therefore, the aim of this chapter was to carry out a detailed site-specific analysis of the oral microbiome of denture wearers using high throughput 16S rRNA gene sequencing technology, and to further this with respect to candidal load. A secondary aim was to investigate the relationship between denture plaque composition, oral disease and host related factors, including immunological and behavioural parameters. This is the first study to provide such a detailed microbial analysis of denture biofilms. The following key questions were investigated:

- What is the oral microbial composition of the denture, mucosal tissue and dental plaque of a denture wearer?
- Does the composition vary between sample sites, and if so, to what extent?
- Is microbiome composition different between health and DS?
- Does the presence of natural teeth or the denture type have an impact on the microbiome composition?
- How does candidal load effect the composition of the microbiome?
- Is there a relationship between host factors and microbiome composition?
- How does denture hygiene effect the microbiome on the denture?
- How does denture hygiene effect the microbiome of the mucosa?

The data represented in this chapter has been published in:

O'Donnell LE, Robertson D, Nile CJ, Cross LJ, Riggio M, Sherriff A, et al. The Oral Microbiome of Denture Wearers Is Influenced by Levels of Natural Dentition. PLoS One. 2015;10(9):e0137717.

Work from this chapter has been presented at the following conferences:

L. O'Donnell, E. Zaura, B. Brandt, C. Nile, D. Robertson, G. Ramage. 'Host microbiome interactions in the oral cavity of a denture wearer'. International Association for Dental Research (IADR), Boston, USA, March 2015.

L. O'Donnell, E. Zaura, B. Brandt, C. Nile, D. Robertson, G. Ramage. 'Microbiome Interactions in the Oral Cavity of a Denture Wearer'. British Society for Oral and Dental Research (BSODR), Cardiff, UK, September, 2015.

Illumina sequencing and initial data sequencing analysis was carried out at the department of Preventive Dentistry, Academic Centre for Dentistry Amsterdam, University of Amsterdam and VU University Amsterdam, Amsterdam, the Netherlands. The key individuals involved at ACTA were Mark Buijs, who carried out DNA extractions, qPCR and sequencing and Egija Zaura and Bernd Brandt who were involved in the initial sequencing data analysis. My role was primarily in all the downstream analyses.

3.3 Methods

3.3.1 Sample collection and processing

Samples intended for 16S high throughput gene sequencing, which included the denture swab, mucosal swab and dental plaque sample, were collected as previously described (section 2.3.2). After collection, the samples were transferred to a category II laboratory for processing and storage at - 80 ºC. Upon completion of sample collection 131 denture swabs, 131 mucosal swabs and 79 dental plaque samples were transferred to the department of preventive dentistry, Academic Centre for Dentistry Amsterdam, VU University Amsterdam for DNA extraction and sequencing.

3.3.2 DNA isolation of high throughput sequencing samples

DNA was extracted from 131 denture swabs, 131 mucosal swabs, and 79 dental plaque samples. Dental plaque samples were centrifuged for 15 min @ 13,000 x g the supernatant was removed and the sample resuspended in 150 µl TE buffer. Swab samples were sonicated for 30 sec, and then the sonicated fluid was transferred into a deep well plate and centrifuged for 15 min at 13,000 x g. The precipitate was then resuspended in 150 µl TE buffer. All samples were then transferred to a plate with each well containing 0.25 ml of lysis buffer (AGOWA mag Mini DNA Isolation Kit, AGOWA, Berlin, Germany), 0.3 g zirconium beads (diameter, 0.1 mm; Biospec Products, Bartlesville, OK, USA) and 0.2 ml phenol. The samples were homogenized with a Mini-beadbeater (Biospec Products) for 2 min. DNA was extracted with the AGOWA mag Mini DNA Isolation Kit. During the DNA extraction process not all samples had sufficient DNA to use for sequencing, therefore DNA from 108 denture samples, 87 mucosal samples and 63 dental samples remained for sequencing, collectively all these samples originated from 123 patients. This process was carried out by Mark Buijs (ACTA).

3.3.3 Quantitative PCR of high throughput sequencing samples

Real time qPCR was performed to determine the concentration of DNA per sample. Primers and a probe for the 16S rRNA gene were used, (F: TCCTACGGGAGGCAGCAGT, R: GGACTACCAGGGTATCTAATCCTGTT Probe: 6FAM-CGTATTACCGCGGCTGCTGGCAC-BBQ). As an internal control for PCR inhibition a

qPCR of PhHV (Phocid herpesvirus type 1 gB gene) was also performed as described by Watzinger et al (2004). The total reaction volume was 20 µl, including 3 µl of DNA. Reactions contained a 26PCR Probe Master Mix (Roche), for 16S rRNA, 7.5 pmol primers and 3.8 pmol probe. For PhHV 1.8 pmol primers and 0.4 pmol probe of each primer was used. qPCR was carried out using the Light cycler LC480-II (Roche Diagnostics, Switzerland) under the following conditions: an activation step of 10 min at 95ºC, followed by 50 cycles consisting of a denaturation step at 95ºC for 30 sec, an annealing step at 60ºC for 30 sec, and an extension step at 72ºC for 30 sec. Bacterial 16S rDNA concentrations (CFU/mL) were determined from standard curves of E. coli K12 cultures.

3.3.4 PCR amplification and Illumina sequencing

Amplicon libraries of the V4 hypervariable region of the 16S rRNA gene were generated for each of the individual samples. PCR was performed using the forward primer 515F (GTGCCAGCMGCCGCGGTAA) and the reverse primer 806R (GGACTACHVGGGTWTCTAAT) (Caporaso et al., 2011). The primers included Illumina adapters and a unique 8-nt sample index sequence key (Kozich et al., 2013). The amplification mix contained 2 units of Phusion HotStart II High fidelity polymerase (Thermoscientific), 1 unit Buffer Phusion HS II (5x), including 1.5 mM MgCl2 (Thermoscientific), 0.2 mM dNTP (Thermoscientific, Germany) and 1 µM of each primer. To each reaction 1 ng of DNA template was added. After denaturation (98ºC; 30 sec), 35 cycles of denaturation (98ºC; 10 sec), annealing (55ºC; 30 sec), and extension (72ºC; 30 sec) were performed. Individual amplicon libraries were analyzed for DNA content with the fluorescent Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen). The libraries were pooled in equimolar amounts. The amplicons were purified by means of the IllustraTM GFXTM PCR DNA and Gel Band Purification Kit (GE Healthcare, Eindhoven, the Netherlands). The quality and the size of the amplicons were analyzed on the Agilent 2100 (Santa Clara, CA, USA). The amplicon was sequenced in paired end mode on a MiSeq sequencing system (Illumina, Eindhoven, the Netherlands) with the v2 kit (Illumina) (Caporaso et al., 2012, Kozich et al., 2013).

3.3.5 Sequencing data analysis

Reads were first quality filtered using Trimmomatic v0.32, (Bolger et al., 2014). Next, the reads were merged using fastq-join implemented in QIIME v.1.8.0 (Bolger et al., 2014). Sequences were clustered into operational taxonomic units (OTUs) using USEARCH v7.01090 (Edgar, 2013), after being quality filtered with usearch (maxee 0.5). The representative sequence of each cluster was assigned a taxonomy using the RDP classifier (ref: Cole JR, Wang Q, Cardenas E, Fish J, Chai B, et al. (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucl Acids Res 37: D141–145. doi: 10.1093/nar/gkn879) (QIIME v.1.8.0) (Greengenes v13.8 97_otus set) with a minimum confidence of 0.8. All bioinformatics analysis was carried out by Bernd Brandt (ACTA).

3.3.6 *Candida* CFU quantification

The denture sonicate was processed and plated out onto *Candida* colorex agar plates, the colonies were counted and the abundance per mL was quantified, as previously described (section 2.3.4).

3.3.7 ELISA testing

Clarified saliva samples were used to assess the presence of AMPs within saliva by enzyme-linked immunosorbant assay (ELISA). ELISA kits for LL-37 (Hycult biotech, The Netherlands), Calprotectin (Hycult biotech), Lactoferrin (Hycult biotech), HNP1-3 (Hycult biotech), Histatin 5 (Stratech scientific, Suffolk, UK) and Beta defensin 1 (BD1) (Peprotech, London, UK) were used according to manufacturer's instructions. Clarified saliva samples were diluted 1:5 in assay buffer (PBS, 0.5% BSA, 0.1% Tween20).

3.3.7.1 LL-37, Calprotetin, Lactoferrin and HNP1-3 ELISA

For the Hycult biotech kits, LL-37, Calprotectin, Lactoferrin and HNP1-3, all reagents within the kit were brought to room temperature (20-25^{o}C) before use. Plates were pre-coated with capture antibody and therefore were ready for use directly from the kit. 100 µL of each sample and standards of known concentration were loaded into the plate provided in duplicate. Standard concentrations varied

depending on the peptide being detected. Plates were left to incubate for 1 h at room temperature. Contents were then discarded and washed with 200 µL of the wash buffer provided, this was repeated a further two times. 100 µL of diluted tracer antibody was added and the plate was incubated at room temperature for 1 h. The plates were washed as previous and 100 µL of streptavidin-peroxidase was added. Plates were incubated for 1 h at room temperature and then washed. 100 µL of 3,3',5,5'-tetramethylbenzide (TMB) substrate was added and the plates were incubated in the dark for 30 min. The reaction was stopped by adding 100 µL of the stop solution provided. Absorbance was read using a plate reader (FLUOstar Omega BMG Labtech, VA, USA) at 450 nm with a 650 nm wavelength correction. A standard curve was constructed by plotting the mean absorbance for each standard against the appropriate protein concentration and the R-squared calculated using a computer program (Omega analysis software, VA, USA). Results were calculated using a 4-parameter curve fit to determine the concentration of protein release in samples tested.

3.3.7.2 Beta defensin 1 ELISA

For the BD-1 ELISA, Capture antibody (1 µg/mL) was diluted in PBS to a concentration of 0.5µg/mL and 100 µL added to each well of a Nunc™ Maxisorp® flat bottomed microtitre plate (Fisher, Loughborough, UK). Plates were sealed and incubated overnight at room temperature. Contents were then discarded and washed 4 times with 300 µL of wash buffer per well. Plates were then blocked with 300 µL of the block buffer provided for 1 h at room temperature to block non-specific binding. Plates were washed as previously described (section 3.3.7.1). Standards were serially diluted in diluent with concentrations ranging from 0 - 1 ng/mL. 100 µL of samples and standards were added to each well and incubated at room temperature for 2 h. Plates were washed and 100 µL of detection antibody, which had been diluted to a concentration of 0.5 µg/mL, was added. Plates were incubated for 2 h at room temperature and were washed as previous. The avadin-HRP conjugate was diluted 1:2000 in diluent and 100 µL was added to each well and left to incubate for 30 min at room temperature. Plates were washed and 100 µL of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) substrate was added to each well. Colour development was monitored and absorbance was read using a plate reader (FLUOstar Omega BMG Labtech) at 405

nm with a 650 nm wavelength correction. Standard curves were prepared and results calculated as previously described (section 3.3.7.1).

3.3.7.3 Histatin 5 ELISA

All reagents were prepared for use as per manufacturer's instructions. Plates were pre-coated with the capture antibody. 100 µL of samples and standards, were added to the appropriate wells with concentrations ranging from 800 to 0 ng/mL. Plates were sealed and incubated for 2 h at 37°C. Liquid was removed from each well but the plate was not washed. 100 µL of biotin-antibody was added to each well, which was then incubated for 1 hour at 37°C. Plates were washed with 200 µL per well of the provided wash buffer, this was repeated a total of three times. 100 µL of HRP-avidin was added per well, plates were sealed and incubated for 1 h at 37°C. Plates were washed 5 times. 90 µL of TMB substrate was added to each well and incubated in the dark for 15-30 min. 50 µL of stop solution was added per well. The absorbance was read using a plate reader (FLUOstar Omega BMG Labtech) at 450 nm with a 570 nm wavelength correction. Standard curves were prepared and results calculated as previously described (section 3.3.7.1).

3.3.8 Analysis of oral hygiene statistics

The data collected from the clinical information sheet and oral health questionnaire was collected and analysed, as previously described (section 2.3.3). In order to assess the effect of denture hygiene on the microbiome, three categories were selected:

1. Oral hygiene, classed as either good or poor.

2. Frequency of denture cleaning. Participants were separated in two groups, those that cleaned their denture once per day and those that cleaned twice.

3. Sleeping with or without a denture *in situ*. Participants were separated into those that slept with their denture in situ and those that did not.

Patient demographics regarding oral hygiene status, denture cleaning frequency and denture sleeping habits are shown in table 2.2 of the patient demographics chapter 2.

3.3.9 Statistical analyses

The data set was randomly sub-sampled to 770 reads per sample (minimum number of reads per sample was 776) to include the maximum number of samples for analysis. OTU datasets were reduced by log2 transformation so as to carry out principal component analysis (PCA) and diversity statistics (Shannon diversity index and Dominance index). Analysis was carried out using PAST software (Hammer O, 2001).

A one-way ANOVA test was applied to compare diversity statistics at oral microbiome sites using GraphPad Prism software (version 4; La Jolla, CA, USA). Principal componant analysis (PCA) was used to reduce the dimensionality of the OTU dataset. 258 items were entered into the PCA. A scree plot was used to determine how many components emerged. Factor loadings above 0.15 on a component were considered to have a strong association with that component and were deemed to be the most informative in describing the microbiome components. To determine if distinct clusters formed for each group on the PCA plots, new variables were created for each principle component by using the factor loadings as regression coefficients, producing a score for each sample. These scores were then used as outcome variables to compare between groups (using t-tests where appropriate- dentate/edentate and complete/partial dentures groups).

The contribution of each bacterial class was calculated in terms of proportion to the overall sample and an unpaired T-test was used to compare health and DS and smoker and non-smoker groups. Diversity statistics were compared via a t-test with GraphPad Prism v5. Spearman's rank correlation was used to assess correlations between the abundance of individual bacterial classes or genera with the proportion of *Candida* spp. found on dentures (CFU counts), using SPSS version 20.

Salivary concentrations of AMPs were compared between healthy and DS groups, dentate/edentate groups and complete/partial groups. Data were log transformed and analysed using a t-test with GraphPad Prism. Furthermore Spearman's rank correlation was used to assess correlations between the abundance of individual bacterial classes or genera found on dentures with the concentrations of salivary AMPs.

To visualize the relationships and associations of the microbiomes with environmental variables, canonical correspondence analysis (CCA) was applied. This form of analysis, carried out using PAST, allows the visualisation of OTU distribution and sample group distribution in relation to a number of environmental variables. Environmental variables included were *Candida* CFU counts and salivary concentration of a number of AMPs. The significance of each of the CCA axes was calculated by permuting the data 999 times.

Candida CFU counts were compared between healthy and DS groups, dentate/edentate groups and complete/partial groups. Data were log transformed and analysed using a t-test with GraphPad Prism v5, as indicated by the mean and median values shown throughout the results section.

The contribution of each bacterial class was calculated in terms of proportion to the overall sample, a Mann-Whitney test was used to compare between denture hygiene status, cleaning frequency and sleeping with denture *in situ* to determine statistical significance. Diversity statistics used a Mann Whitney test to compare between the same groups. A Kruskall-Wallis test with Dunns post-hoc test was employed to compare differences when the groups were further split into healthy and DS groups to account for multiple pair-wise comparisons.

3.3.10 Study Design

The study was designed as a pilot study and was initially only powered to detect a biologically meaningful association between health and DS and microbiome composition, and therefore was not originally powered to detect differences between additional variables including, denture type, dentate status, *Candida*

spp. levels and salivary AMP levels. Thus, non-significant results between these variables are not necessarily absence of effect, but a result of not achieving the full sample size required.

3.4 Results

3.4.1 Illumina sequencing output

Illumina high throughput gene sequencing was carried out on 108 denture swabs, 87 mucosal swabs and 63 of the dental plaque clinical samples that were collected. Across all of the samples 632 OTUs were identified, with an average of 94 OTUs per sample (SD 39; min 25, max 254), 536 of which contained a minimum of 5 reads. The data was sub-sampled to 770 reads per sample in order to avoid bias of variable sample size. After sub-sampling 502 OTUs remained with an average of 46 OTUs per sample (SD 18; min 11, max 121). The samples were categorised into five main phyla which represented 99.6% of the reads: Firmicutes (40.6%), Actinobacteria (23%), Bacteriodetes (22.2%), Proteobacteria (9.8%) and Fusobacteria (4%). Samples were categorised into groups according to sample site: denture (n=108), mucosal (n=87) and dental (n=63), with 337, 414 and 306 OTUs identified, respectively.

3.4.2 Oral microbiome by sample site

Clinical samples were separated into groups according to sample site: denture (n=108), mucosal (n=87) and dental (n=63), with 337, 414 and 306 OTUs identified, respectively. On average, denture samples had 42 OTUs (SD 14; min 13, max 87), mucosal 49 OTUs (SD 20; min 11, max 121) and dental 51 OTUs (SD 37; min 16, max 306) (Figure 3.1A). Interestingly, denture plaque samples were found to have significantly less OTUs compared to dental, ($p<0.01$), and mucosal, ($p<0.05$), plaque. Figure 3.1B shows the relative abundance of bacterial taxa at the class level present in each sample type, and demonstrates considerable variation in taxonomic profiles between sites. *Actinobacteria* and *Bacilli* were the two predominant classes found at denture and mucosal sites, comprising 75.2% and 66.4%, respectively, of the overall composition when combined. However, *Actinobacteria* and *Bacilli* only contributed only 30% to dental plaque. Diversity statistics applied across samples revealed that dental plaque is more diverse (Figure 3.2A) than both denture plaque (Mean: 2.35 v 2.89, $p<0.001$), and mucosal surfaces (Mean: 2.44 v 2.89, $p<0.001$), with less dominant taxa according to the dominance index ($p<0.001$) (Figure 3.2B.).

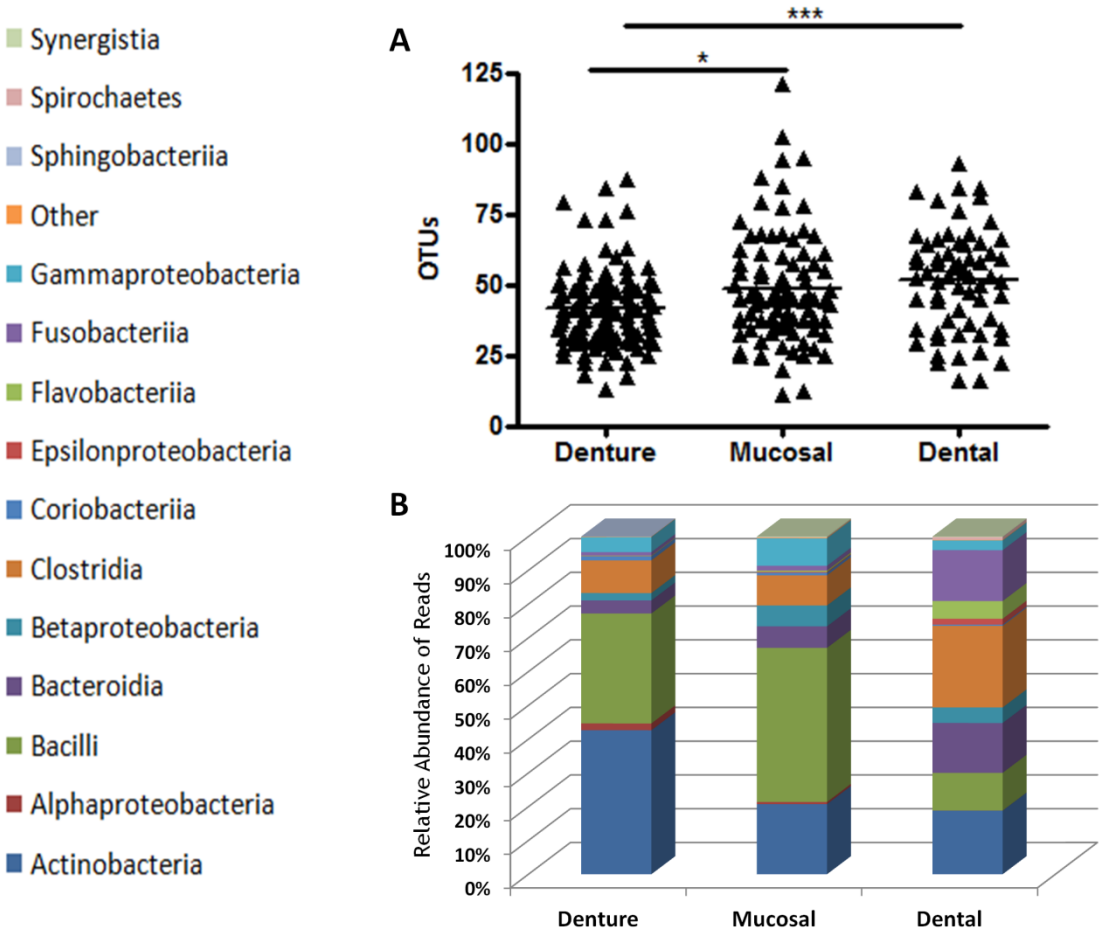


Figure 3.1: Microbiome composition of various sample sites in the oral cavity of denture wearers.
108 denture swabs, 87 mucosal swabs and 63 dental plaque samples underwent pyrosequencing of the 16S hypervariable V4 region to determine the bacterial composition of denture, palatal mucosal and dental plaque. **(A)** Number of OTUs identified per sample at each sample site **(B)** Relative abundance of bacterial taxa at the class level per sample site. Data represents the mean (*p<0.05, *** p<0.001). Statistical analysis was performed using a one-way ANOVA with Bonferonni post-test to compare all groups to each other.

PCA was applied to reduce the multidimensionality of the dataset. Two primary principal components emerged from this analysis. On average, dental samples scored higher on PC1 than denture or mucosal samples (p<0.0001) (Fig 3.3A), indicating higher frequencies of *Fusobacterium, Corynebacterium, Selenomonas, Campylobacter* and *Prevotella*, and lower of *Streptococcus* and *Rothia*. Furthermore, when denture and mucosal samples were directly compared, denture samples had a higher average score on PC1 than mucosal samples (p=0.0006) (Fig 3.3B). This indicated higher frequencies of *Lactobacillus*,

Actinomyces, *Atopobium* and *Scardovia,* and lower frequencies of *Streptococcus*, *Rothia* and *Haemophilus*.

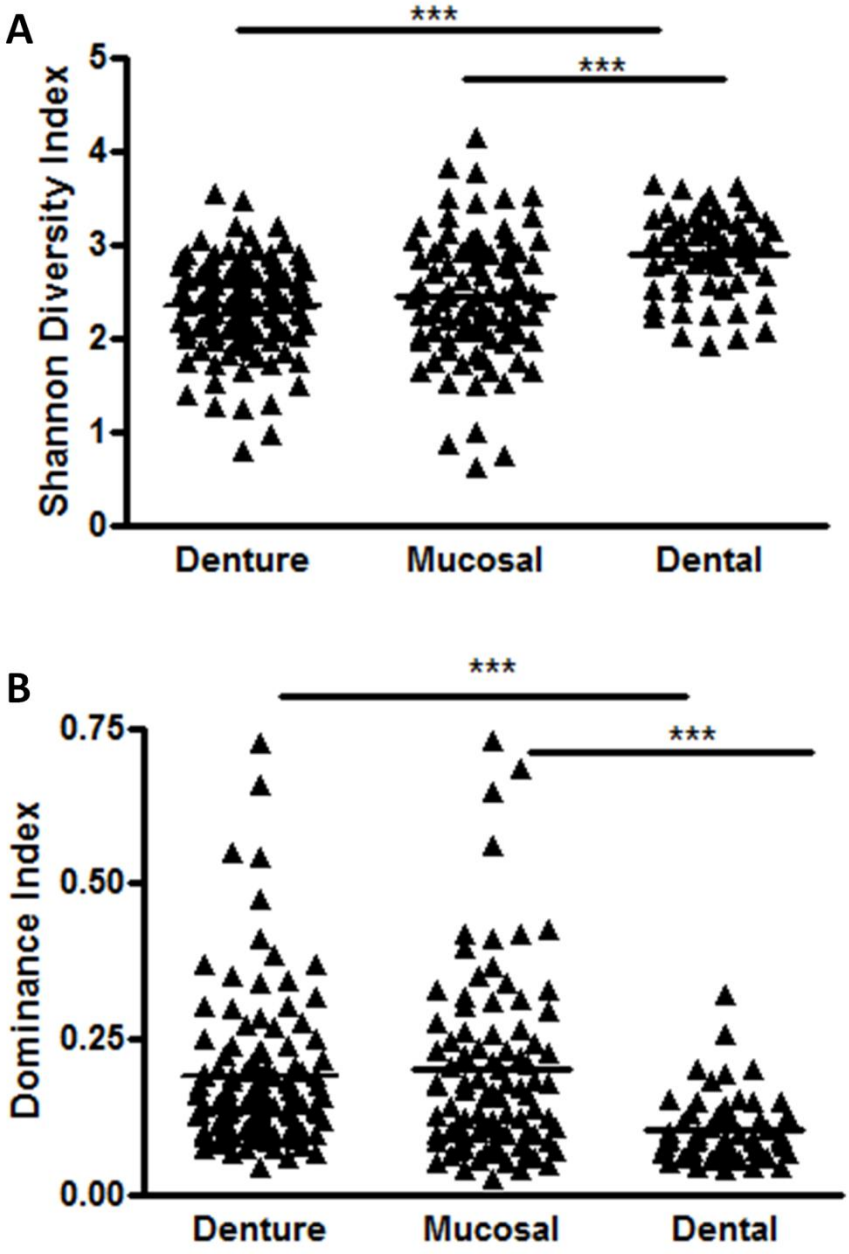


Figure 3.2: Microbial diversity varies between sample sites in the oral cavity of denture wearers.
The taxonomic diversity and dominance of each group was analysed and compared via a (**A)** Shannon Diversity Index and (**B)** Dominance Index. Data represents the mean (*** $p<0.001$). Statistical analysis was performed using a one-way ANOVA with Bonferonni post-test to compare all groups to each other.

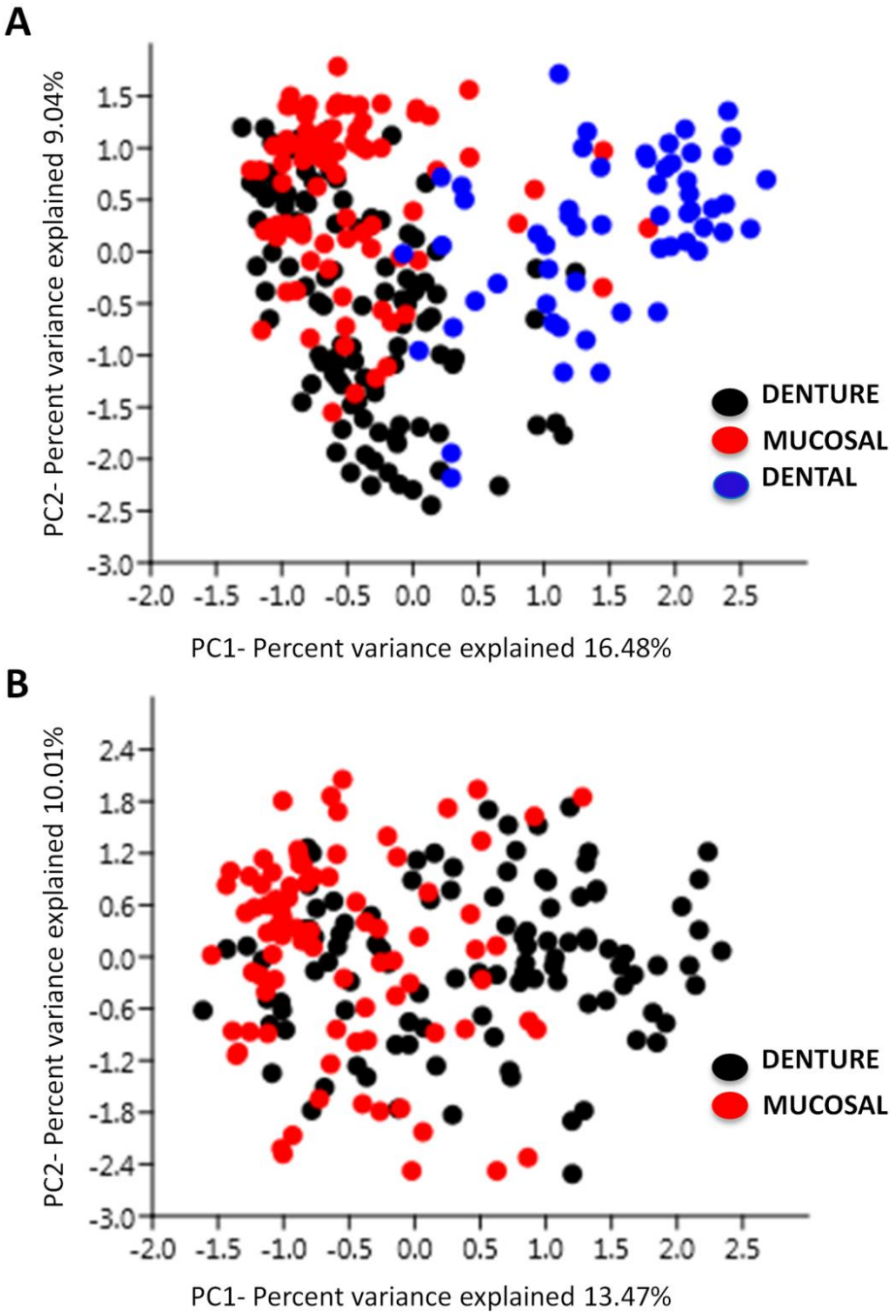


Figure 3.3: Principal component analysis of denture, mucosal and dental samples.
PCA was applied to all sample types to reduce the multidimensionality of the data and to determine variances between **(A)** Denture, Mucosal and Dental groups and **(B)** Denture and Mucosal groups. For statistical analysis new variables were created for each principle component by using the factor loadings as regression coefficients, producing a score for each sample. These scores were then used as outcome variables to compare between groups using a two-tailed unpaired t test.

3.4.3 Dentate v Edentate and partial v complete denture microbiome

Samples were separated into groups depending on denture type (complete/partial) and on the presence or absence of teeth (dentate/ edentate), and the microbiome were compared at the denture and mucosal sites. PCA was applied to the data, and as previous, two main principal components emerged from this analysis. When comparing denture type on the denture samples (Fig 3.4A), partial dentures scored higher on PC2 than complete denture samples ($p=0.0073$), revealing higher frequencies of *Actinomyces, Haemophilus, Corynebacterium* and *Veillonella* and lower frequencies of *Lactobacillus* and *Streptococcus*. Denture samples were then split into dentate and edentate groups (Fig 3.4B). Dentate samples scored higher along PC2 than edentate ($p=0.0194$), and as with the partial denture group, they had higher frequencies of *Actinomyces, Haemophilus, Corynebacterium* and *Veillonella* and lower frequencies of *Lactobacillus* and *Streptococcus*. At the mucosal microbiome partial dentures scored higher on PC1 than complete denture samples (Fig 3.4C) ($p=0.0001$), and showed higher frequencies of *Actinomyces, Prevotella, Haemophilus* and *Neisseria* and lower frequencies of *Streptococcus, Lactobacillus* and *Jathinobacterium*. No differences in scores were found between dentate and edentate samples (Fig 3.4D).

The diversity of the same sample groups were compared and revealed that partial denture samples were significantly more diverse than those from complete dentures at both the denture (Mean: 2.29 v 2.52, $p<0.05$) and mucosal surfaces (Mean: 2.34 v 2.67, $p<0.05$], (Fig 3.5A and 3.5E). Furthermore, the dentate patients had a significantly more diverse microbiome than the edentate, (Fig 3.5C and 3.5G); these results were found at both the denture (Mean: 2.45 v 2.25, $p<0.05$) and mucosal surfaces (Mean: 2.6 v 2.25, $p<0.05$). In terms of microbial dominance of samples, the dominance index showed no significant differences between groups at the denture (Fig 3.5B and 3.5D) and mucosal surfaces (Fig 3.5F and 3.5H) for both complete/partial and dentate/edentate groups.

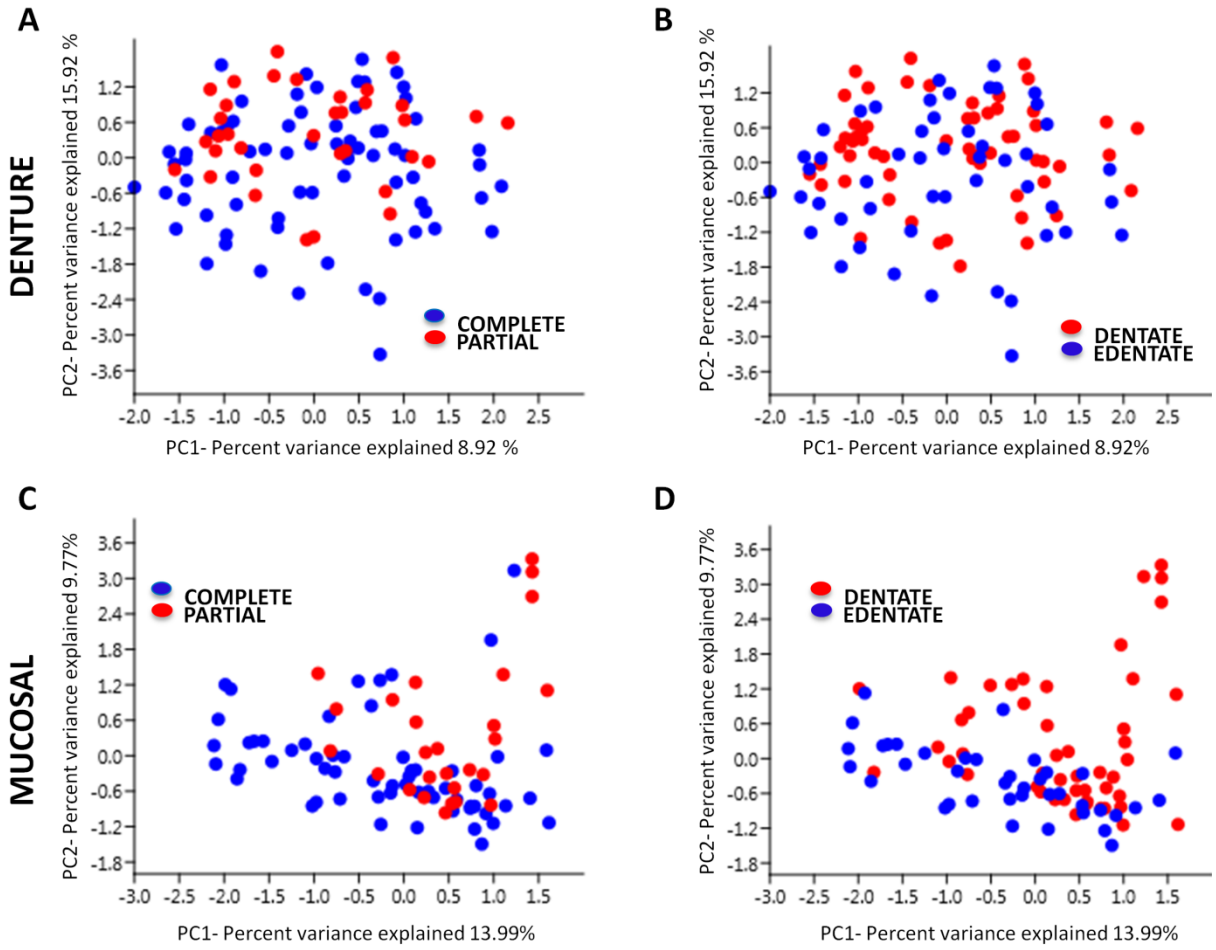


Figure 3.4: Principal component analysis comparing dentition status and denture type.
Samples were separated into groups based on denture type, (complete or partial) and dentition status (dentate or edentate) and principal component analysis was applied to the data for the denture **(A-B)** and mucosal microbiome **(C-D)**. For statistical analysis new variables were created for each principle component by using the factor loadings as regression coefficients, producing a score for each sample. These scores were then used as outcome variables to compare between groups using a two-tailed unpaired t test.

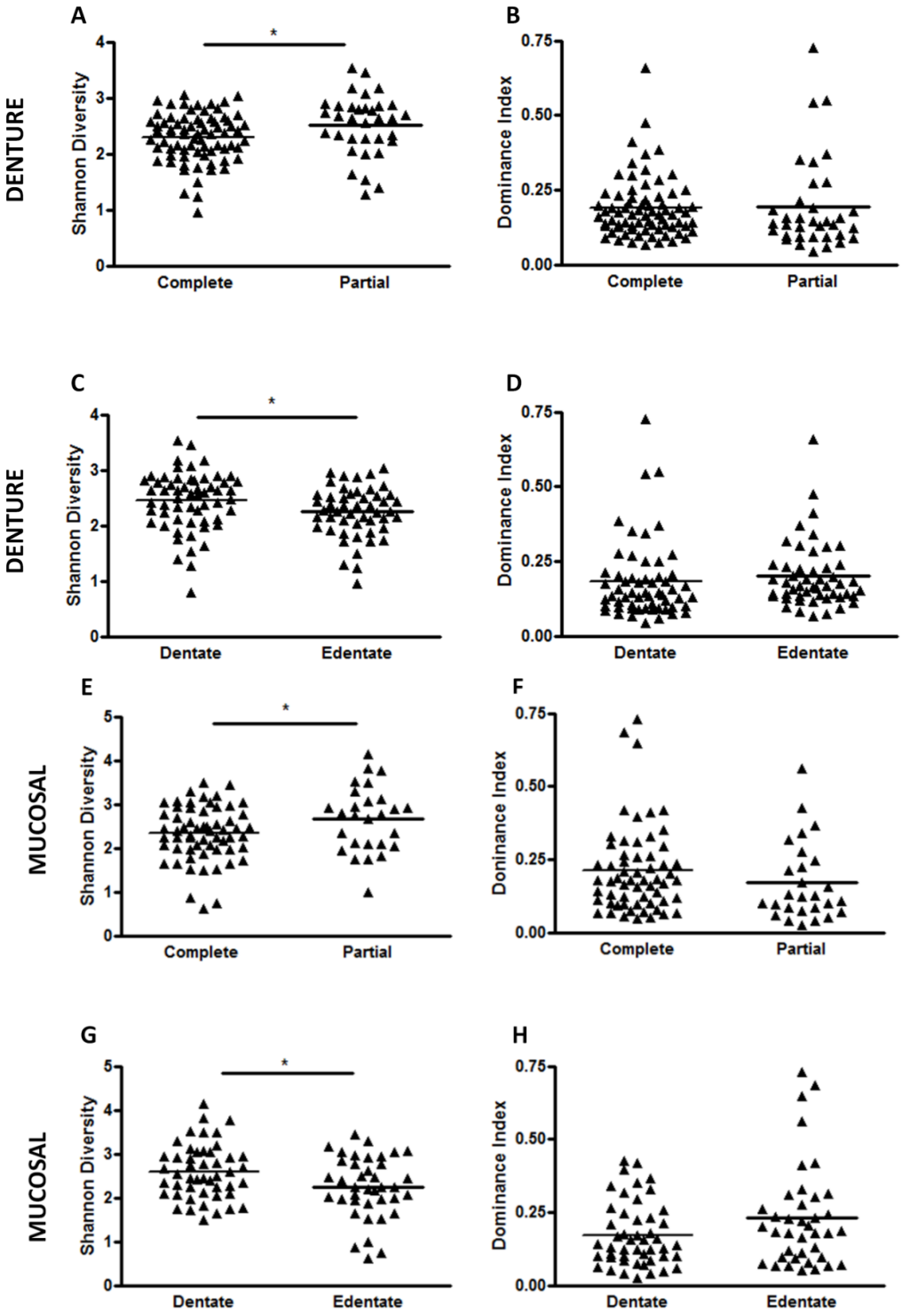


Figure 3.5: Diversity statistics comparing dentition status and denture type.

Samples were separated into groups based on denture type, (complete or partial) and dentition status (dentate or edentate) and the taxonomic diversity of each group was analysed and compared via a Shannon Diversity Index for the denture **(A and C)** and mucosal microbiome **(E and G)**. Sample dominance was assessed by dominance index at the denture **(B and D)** and mucosa **(F and H).** Data represents the mean (* $p<0.05$). Statistical analysis was performed using a two-tailed unpaired t test.

3.4.4 Health v Disease

Based on Newton's inflammation scores, patients were separated into health and DS groups, and their microbiomes analysed. When comparing the relative abundance (%) of bacterial classes between health and DS groups, there were no differences found in dental plaque (Fig 3.6A) (Appendix V) or when comparing genera (Appendix VI). Denture plaque showed significantly higher proportion of *Bacteroidia* (Mean: 2.97 v 5.18, $p<0.05$) and *Clostridia* (Mean: 8.65 v 11.24, $p<0.05$), in DS patients (Fig 3.6C) (Appendix VII), and when investigated at the genus level (Appendix VIII), further increases in the DS group could be attributed to *Prevotella* spp. (Mean: 2.81 v 4.85, $p<0.01$) and *Veillonella* spp (Mean: 6.40 v 7.53, $p<0.05$), respectively. The mucosal microbiome showed an altered composition between health and disease, in classes *Actinobacteria* (Mean: 17.56 v 23.83, $p<0.05$), *Bacteroidia* (Mean: 4.85 v 8.64, $p<0.05$), *Clostridia* (Mean: 8.17 v 10.07, $p<0.05$) and *Fusobacteria* (Mean: 1.87 v 1.07, $p<0.05$) (Fig 3.6E) (Appendix IX). At the genus level an increase in *Prevotella* (Mean: 4.19 v 6.85, $p<0.05$) was seen in DS patients (Appendix X). PCA was applied to the data, and as previous, two primary principal components emerged from this analysis, which explained the largest proportion of variance within the data. Scores based on factor loadings were used to compare healthy and DS groups for dental, denture and mucosal samples to determine if they formed distinct clusters (Fig 3.6B, 3.6D, and 3.6F). No significant differences were found between health and DS along PC1 or PC2 at any of the sample sites. Furthermore, Shannon diversity index and dominance index statistics indicated that mucosal samples from DS patients were significantly more diverse (Mean: 2.27 v 2.69, $p<0.01$) (Fig 3.7E) with less dominant taxa (Mean: 0.23 v 0.16, $p<0.05$) (Fig 3.7F) than their healthy counterparts. No differences were found in dental (Fig 3.7A-B) and denture plaque (Fig 3.7C-D). Further analysis of diversity was carried out by sub-grouping into mild inflammation (Newton's grade 1) and severe inflammation (Newton's grade 2&3); however, no differences that were statistically significant were observed.

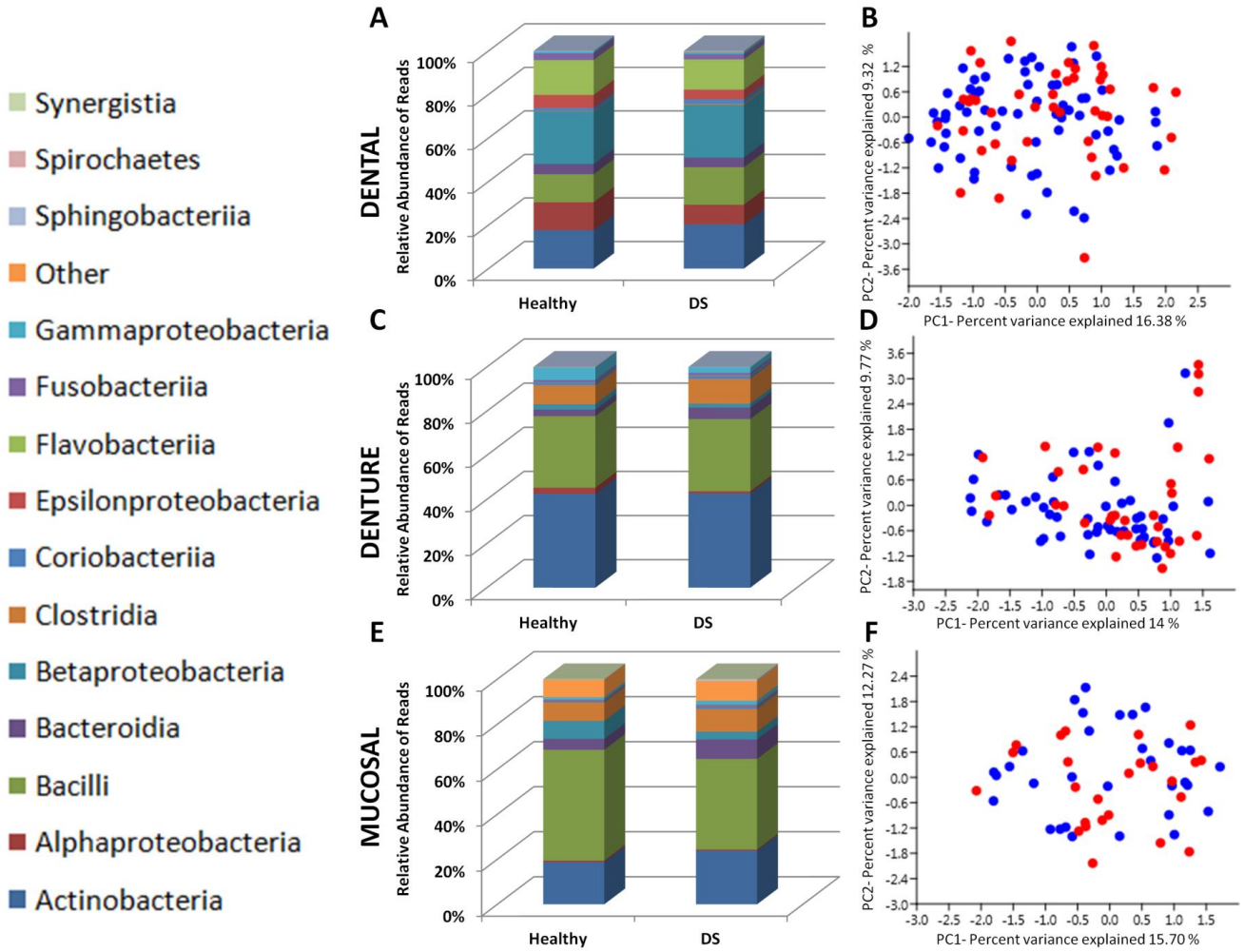


Figure 3.6: Microbiome composition and principal component analysis of healthy and DS samples.
The microbiome composition was assessed for differences between health and disease in (**A**) Dental Plaque, (**C**) Denture Plaque and (**E**) Mucosal Plaque at the class level. Principal component analysis was applied to the data for the dental (**B**), denture (**D**) and mucosal samples (**F**). Statistical analysis was performed using a two-tailed unpaired t test. For PCA, new variables were created for each principle component by using the factor loadings as regression coefficients, producing a score for each sample. These scores were then used as outcome variables to compare between groups using a two-tailed unpaired t test.

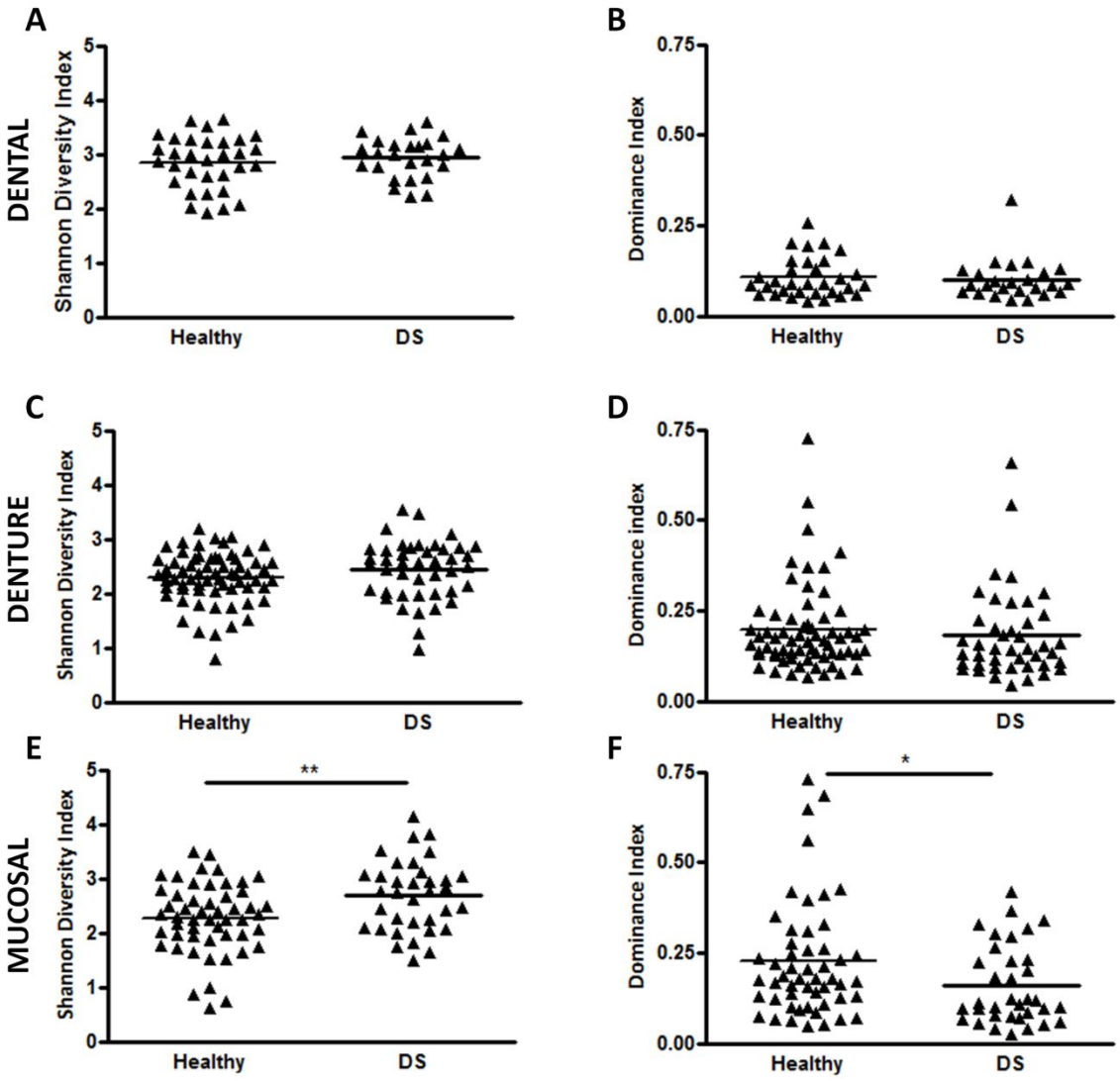


Figure 3.7: Diversity statistics comparing health and DS.
The taxonomic diversity of health and DS groups was analysed and compared via a Shannon Diversity Index in (**A**) Dental Plaque, (**C**) Denture Plaque and (**E**) Mucosal Plaque. Sample dominance was assessed by dominance index in (**B**) Dental Plaque, (**D**) Denture Plaque and (**F**) Mucosal Plaque. Data represents the mean (* $p<0.05$, ** $p<0.01$). Statistical analysis was performed using a two-tailed unpaired t test.

3.4.5 Impact of smoking on the microbiome

The dental, denture and mucosal microbiomes of smokers and non-smokers were compared to assess for any differences in composition and diversity. When comparing the relative abundance (%) of bacterial classes between health and DS groups, there were no differences found in dental plaque classes (Fig 3.8A) (Appendix XI) or genera (Appendix XII). Denture plaque showed no differences in terms of classes (Fig 3.8C) (Appendix XII), but showed significantly higher proportion of *Atopbium* (Mean: 27.03 v 1.18, $p<0.001$) and lower proportions of Selenomonas (Mean: 0.14 v 0.81, $p<0.01$) in smokers at the genus level (Appendix XIV). The mucosal microbiome found no differences at the class (Fig 3.8E) (Appendix XV) or genus levels (Appendix XVI). PCA was applied to the data, and as previously described, and two primary principal components emerged from this analysis for the dental (Fig3.8B), denture (Fig 3.8D) and mucosal (Fig 3.8F) microbiomes. No significant differences were found between smokers and non-smokers along PC1 or PC2 at any of the sample sites. Furthermore, Shannon diversity index and dominance index statistics indicated that no differences were found at the dental (Fig 3.9A-B), denture (Fig 3.9C-D) and mucosa (Fig3.9E-F).

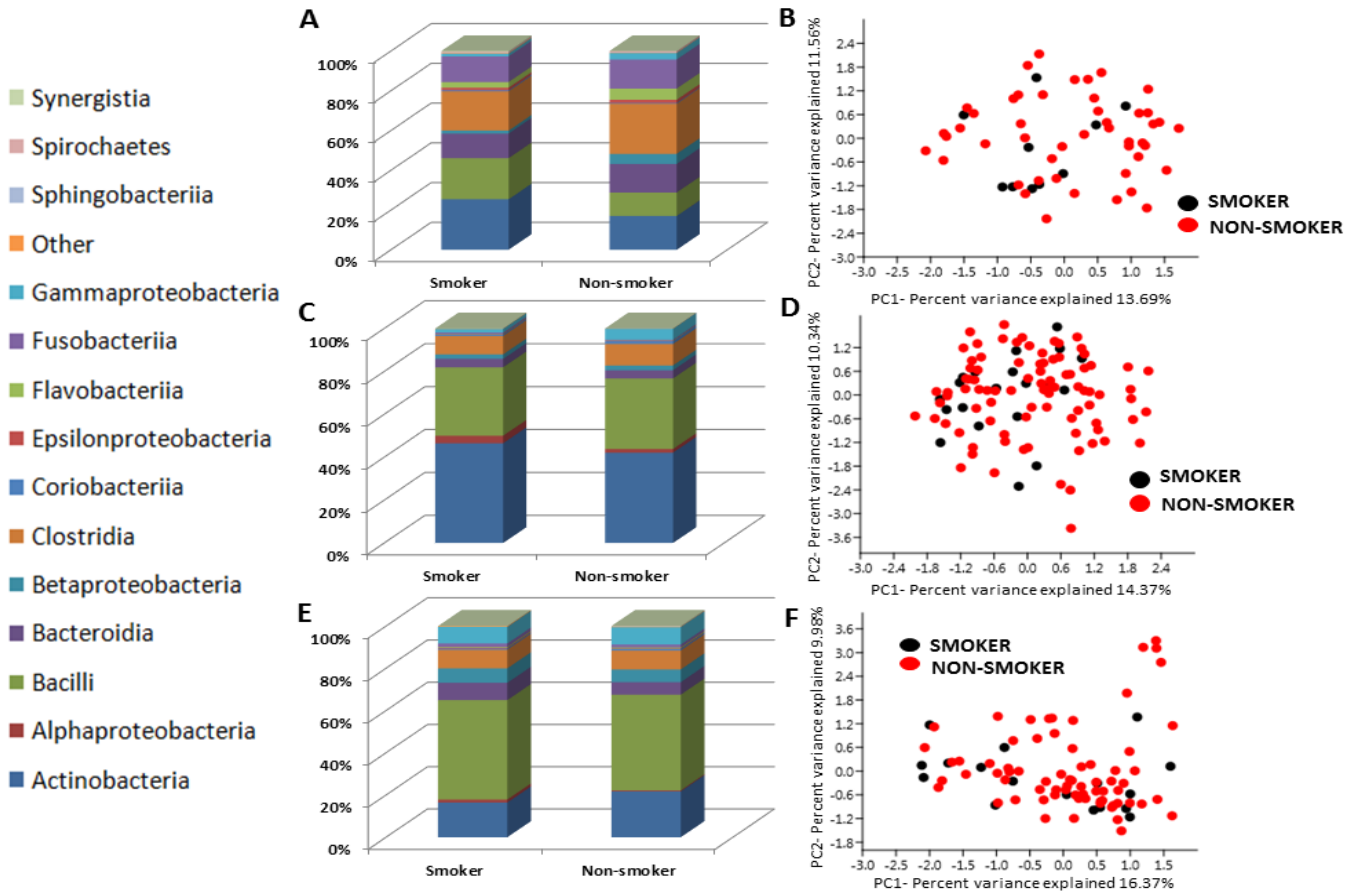


Figure 3.8: Microbiome composition and principal component analysis of smokers and non-smokers.
The microbiome composition was assessed for differences between smokers and non-smokers in (**A**) Dental Plaque, (**C**) Denture Plaque and (**E**) Mucosal Plaque at the class level. Principal component analysis was applied to the data for the dental (**B**), denture (**D**) and mucosal samples (**F**). Statistical analysis was performed using a two-tailed unpaired t test. For PCA, new variables were created for each principle component by using the factor loadings as regression coefficients, producing a score for each sample. These scores were then used as outcome variables to compare between groups using a two-tailed unpaired t test.

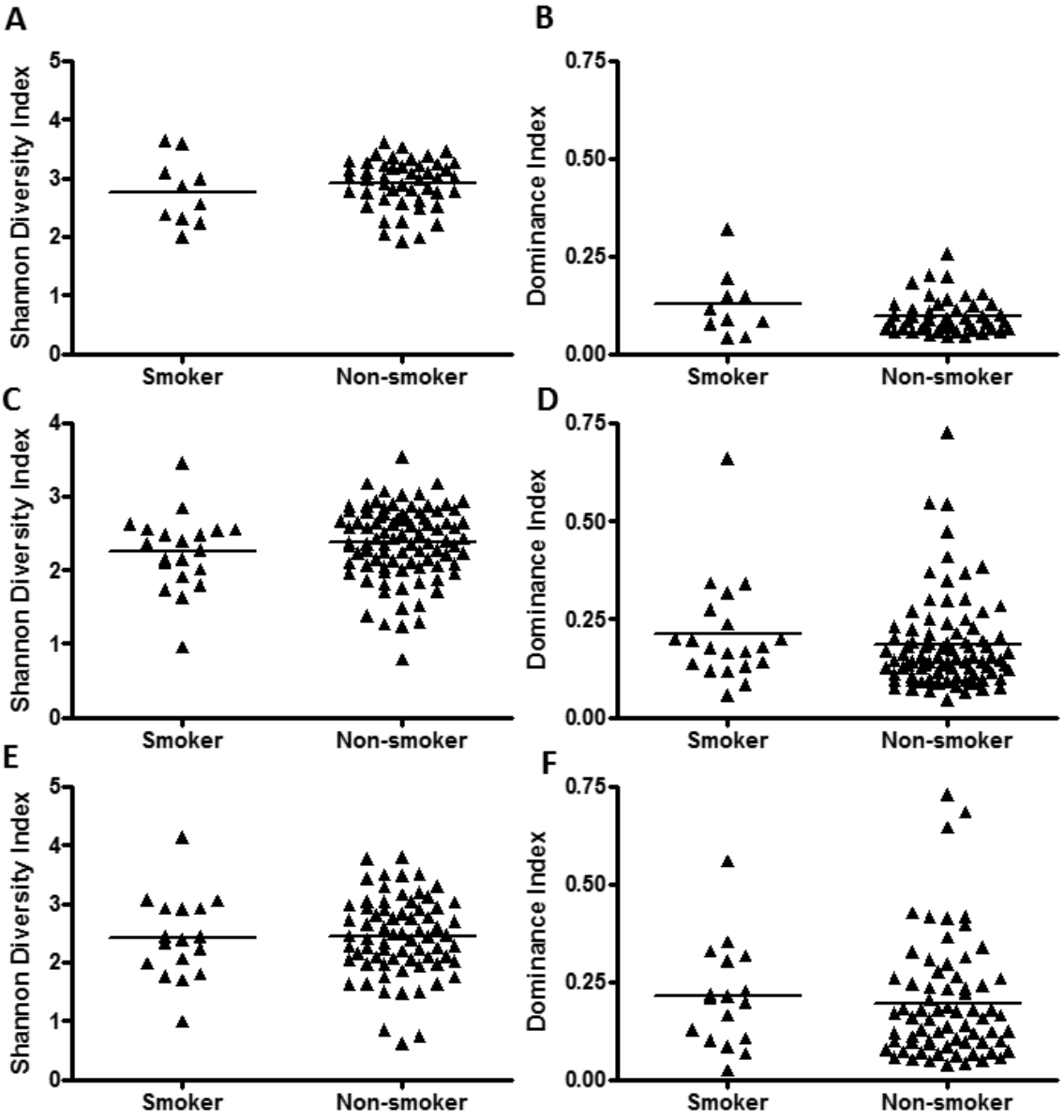


Figure 3.9: Diversity statistics comparing smokers and non-smokers.
The taxonomic diversity of smoker and non-smoker groups were analysed and compared via a Shannon Diversity Index in (**A**) Dental Plaque, (**C**) Denture Plaque and (**E**) Mucosal Plaque. Sample dominance was assessed by dominance index in (**B**) Dental Plaque, (**D**) Denture Plaque and (**F**) Mucosal Plaque. Data represents the mean.

3.4.6 Impact of *Candida* on the microbiome

The impact of increasing *Candida* CFU on the bacterial microbiome of each individual was assessed at the denture and mucosal surfaces. A positive correlation was found between *Candida* CFU and *Bacilli* class on dentures (p=0.01; Spearman's rho 0.387), but *Candida* CFU negatively correlated with *Fusobacteria* (p=0.01; Spearman's rho -0.470) (Fig 3.10). This was reflected at the genus level with *Lactobacillus* (p=0.0001; Spearman's rho 0.502) and *Fusobacteria* (p=0.025; Spearman's rho -0.417). No significant correlations were found between bacterial classes and *Candida* on the mucosal surface when assessing the complete cohort.

In addition to the effect of denture type and edentulism on microbiome composition and diversity, other factors were also important, including *Candida* CFU count. The average CFU *Candida* count found on dentures was significantly higher in individuals with a complete denture (Mean: 5.77 v 5.02, $p>0.01$) (Fig 3.11A) and in edentate individuals (Mean: 5.86 v 5.25, $p<0.05$) (Fig 3.11B), when compared to partial dentures and dentate individuals respectively.

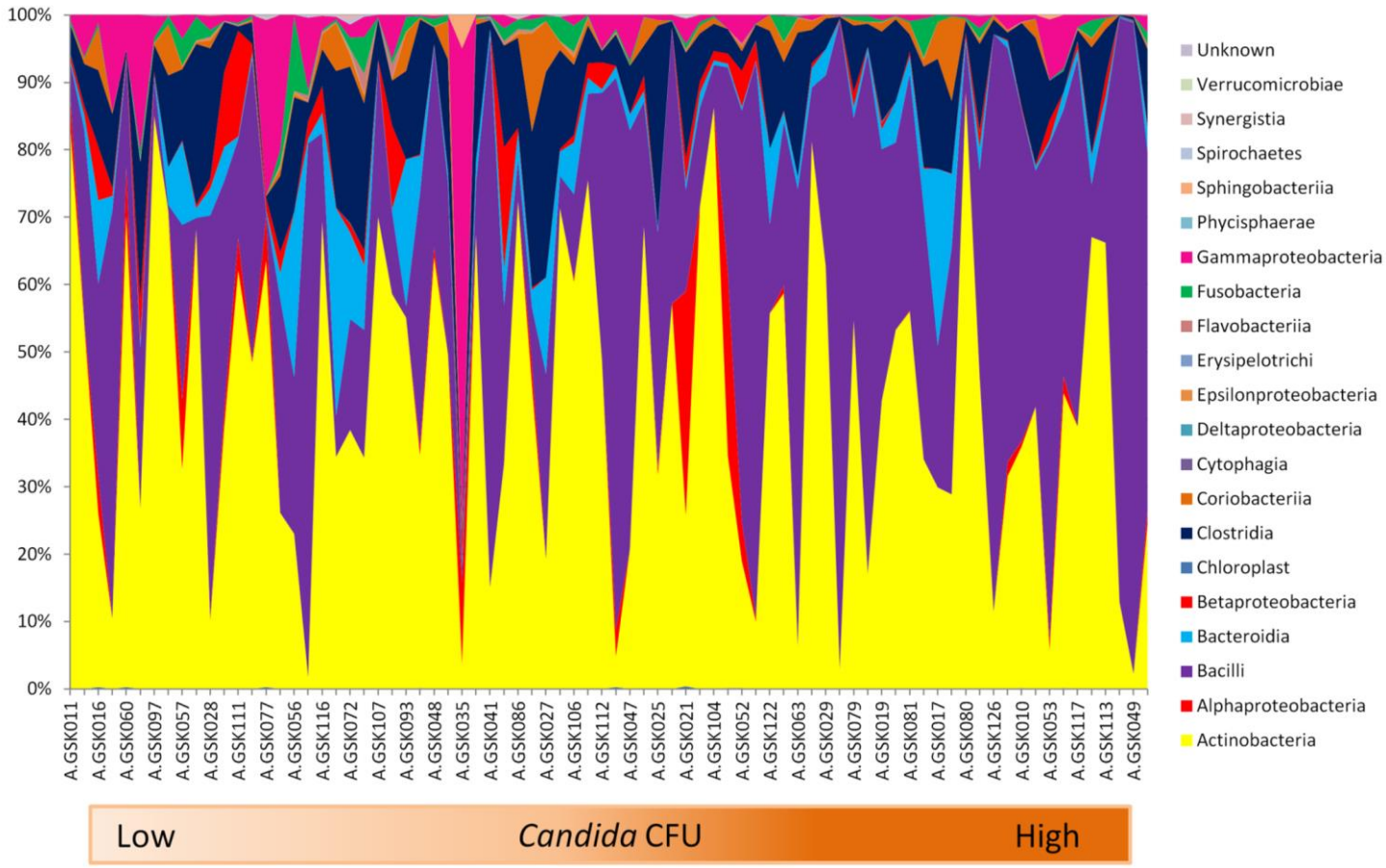


Figure 3.10: The impact of increasing Candida spp. CFU on the microbiome of the denture.
At the denture site the relative abundance of bacterial taxa within each sample was measured against increasing *Candida* CFUs at the class level. Statistical analysis was carried out using Spearman's rank correlation.

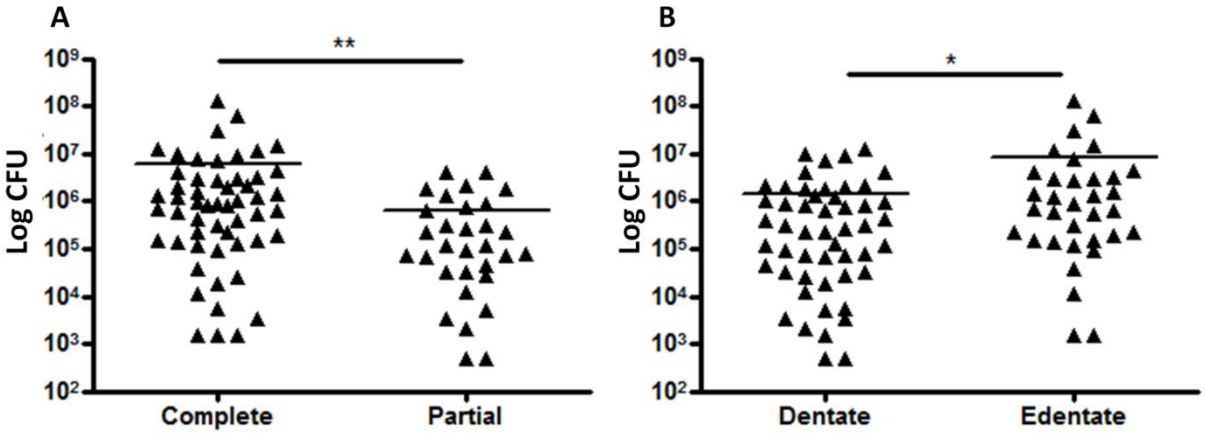


Figure 3.11: Abundance of Candida on dentures is affected by denture type and presence of natural teeth.
The average CFU *Candida* counts found on dentures were compared between complete and partial (**A**) and dentate and edentate (**B**) patients. *p<0.05, **p<0.01. Statistical analysis was performed using a two-tailed unpaired t test.

3.4.7 Antimicrobial peptide detection in the saliva of healthy and diseased patients

The levels of each AMP were compared between healthy and DS groups, and only LL-37 showed significantly elevated levels in DS patients (Mean: 1.04 v 1.28, p<0.05) (Fig 3.12 A). HNP 1-3, lactoferrin, calprotectin, Histatin 5 and BD1, showed no statistically significant differences between groups (Fig 3.12 B-F). Furthermore, certain salivary AMPs, namely LL37, HNP 1-3 and Histatin 5 all showed significantly higher concentrations in patients with partial dentures (Fig 3.13 A-C), and also in dentate individuals (Fig 3.13 G-I). Calprotectin, Lactoferrin and BD1 all showed no differences between complete/partial (Fig 3.13 D-E) and dentate/edentate groups (Fig 3.13 J-L) (3.1).

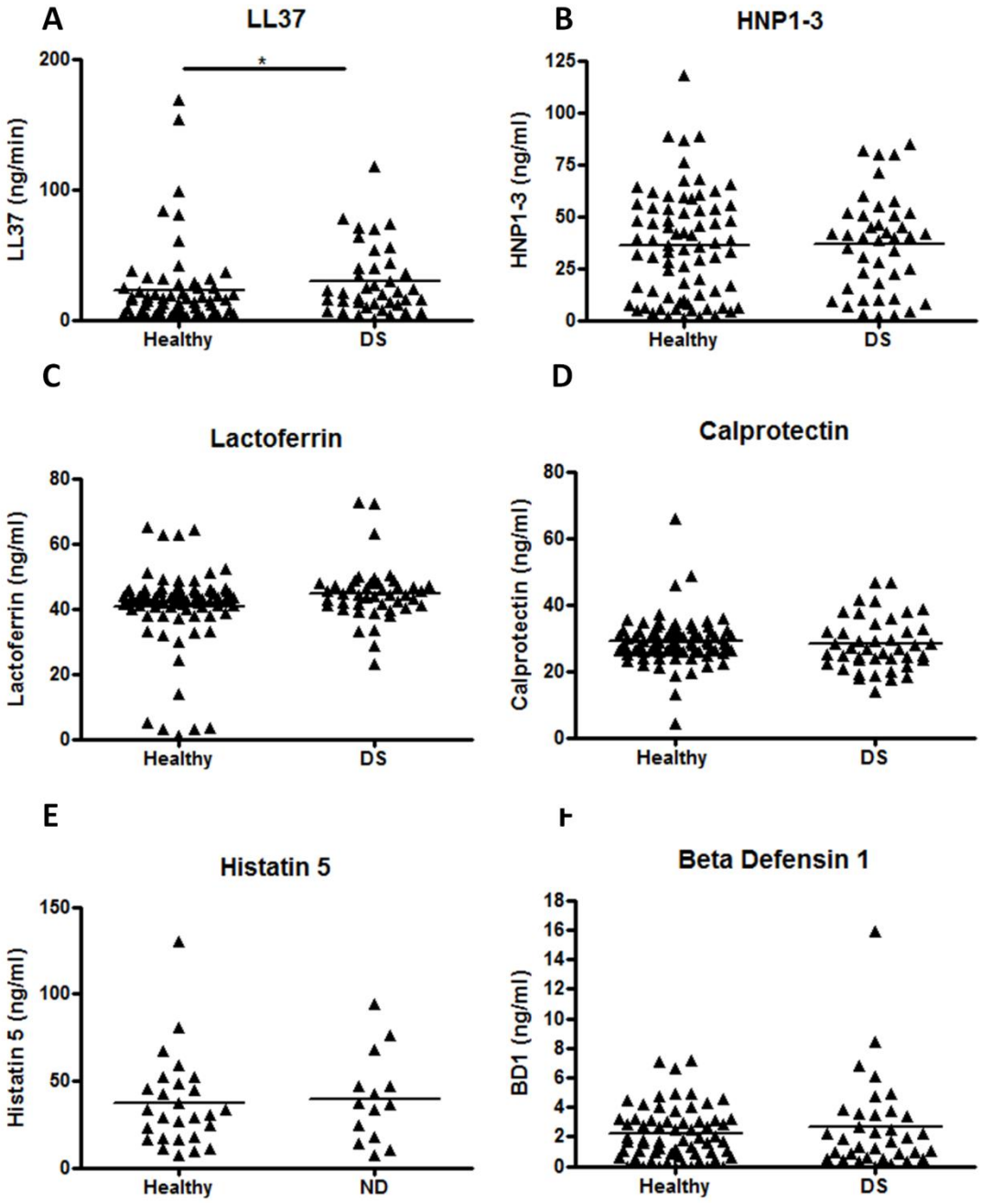


Figure 3.12: Concentrations of salivary AMPs increases with severity of inflammation.
The salivary concentrations of (**A**) LL37, (**B**) HNP1-3, (**C**) Lactoferrin, (**D**) Calprotectin, (**E**) Histatin 5 and (**F**) BD1 were compared between healthy and diseased individuals. Data represents the mean (*$p<0.05$). Statistical analysis was performed using a two-tailed unpaired t test.

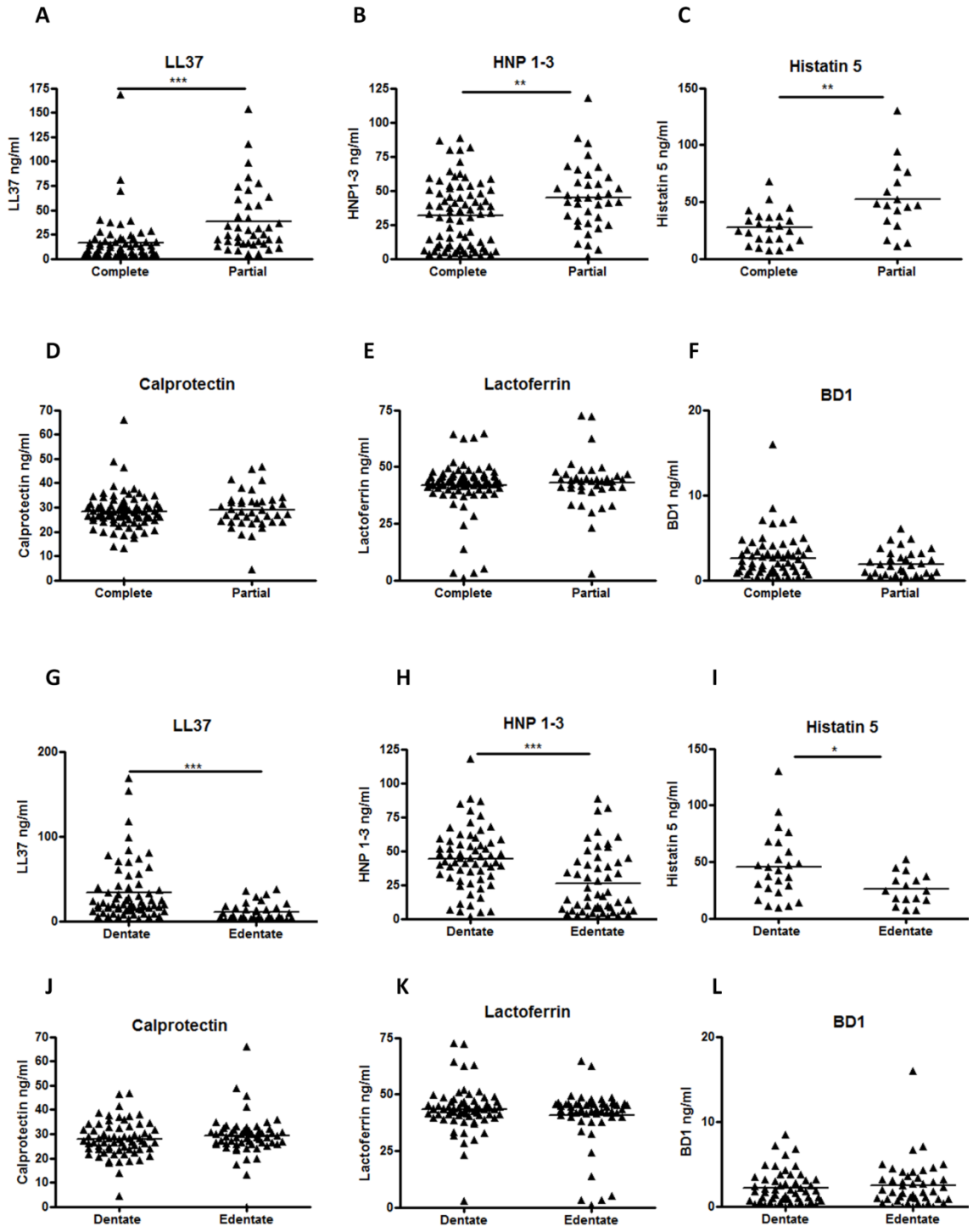


Figure 3.13: Concentration of salivary AMPs is affected by denture type and presence of natural teeth

The average AMP concentrations found in saliva was compared between complete and partial (**A - F)** and dentate and edentate (**G - L)** for LL37, HNP 1-3, Histatin 5, Calprotectin, Lactoferrin and BD1 respectively. Data represents mean (*p<0.05, **p<0.01, **p<0.001). Statistical analysis was performed using a two-tailed unpaired t test.

Table 3.1: Patients screened for salivary antimicrobial peptides.

AMP (n)	Healthy n (%)	DS n (%)	Dentate n (%)	Edentulous n (%)	Partial n (%)	Complete n (%)
LL37 (103)	64 (62)	39 (38)	66 (64)	37 (36)	63 (61)	40 (39)
Lactoferrin (125)	81 (65)	44 (35)	68 (55)	57 (45)	40 (32)	85 (68)
Calprotectin (124)	81 (65)	43 (35)	67 (54)	57 ((46)	41 (33)	83 (67)
HNP 1-3 (116)	74 (64)	42 (36)	63 (54)	53 (46)	79 (68)	37 (32)
BD1 (100)	65 (65)	35 (35)	54 (54)	46 (46)	65 (65)	35 (35)
Histatin 5 (40)	27 (68)	13 (32)	25 (63)	15 (37)	23 (58)	17 (42)

3.4.8 Impact of AMP's on the microbiome

Next, we investigated the impact of the AMPs on the denture and mucosal microbiome. The relative abundance of bacterial classes and genera for the complete patient cohort was compared against salivary levels of each AMP. However, no significant AMP/bacterial correlations were found at the denture or mucosal surface. The AMP saliva concentration was also compared against increasing Candida CFU, but again no significant correlations were identified. Nonetheless, CCA analysis was performed and demonstrated that at the mucosal microbiome distinct clusters formed and showed that the dentate group related stronger to the AMPs (Fig 3.14).

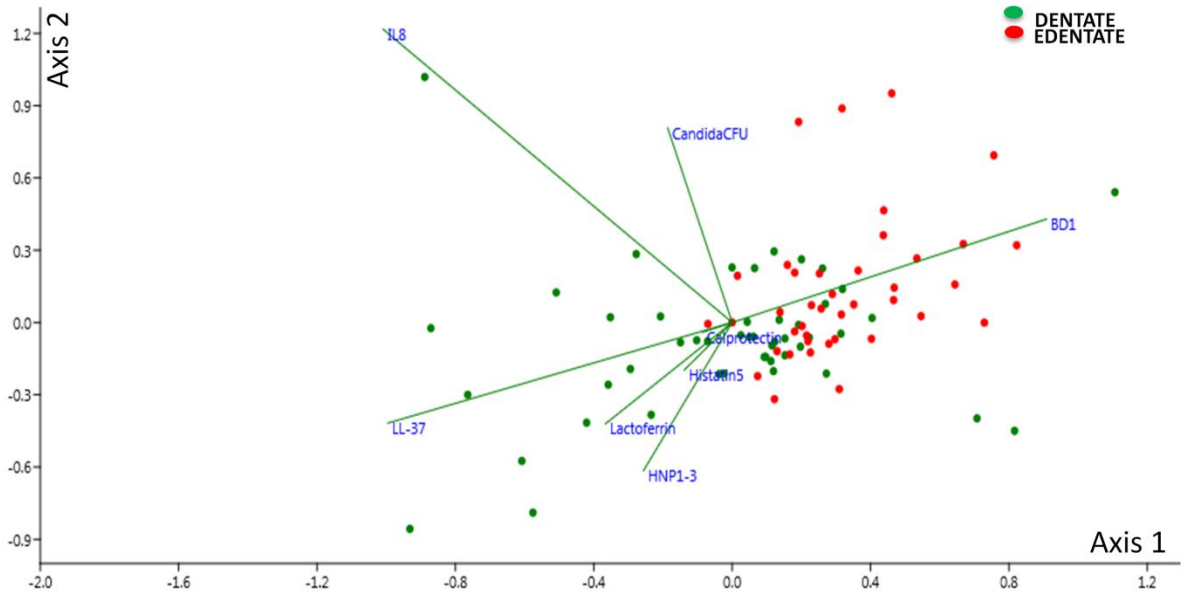


Figure 3.14: Cannonical Correspondence Analysis of the denture microbiome. CCA biplot allowing visualisation of dentate and edentate samples, represented by dots, in relation to environmental factors, including Candida CFU and salivary AMPs, which are represented by vectors. The variability in the samples was explained on axis 1 with 26.5% and on axis 2 with 19.3%.

3.4.9 Impact of oral hygiene on the denture microbiome

The Shannon diversity and Dominance index were employed to compare the diversity and dominance of the denture microbiome for the different patient and denture hygiene practices (Fig 3.15). As for hygiene status, no statistical significance was observed between good and poor hygiene groups in terms of diversity (Fig 3.15A) [Median: 2.36 (min: 0.79, max: 3.46) v 2.49 (min: 1.53, max: 3.54), $p>0.05$] or dominance (Fig 3.15B) [Median: 0.17 (min: 0.06, max: 0.73) v 0.14 (min: 0.05, max: 0.37), $p>0.05$]. Whether dentures were cleaned once or twice per day appeared to have no effect on the diversity (Fig 3.15D) [Median: 2.44 (min: 0.96, max: 3.46) v 2.37 (min: 0.79, max: 3.54), $p>0.05$] or dominance (Fig 3.15E) [Median: 0.16 (min: 0.06, max: 0.66) v 0.15 (min: 0.05, max: 0.73), $p>0.05$] of the denture microbiome, this also applied to sleeping with or without a denture *in situ*, (Fig 3.15G) [Median: 2.49 (min: 0.96, max: 3.54) v 2.35 (min: 0.79, max: 3.08), $p>0.05$] and (Fig 3.15H) [Median: 0.14 (min: 0.05, max: 0.66) v 0.17 (min: 0.06, max: 0.73), $p>0.05$], respectively. Comparison of the average bacterial class proportion between the groups revealed few changes other than elevated levels of *Alphaproteobacteria* [Median: 0 (min: 0, max: 50.52) v 0.13 (min: 0, max: 30.65), $p<0.05$], at the denture in those with poor oral hygiene (Fig.3.15C) (Appendix XVII). In addition, comparison at the genus level showed elevated levels of *Scardovia* [Median: 0 (min: 0, max: 16.75) v 0.32 (min: 0, max: 27.27), $p<0.05$] in the poor hygiene group (Appendix XVIII). As for cleaning frequency, only a few significant changes in bacterial classes were identified between groups, including an increase in *Bacilli* [Median: 18.57 (min: 0.39, max: 81.95) v 32.34 (min: 0.91, max: 96.92), $p<0.01$] in those that clean twice a day (Fig.3.15F) (Appendix XIX), no differences were found in terms of genus (Appendix XX). The denture microbiome of those that slept with their dentures *in situ* had significantly higher levels of *Bacteroidia* [Median: 2.6 (min: 0, max: 40.65) v 1.49 (min: 0, max: 12.34), $p<0.05$] *Clostridia* [Median: 14.94 (min: 0.13, max: 41.17) v 4.42 (min: 0.26, max: 21.43), $p<0.001$] Elipsonproteobacteria [Median: 0 (min: 0, max: 1.56) v 0 (min: 0, max: 0.26), $p<0.05$] and *Fusobacteria* [Median: 1.04 (min: 0, max: 11.04) v 0.26 (min: 0, max: 4.42), $p<0.001$], and significantly lower levels of *Bacilli* [Median: 21.3 (min: 0.91, max: 86.88) v 34.61 (min: 0.39, max: 82.86), $p<0.01$] and *Betaproteobacteria* [Median: 0.39 (min: 0.39, max: 18.05) v 1.36 (min: 0.91, max: 17.66), $p<0.05$] (Fig.3.15I) (Appendix XXI). As for the genus,

elevated levels of *Actinomyces* [Median: 23.44 (min: 0, max: 83.25) v 10.58 (min: 0.39, max: 86.88), p<0.05], *Leptotrichicia* [Median: 0.58 (min: 0, max: 9.22) v 0.13 (min: 0, max: 3.64), p<0.001], *Nessieria* [Median: 0.13 (min: 0, max: 8.70) v 0.26 (min: 0, max: 16.62), p<0.01] and *Veillonella* [Median: 6.36 (min: 0, max: 41.17) v 3.05 (min: 0, max: 20.39), p<0.01] were seen in those that sleep with their dentures in situ, as well as decreased levels of *Haemophilus* [Median: 0.39 (min: 0, max: 20.13) v 0.78 (min: 0, max: 36.62), p<0.05], *Rothia* [Median: 2.21 (min: 0, max: 40.26) v 8.38 (min: 0.13, max: 67.27), p<0.001] and *Streptococcus* [Median: 13.64 (min: 0, max: 86.62) v 25.65 (min: 0, max: 80.91), p<0.05] (Appendix XXII).

The impact of disease regarding patient and denture hygiene practices was further assessed by separating each group into healthy and DS sufferers. Diversity and dominance was assessed across the hygiene status, cleaning frequency and sleeping with the denture in situ categories, however, no statistically significant changes were noted between any of the groups, across any of the categories (Fig 3.16). Changes in the average abundance of each bacterial class were compared between the healthy and DS groups (Fig 3.17). No significant changes were detected across the groups within the hygiene category for any classes of bacteria. Within the cleaning category, there was a significant reduction in the level of *Fusobacteria* [Median: 2.08 (min: 0, max: 70.52) v 1.04 (min: 0, max: 14.42), p<0.05] between both healthy groups when the denture was cleaned twice per day, this was also seen between the healthy (once) group and the DS (twice) group [Median: 18.57 (min: 0, max: 70.52) v 1.04 (min: 0, max: 20.13), p<0.05]) (Fig 3.17B). As for sleeping with a denture *in situ*, the abundance of *Clostridia* was significantly lower in healthy individuals that do not wear their denture whilst sleeping when compared to healthy individuals that sleep with their denture *in situ* [Median: 14.29 (min: 0.26, max: 41.17) v 4.03 (min: 0.26, max: 21.43), p<0.01] as well as diseased individuals that sleep whilst wearing their denture [Median: 4.03 (min: 0.26, max: 21.43) v 12.47 (min: 0.13, max: 36.75), p<0.001] (Fig 3.17C).

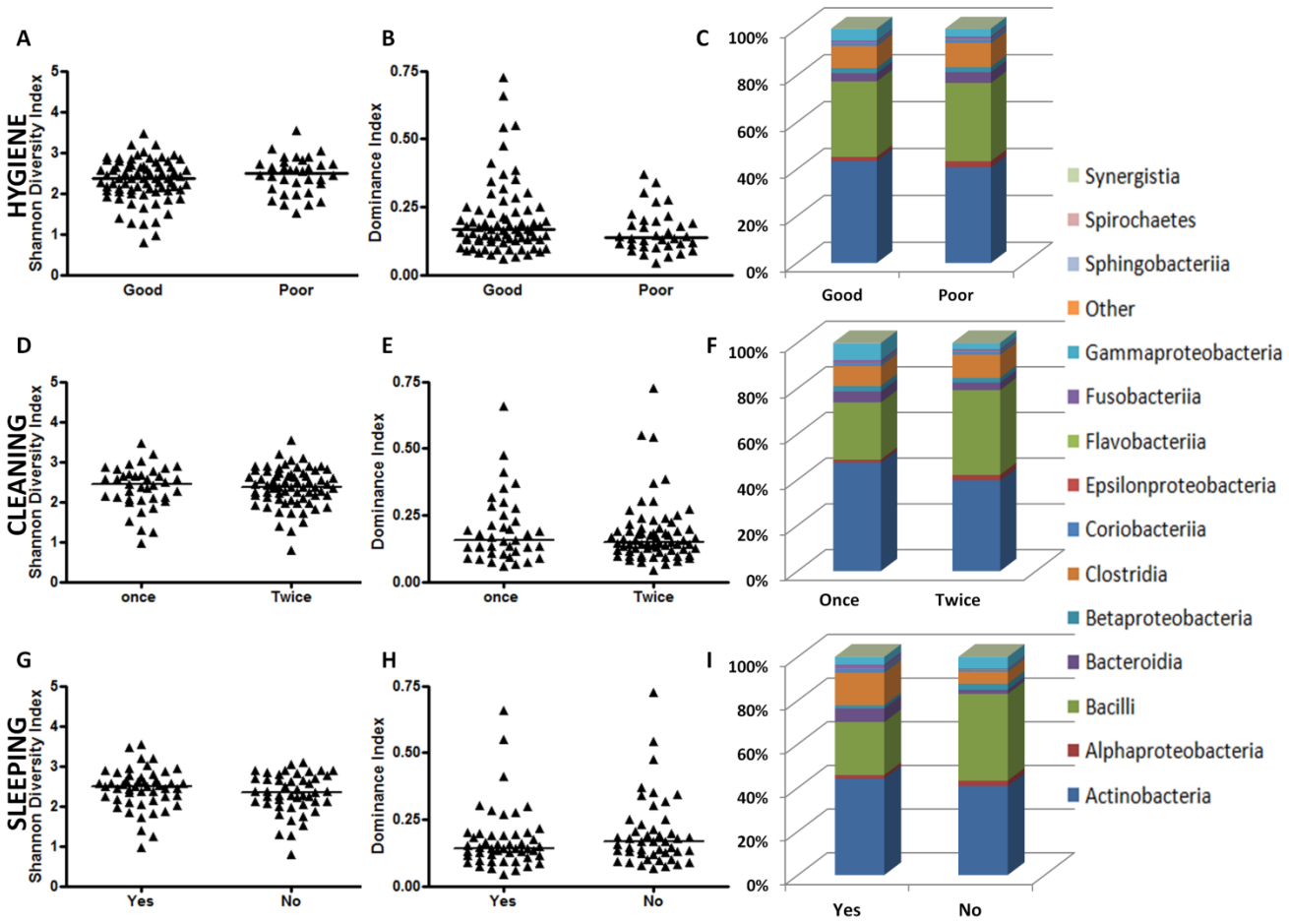


Figure 3.15: Comparison of changes in the denture microbiome as a result of oral hygiene practices.
The microbiome was assessed for differences in taxonomic diversity and dominance of denture plaque for hygiene status (**A** -**B**), denture cleaning frequency (**D** -**E**) and sleeping with a denture *in situ* (**G** -**H**). Differences in denture microbiome composition for hygiene status (**C**), denture cleaning frequency (**F**) and sleeping with a denture *in situ* (I) was also compared. Data represents the median. Statistical analysis was performed using a Mann Whitney test as data did not conform to a normal distribution.

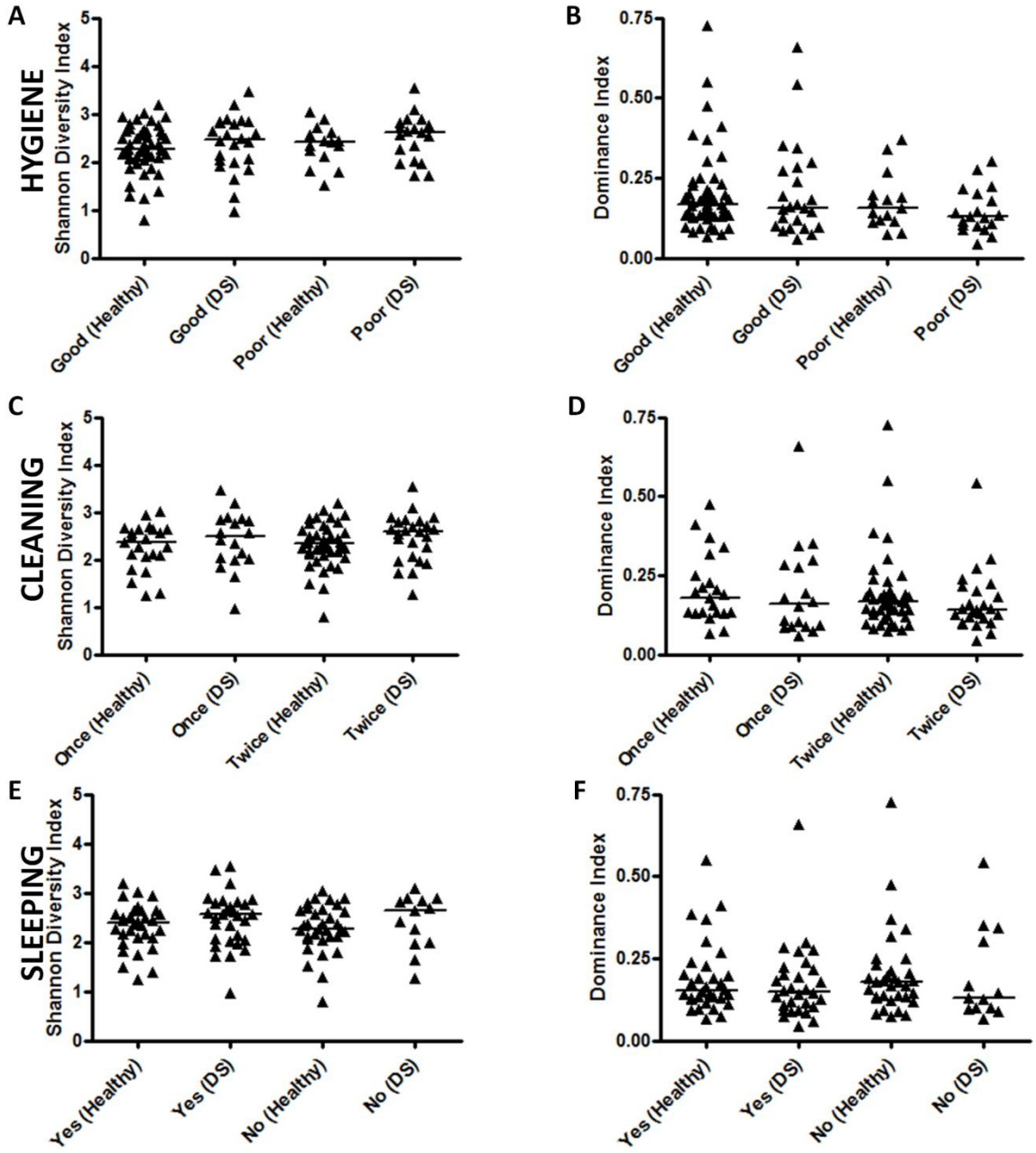


Figure 3.16: Comparison of changes in diversity of the denture microbiome as a result of oral hygiene practices.
The microbiome was assessed for differences in taxonomic diversity and dominance of denture plaque, healthy and diseased patient groups were further split based on hygiene status (**A**-**B**), denture cleaning frequency (**D** -**E**) and sleeping with a denture *in situ* (**G** -**H**). Data represents the median. Statistical analysis was performed using a Kruskall-Wallis test with Dunn's post-test to compare all groups to each other.

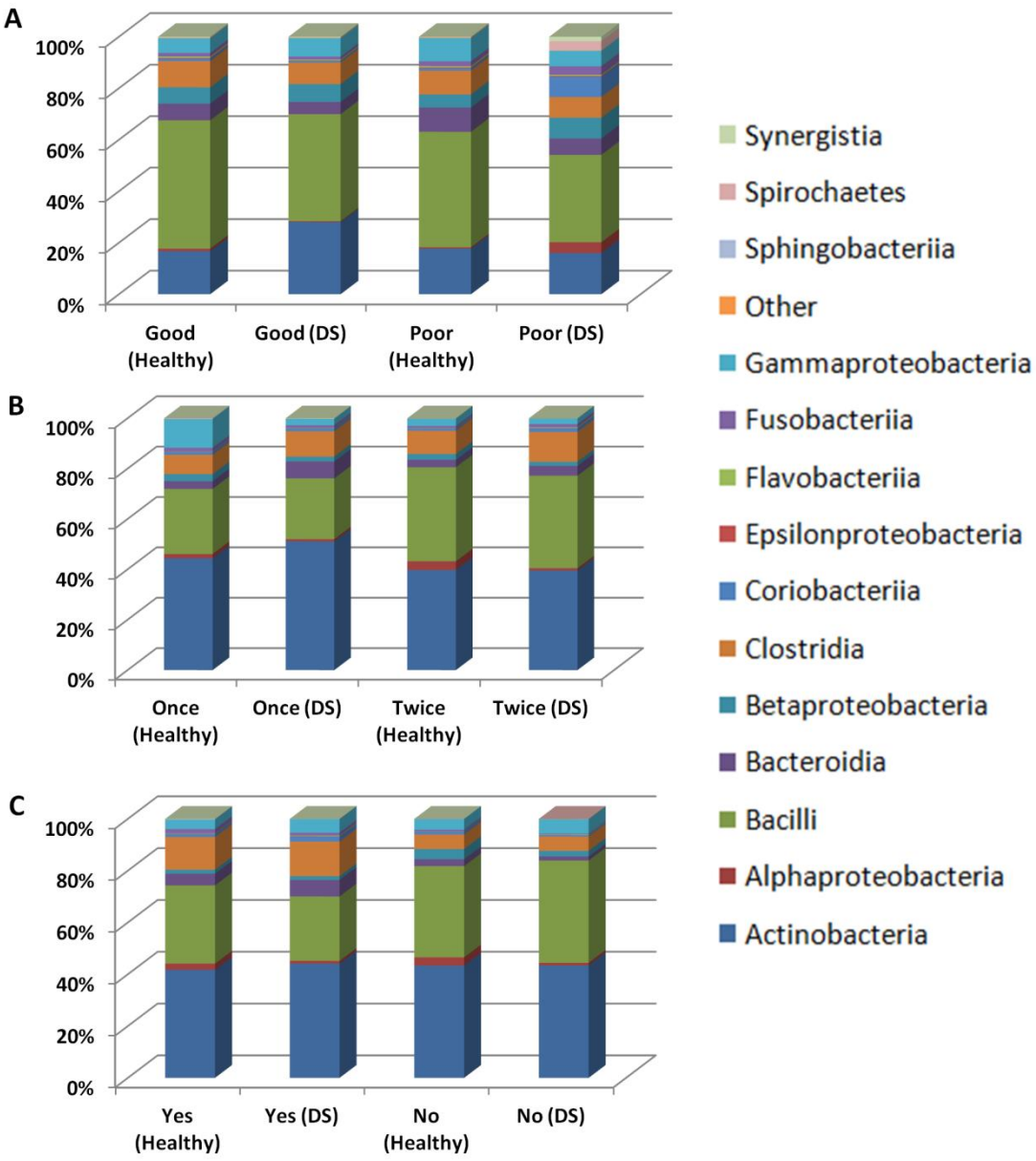


Figure 3.17: The effect of disease status on the denture microbiome as a result of oral hygiene practices.
The microbiome was assessed for taxonomic differences in denture plaque, healthy and diseased patient groups were further split based on hygiene status (**A**), denture cleaning frequency (**B**) and sleeping with a denture *in situ* (**C**). Statistical analysis was performed using a Kruskall-Wallis test with Dunn's post-test to compare all groups to each other.

3.4.10 Impact of oral hygiene on the mucosal microbiome

Differences in the diversity and dominance of the mucosal microbiome were assessed using the Shannon diversity index and dominance index for each of the patient and denture hygiene categories being measured (Fig 3.18). Comparison of good and poor oral hygiene at the mucosa revealed that those with poor oral hygiene have a significantly more diverse mucosal microbiome than those classed as having good oral hygiene [Median: 2.25 (min: 0.62, max: 3.81) v 2.66 (min: 1.49, max: 4.15), $p<0.05$]) (Fig 3.18A). Furthermore, the poor hygiene group had significantly less dominant taxa than the good hygiene group [Median: 0.18 (min: 0.04, max: 0.73) v 0.12 (min: 0.03, max: 0.42), $p<0.05$] (Fig 3.18B). Cleaning dentures either once or twice per day appeared to have no significant effect on the diversity [Median: 2.50 (min: 0.75, max: 3.81) v 2.34 (min: 0.62, max: 4.15), $p>0.05$] (Fig 3.18D) or dominance [Median: 0.14 (min: 0.04, max: 0.65) v 0.18 (min: 0.03 max: 0.73), $p>0.05$] (Fig 3.18E) of the mucosal microbiome. The mucosal microbiome was also unaffected by sleeping with a denture *in situ* in terms of diversity [Median: 2.31 (min: 0.62, max: 4.15) v 2.41 (min: 0.87, max: 3.29), $p>0.05$] (Fig 3.18G) or dominance [Median: 0.18 (min: 0.03, max: 0.69) v 0.17 (min: 0.06, max: 0.73), $p>0.05$] (Fig 3.16H). Bacterial class abundance levels showed no significant variation between groups in terms of hygiene status (Fig 3.18C) (Appendix XXIII), yet in terms of genera *Bifidobacterium* [Median: 0 (min: 0, max: 15.84) v 026 (min: 0, max: 5.71), $p<0.01$], *Kingella* [Median: 0 (min: 0, max: 0.78) v 3.9 (min: 0, max: 1.43), $p<0.01$] and *Scardovia* [Median: 0 (min: 0, max: 0.52) v 0.13 (min: 0, max: 6.10), $p<0.001$] were significantly elevated in those with poor hygiene (Appendix XXIV). No differences were seen at the class (Fig 3.18F) (Appendix XXV) or genus (Appendix XXVI) levels for cleaning frequency. However, sleeping whilst wearing a denture revealed significantly elevated levels of *Bacteroidia* [Median: 6.95 (min: 0, max: 37.92) v 3.9 (min: 0, max: 15.19), $p<0.05$], *Coriobacteria* [Median: 0.39 (min: 0, max: 6.23) v 0.13 (min: 0, max: 3.51), $p<0.05$], *Epsilonproteobacteria* [Median: 0.19 (min: 0, max: 1.82) v 0.13 (min: 0, max: 0.91), $p<0.05$] and *Fusobacteria* [Median: 1.17 (min: 0, max: 7.27) v 0.26 (min: 0, max: 6.88), $p<0.01$] (Fig.3.18I). Elevated levels of the genera *Leptotrichicia* [Median: 0.71 (min: 0, max: 7.27) v 0.13 (min: 0, max: 6.49), $p<0.05$] and *Rothia* [Median: 7.27 (min: 0.65, max: 50.52) v 5.65 (min: 0, max: 28.70), $p<0.05$] were found in those that sleep with their denture *in situ* (Appendix XXVIII).

As previous, the groups within the hygiene, cleaning and sleeping categories were further separated into healthy and DS groups to investigate the impact of disease. Microbiome diversity and dominance were compared for each category. The diversity of the mucosal microbiome was significantly higher in the poor hygiene group with DS when compared to the healthy group with good hygiene [Median: 2.23 (min: 0.62, max: 3.44) v 2.92 (min: 1.49, max: 4.15), $p<0.05$] (Fig 3.19A); however no significant changes in dominance were detected (Fig 3.19B). Neither diversity (Fig 3.19C) nor dominance (Fig 3.19D) showed significant variation between groups in the cleaning frequency category, this also applied to sleeping with the denture in situ (Fig 3.19E-2F). There were no significant changes were observed when comparing the abundance of individual classes of bacteria across the healthy and DS groups within the hygiene (Fig 3.20A), cleaning (Fig 3.20B) or sleeping categories (Fig 3.20C).

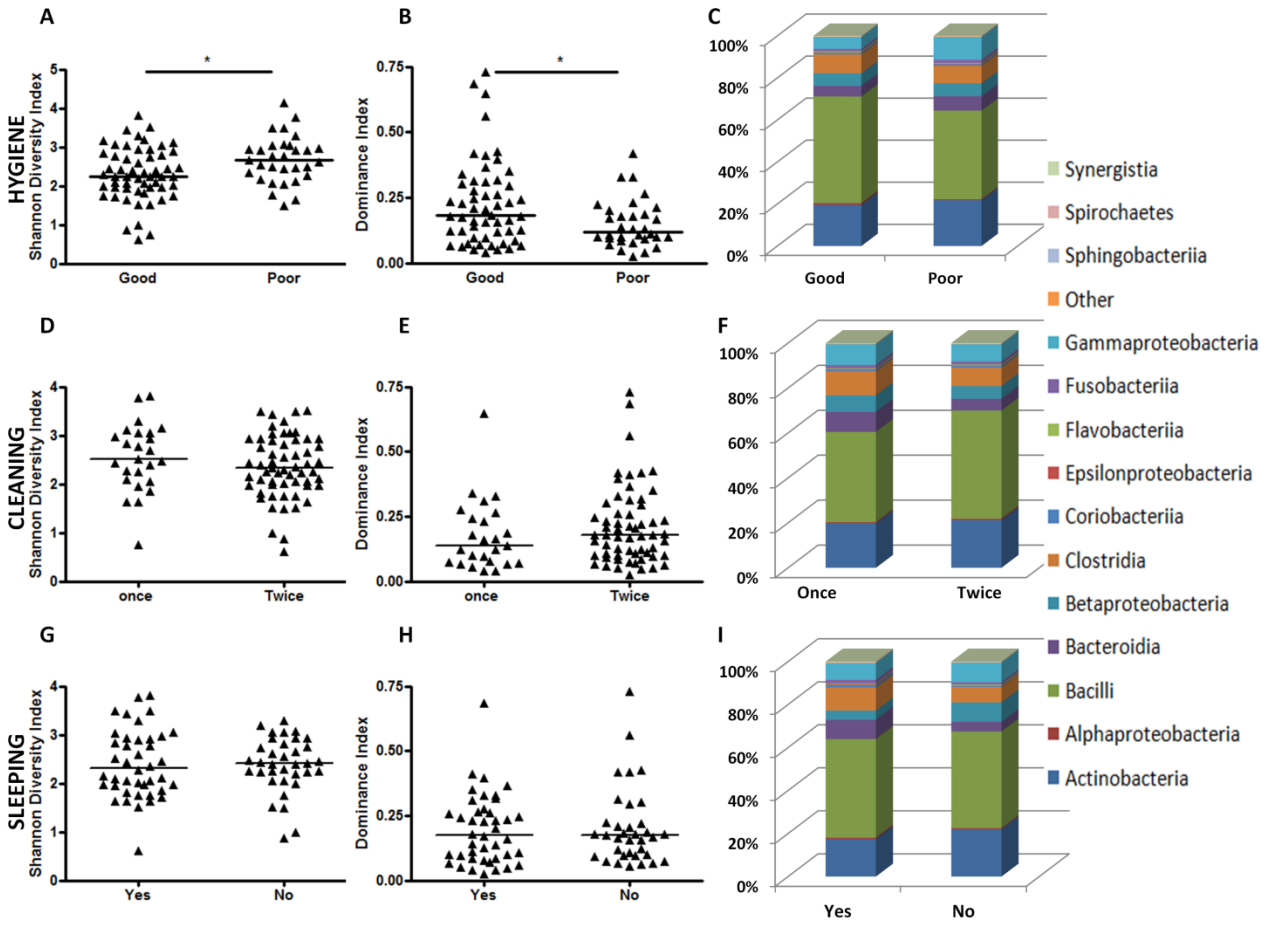


Figure 3.18: Comparison of changes in the mucosal microbiome as a result of oral hygiene practices.
The microbiome was assessed for differences in taxonomic diversity and dominance of mucosal plaque for hygiene status **(A)** and **(B)**, denture cleaning frequency **(D)** and **(E)** and sleeping with a denture in situ **(G)** and **(H)**. Differences in mucosal microbiome composition for hygiene status **(C)** denture cleaning frequency **(F)** and sleeping with a denture *in situ* were compared **(I).** Data represents the median (* $p<0.05$). Statistical analysis was performed using a Mann Whitney test as data did not conform to a normal distribution.

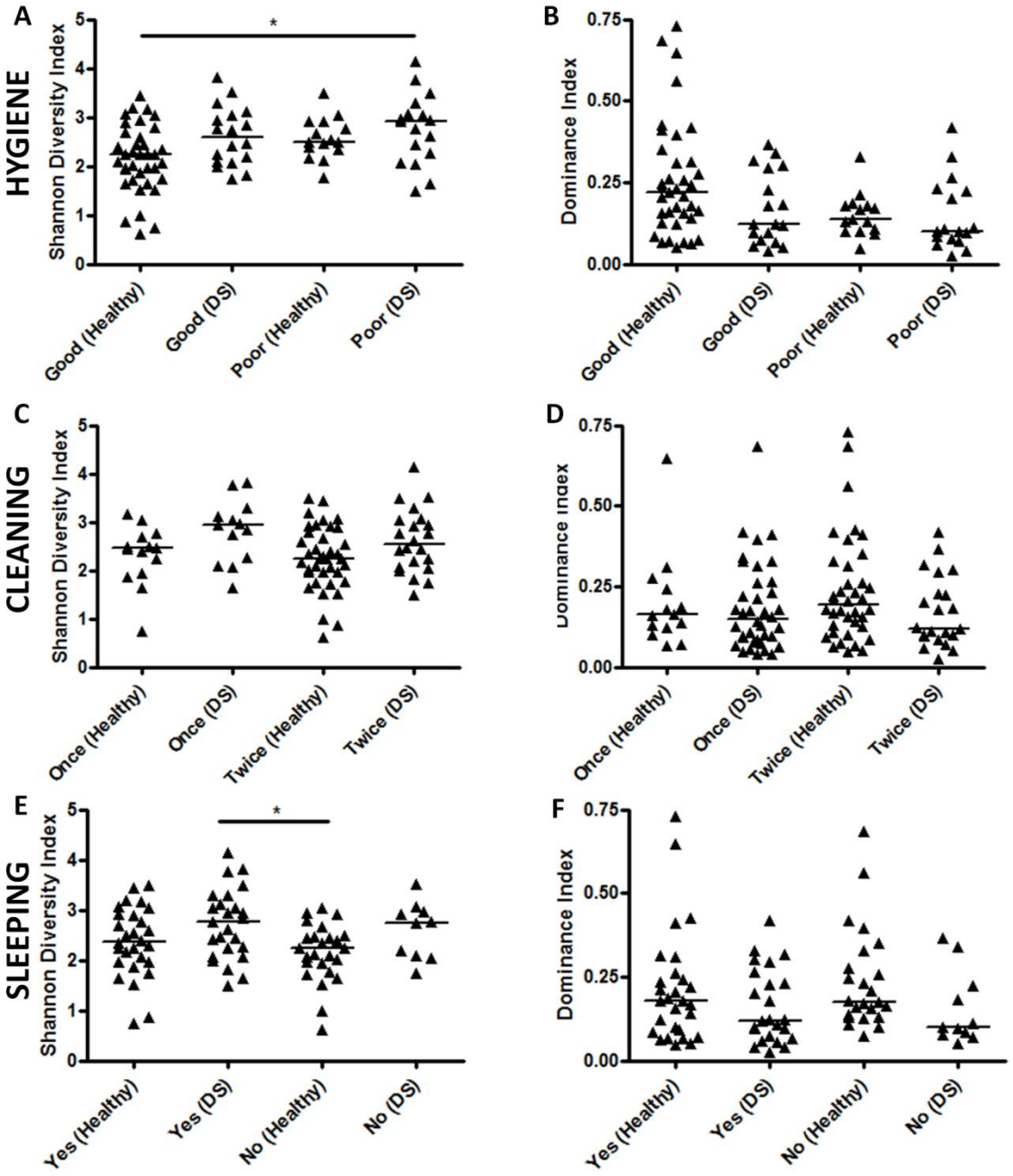


Figure 3.19: Comparison of changes in diversity of the mucosal microbiome as a result of oral hygiene practices.
The microbiome was assessed for differences in taxonomic diversity and dominance of mucosal plaque, healthy and diseased patient groups were further split based on hygiene status **(A)** and **(B)**, denture cleaning frequency **(C)** and **(D)** and sleeping with a denture *in situ* **(E)** and **(F)**. Data represents the mean (*$p<0.05$). Statistical analysis was performed using a Kruskall-Wallis test with Dunn's post-test to compare all groups to each other.

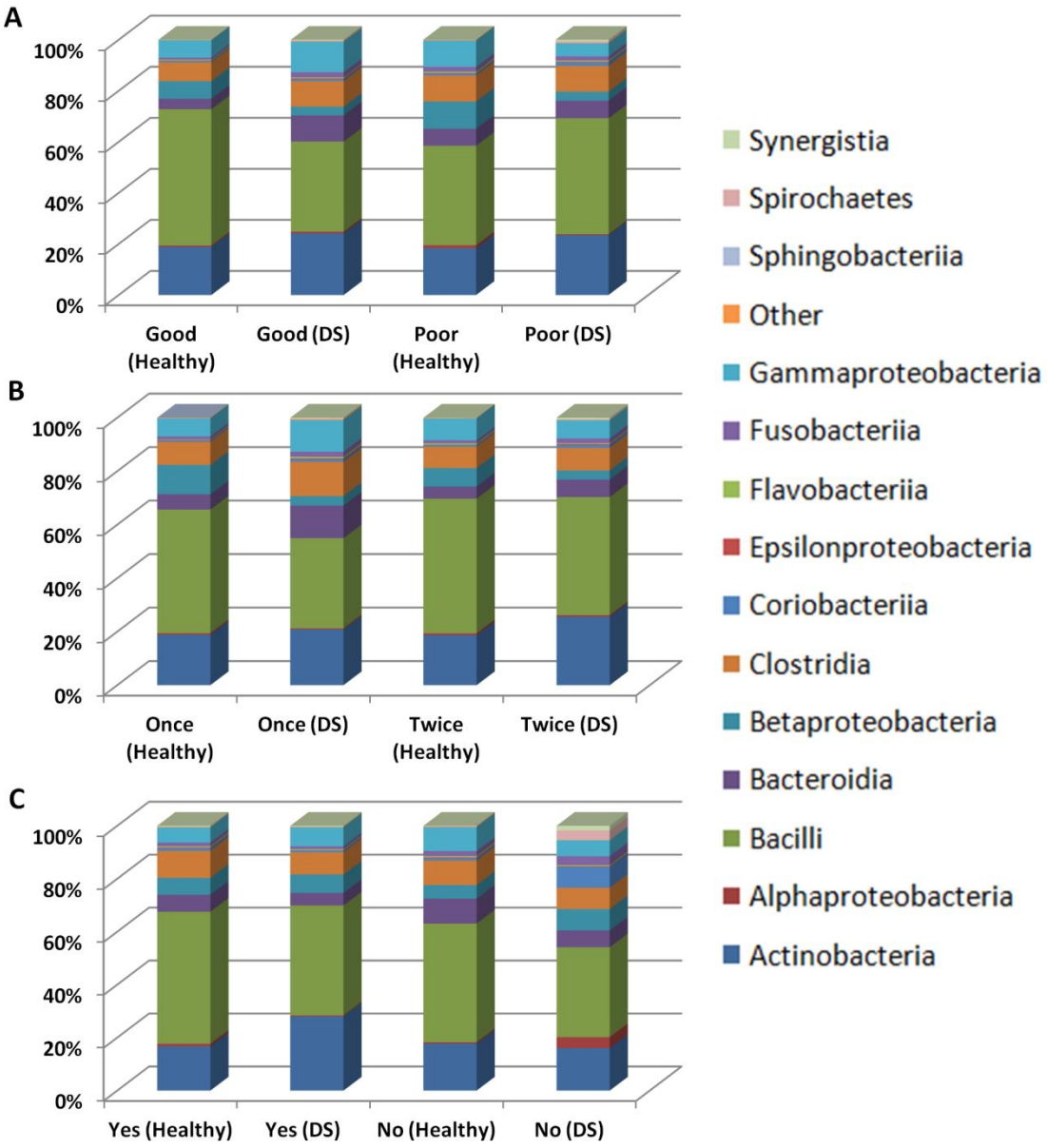


Figure 3.20: The effect of disease status on the mucosal microbiome as a result of oral hygiene practices.
The microbiome was assessed for taxonomic differences in mucosal plaque, healthy and diseased patient groups were further split based on hygiene status **(A)**, denture cleaning frequency **(B)** and sleeping with a denture *in situ* **(C)**. Statistical analysis was performed using a Kruskall-Wallis test with Dunn's post-test to compare all groups to each other.

3.5 Discussion

Improving oral health has become a primary focus in medical research, and thus significant amount of time and money has been spent on investigating the pathogenesis and prevention of oral disease (Petersen et al., 2005, Petersen, 2003). This is driven because of the increasing associations between oral health and systemic diseases, which includes rheumatoid arthritis, cardiovascular disease and respiratory disease (Inaba and Amano, 2010, Farquharson et al., 2012). However, this research has focused primarily on dental-related disease, whereas, denture-related disease, a problem affecting a large proportion of the ever-increasing elderly population (Divaris et al., 2012, Griffin et al., 2012), has been relatively understudied. Moreover, the oral cavity is a complex anatomical structure and the presence of prostheses, such as a denture, may influence the ecological balance within the oral cavity. Therefore, a detailed investigation was required to gain a more in depth understanding of the oral microbiology of a denture wearer.

Historically, denture-related research has a disproportionate focus on yeast associated infection (Dagistan et al., 2009a, Pereira-Cenci et al., 2008, Coco et al., 2008a), yet there is a distinct lack in understanding of the bacterial microbiome within the oral cavity of the denture wearer. The majority of studies investigating DS focus primarily on the role of *Candida* spp. (Pereira-Cenci et al., 2008, Gendreau and Loewy, 2011), however, given that up to 10^{11} microbes are capable of colonising the denture surface (Nikawa et al., 1998), then it is likely that bacteria also play a role in disease. Other studies have investigated the bacterial composition of denture plaque, however, these studies have been limited by the method of investigation, such as culturing and DNA-DNA hybridisation, which limit the number of bacterial species that can be identified (Lamfon et al., 2005, Campos et al., 2008, Sumi et al., 2003, Sachdeo et al., 2008, Teles et al., 2012). For example, Campos et al (2008) used PCR and identified 82 bacterial species, this however only made up approximately 50% of the bacterial flora detected in the samples, as the remainder were not-yet cultivated phylotypes, and thus remained unidentified (Campos et al., 2008). This study however has for the first time used detailed high-throughput 16S rRNA gene

analysis of the oral microbiomes of a range of denture wearers, which gives a more comprehensive and global representation of the bacterial microbiome.

We first assessed the bacterial microbiome of three independent sites from the oral cavity of denture wearers, the denture, the palatal mucosa and dental plaque. Comparing the relative abundance of bacterial classes at the different sites showed the more diverse nature of the dental plaque in comparison to the denture and mucosal sites, both of which were predominantly composed of two classes, *Actinobacteria* and *Bacilli*. This result is unsurprising as both *Actinobacteria* and *Bacilli* are associated with being amongst the primary colonisers within the oral cavity, particularly *Actinomyces* and *Streptococcus* species (Wade, 2013, Aas et al., 2005, Diaz et al., 2006). The diversity statistics further substantiated this finding, showing that the dental microbiome was significantly more diverse and less dominant than both the denture and mucosal microbiome. These results are supported by a recent study comparing biofilms forming on natural teeth against those forming on denture teeth, which demonstrated that biofilms forming on natural teeth develop quicker and have a more abundant proportion of species present (Teles et al., 2012). The PCA analysis plots indicated distinct groups formed in which denture and mucosal groups clustered together and away from the dental group, thus indicating not only are they distinct in terms of diversity, but also compositionally separate. Moreover, direct comparison of denture and mucosal groups indicates that they also form their own distinct groups, and the reason for them clustering together previously was likely because of their similarity to one another as opposed to the dental microbiome. The different anatomical locations of these microbiomes likely explains these differences, as the adherent surfaces and surrounding environment of dental plaque may be more suitable for the growth of a more diverse range of microorganisms (Zaura et al., 2009a). For example, saliva is an important source of nutrients for microbes in the oral cavity (Humphrey and Williamson, 2001), however, the presence of a denture, acts as a barrier, thus with reduced nutrients available, fewer organisms can form a sustainable niche.

This study has shown that the presence of natural dentition has a profound impact on the composition and diversity of the oral microbiome of a denture wearer. In terms of diversity, both the denture and mucosal microbiome is significantly more

diverse in dentate patients compared to edentate and in those with partial dentures versus those with complete. This suggests that this increased microbial diversity is not restricted to the anatomical sample site of the tooth, but also appears to affect the entire oral cavity. Patients included in this study that still had natural teeth remaining, ranged from 1 to 28 teeth, yet despite this wide range, PCA analysis indicated that they formed distinct groups from one another, indicating that even the presence of only one tooth is sufficient to have a profound impact of the microbiome composition. Distinct groups formed for the denture microbiome of both the complete/partial and dentate/edentate groups; however, for the mucosal microbiome distinct groups could only be distinguished between denture types. This could simply be a result of lack of statistical power, which is likely, given the similarity to the complete/partial PCA plot. Nonetheless, the lack of obvious groups between dentate and edentate samples at the mucosa could perhaps be explained by the nature of the surface. As we have already shown, the microbiome composition within the oral cavity is very much dependant on the location, therefore as the mucosal surface is biotic, microbes in contact are likely to be exposed to pathogen recognition receptors (PRRs) or host defence peptides of the epithelial cells (Diamond et al., 2008a, Bingle and Gorr, 2004, Dickinson et al., 2011). As many of the dentate associated bacteria are not traditional commensals, they may have a limited ability to colonise such a niche as well as the potential to initiate an immune response (Signat et al., 2011, Kinane et al., 2008). Dentures, on the other hand are an abiotic surface, therefore biofilms from dentate individuals forming on this surface are likely to have less exposure to host defences and thus bacteria may grow unimpeded and have sufficient time to develop into a compositionally distinct plaque microcosm.

One of the primary aims of this study was to identify any compositional changes in the microbiome between health and disease. However, no significant differences were shown between the two groups in dental plaque in terms of abundance of bacterial classes or diversity. Denture plaque revealed a number of notable changes between health and disease, with significantly higher proportions of *Prevotella* and *Veionella* species found in denture stomatitis sufferers. These bacteria are more commonly associated with natural dentition, which suggests that DS microbiomes have a composition more comparable to that of dental plaque, however it could be due to the fact that the majority of our DS sufferers

had some natural dentition remaining. The virulence of both *Prevotella* and *Veillonella* species are well known in terms of oral disease, more specifically PD (Millhouse et al., 2014). In PD, *Prevotella intermedia* intitiates the production of degradative proteinases, including matrix metalloproteinases and proinflammtory cytokines by host cells, all of which contributes to the destruction of host tissues (Guan et al., 2009). Furthermore, *P. intermedia* has demonstrated resistance to antibiotics including penicillins, cephalosphorins and tetracyclines, thus elimination of this particular organism may be critical for disease prevention (Kulik et al., 2008, van Winkelhoff et al., 1997). *P. intermedia* is found throughout the oral cavity at healthy sites, and studies have shown that the profile of cytokines and proteins released by the bacteria is site dependant, which suggests that the environmental conditions have a strong influence on the behaviour of this particular bacteria (Kuboniwa et al., 2012). Moreover, the environment on the upper surface of the denture, in direct contact with the mucosa is and cut off from the antimicrobial effects of saliva, may support the pathogenicity of *P. intermedia* and enhance DS. Thus, this merits further investigation, in which the incorporation of *P. intermedia* into a denture biofilm model would be beneficial to assess its virulence. As for *Veillonella*, it is not generally considered a pathogen in the oral cavity, however it is implicated in oral disease due to its role as an intermediary in the development of periodontal biofilms by supporting the growth and attachment of the red complex bacteria, that are responsible for disease (Millhouse et al., 2014).

Furthermore, at the mucosa, bacterial classes *Actinobacteria* and *Bacteroidia* also increased significantly in DS patients, further strengthening this hypothesis. A study supporting these findings by Campos *et al* (2008) investigated the microbial biofilm communities of denture stomatitis sufferers (Campos et al., 2008). They found 32 bacterial phylotypes that were unique to denture stomatitis biofilms, a large proportion of which fell under the genera *Atopobium* (16%) and *Prevotella* (11%), both of which fall into the classes *Actinobacteria* and *Bacteroidia,* respectively. Moreover, the mucosal microbiome of DS individuals is significantly more diverse with less dominant tax than their healthy counterparts, unlike the dental or denture microbiomes. Thus these results suggest that the compositional changes that are responsible for disease progression are occurring at the mucosa.

However, whether these changes are cause or effect of disease is an area that merits further investigation.

We have established that the presence of natural teeth alters the composition and diversity of the microbiome, and our results suggest that the DS microbiome contains increased proportions of microbes more commonly associated with a dentate status. Therefore, we looked at the ratio of dentate to edentate within the health and DS groups and we found that both groups were equally represented within the health category (50%); however, the dentate group made up a considerably larger proportion of the DS category, (61%) than the edentate (39%). Therefore, this may explain why we are seeing higher levels of these, normally dentate-associated microbes, within the microbiomes of DS individuals. Within the DS group it is likely that dentate and edentate individuals form two distinct biofilms, both with pathogenic and invasive potential, however, given that the majority of those with severe inflammation had teeth (67%), the presence of natural teeth may exacerbate denture stomatitis infection, creating a more pathogenic biofilm than those found in the edentulous.

Denture related disease is almost always attributed to infection with *C. albicans*, however, given the vast range of bacterial species identified on dentures and the surrounding mucosa in this study; it is unlikely that the infection can be attributed solely to *Candida* spp. Correlation analysis was carried out to investigate the affect of increasing *Candida* load (CFU counts) on the bacterial microbiome both at the class and genus level. At the denture and mucosal microbiome, there was a positive correlation with the class *Bacilli* and a negative correlation with *Fusobacteria* at the denture, these findings are in line with Kraneveld *et al* (2012), where they identified the similar correlations when analysing the effect of increasing *Candida* load on the salivary microbiome of the elderly (Kraneveld et al., 2012). At the genus level, these correlations could be attributed to *Lactobacillus* species. This finding was surprising as the majority of literature regarding these species, indicates that they have an antagonistic relationship (Orsi et al., 2014). The mechanisms by which *Lactobacillus* inhibits growth of *Candida spp*. are not fully understood, investigations have suggested production of hydrogen peroxide by *lactobacilli* leads to anti-candidal activity. Furthermore, *lactobacilli* can modulate the host response, up-regulating cytokines when co-

cultured with *C. albicans*, which could be associated with the clearance of candidal infection. Nevertheless, denture-wearing patients have been shown to have higher levels of *Lactobacillus* species in their saliva, but they are more commonly isolated in the saliva of DS sufferers (87%) in comparison to healthy controls (65%) (Bilhan et al., 2009). Yet, in spite of the vast evidence of an antagonistic interaction, certain species of oral *Lactobacillus* (namely *L. casei*) have demonstrated a stimulatory effect on *C. albicans* hyphal growth (Orsi et al., 2014). Furthermore, it has been shown that *Candida* hyphae can co-aggregate with *lactobacilli* and sustain their levels in patients with advanced oral diseases (Bilhan et al., 2009). Nonetheless, most of these studies have focused on *Lactobacillus* spp. inhibitory effects on *C. albicans*, but their inhibition of other *Candida* species such as *C. glabrata* has proven less effective, as Jiang *et al* (2014) demonstrated that only 1 of six probiotic *Lactobacillus* species used in the study had an inhibitory effect on *C. glabrata* growth (Jiang et al., 2014).

Furthermore, it could be suggested that what is missing from this study is a control group of healthy non denture wearers to which the microbiome can be compared. Whilst this particular control group would be beneficial in terms of observing differences in the mucosal and dental microbiome, it would be impossible for the denture. The key purpose of such a group would be to attribute changes observed to the presence of a denture, however, this study has shown, numerous factors such as the presence of natural teeth, sleeping with a denture in, oral hygiene etc. can all contribute towards changes in the microbiome composition. This, makes understanding how the denture affects the microbiome composition increasingly complex. Unfortunately, the nature of this study did not allow for collection for such a patient cohort, primarily due to time constraints, costs and ethics. Nonetheless, we are able to use data from previous literature looking at the healthy microbiome. Comparison of our mucosal and dental microbiome data to other literature, showed several of the same genera which predominate within the healthy oral microbiome including, *Streptococcus*, *Actinomyces*, *Rothia*, *Veillonella, Fusobacterium, Haemophilus, Porphyromonas Prevotella, Corynebacterium* etc (Aas et al., 2005, Jenkinson and Lamont, 2005, Tuomanen, 2005, Zaura et al., 2009b). Interestingly, our data found that *Lactobacilli* are one of the predominating genera the oral cavity of denture wearers, yet does not seem to be the case for the 'healthy' oral microbiome. Therefore, this again draws

attention to the role of lactobacilli in the oral cavity, emphasising the need for further investigation into its role in the mouth of denture wearers.

As well as the microbial interactions in the oral cavity of denture wearers, we were also interested in gaining a wider understanding of the host-microbial interactions. From the range of AMPs measured, only LL37 showed any significant increases between health and inflammation. This could be explained by the fact that the majority of those with inflammation (66%) were in the dentate category, whereas the healthy group were predominantly edentate (95%), and as studies have shown the concentration of salivary AMPs declines with the loss of natural teeth (Davidopoulou et al., 2013). However, gaining a true understanding of salivary AMP concentration is more difficult with an elderly population, as the vast majority take one or more types of oral medication, many of which have been shown to cause xerostomia and thus there is the potential that they may affect salivary AMP concentrations (Visvanathan and Nix, 2010). Yet, to our knowledge the direct implication of oral medication on AMP production has not yet been elucidated.

Furthermore, the method of collection and handling of saliva samples has to be carefully considered when measuring the levels of a specific protein, as factors such as saliva flow rate, site of sampling or sample storage can all impact the protein concentration (Sjogren et al., 2006). For example, Sjogren et al (2006) took to normalising salivary measurements of a specific protein with the total protein concentration in order to control for changes in saliva flow, which can alter the concentration of particular analytes. However, others regard this method as inconclusive due to the complexity of individual protein secretion from the different saliva glands (Bishop and Gleeson, 2009, Brandtzaeg, 2007). Moreover, consistency of sample location is imperative due to the inconsistency of some salivary proteins at different locations. Several studies have shown differences in the concentration of specific proteins between different types of saliva including, whole saliva, pure parotid saliva and residual saliva (Brennan and Fox, 2000, Ruhl et al., 2004, Lee et al., 2007). Therefore, the current recommendation is collection of whole (mixed) saliva, which was collected in this study, in order to minimise differences in composition seen at different locations and saliva sources. Additionally, some proteins are very sensitive to degradation at room

temperature, therefore aliquoting samples before immediate freezing is recommended to avoid continuous freeze-thawing which can be detrimental to the samples (Groschl, 2008). Thus, although measuring protein levels within saliva is an extremely useful technique, it is not without its caveats and therefore preventative measures should be taken to ensure accurate interpretation of results.

In order to identify any potential relationships or associations between AMPs and the microbiome, correlation analysis was carried out. Each AMP was correlated with the denture and mucosal microbiome at both the class and genus level. However, no significant correlations were identified. Nonetheless, this is not to say that the AMPs are not interacting with specific microbes, it is more likely due to their broad spectrum activity (Radek and Gallo, 2007). Furthermore, Bals *et al* (2000) stated that establishing the individual contribution of an AMP to host defence is extremely difficult due to the complexity of the environment and host-microbial interactions (Bals, 2000), as we have clearly demonstrated from the data described herein.

Moreover, in terms of the microbiome, simply understanding "*what is there?*" is not enough; other factors need to be taken into account, both biological and environmental. Certain 'habits' are commonplace amongst denture wearers, such as poor maintenance of denture hygiene or wearing a denture whilst sleeping (Kulak-Ozkan et al., 2002). Habits such as these may lead to the accumulation of plaque, and could subsequently lead to infection of the mucosa or the aspiration of microbes. Furthermore, they may provide the ideal environment to alter the microbial composition (dysbiosis), leading to the accumulation of fungi and bacteria associated with a diseased phenotype. Therefore, understanding how these factors can affect our microbiome and in turn impact on our general oral health both physically and psychologically, should be taken into account, as their impact may lead to more severe consequences than denture stomatitis.

Poor oral hygiene unfortunately is common place amongst denture wearers, more often than not a result of poor knowledge of the correct hygiene practices and products available (de Castellucci Barbosa et al., 2008). Poor hygiene leads to the accumulation of plaque, subsequently increasing the complexity and diversity of

the microbiome, which supports our findings of a more diverse microbiome, with less dominant taxa at the mucosa. This result reflects findings previously shown in this chapter whereby significant differences in diversity between healthy and diseased patients were only detected at the mucosal. Therefore, as suggested previously, compositional changes related to health/hygiene occur at the mucosal site, but again further investigation is required to determine if these changes are a cause or effect of poor hygiene. The lack of differences in microbial composition and diversity between those with good and those with poor oral hygiene is surprising, particularly given the abundant evidence demonstrating a link between poor oral hygiene and the development of oral diseases, e.g. gingivitis, PD, candidiasis, etcetera (Pihlstrom et al., 2005, Akpan and Morgan, 2002, Darwazeh et al., 2010, Loe, 2000). Yet, it is possible that the lack of changes is due to the environment, as an edentulous oral cavity is less diverse than that of a fully dentate individual. Therefore, it is not to say that there are no significant changes occurring, it likely that in a less complex environment the changes occurring are more subtle and perhaps less likely to lead to a pathogenic outcome.

We have previously shown that there are significant differences in the microbiome between healthy and diseased individuals in terms of microbial composition and diversity, at the mucosal surface in particular. Therefore, we were interested to know if having denture related disease induces changes in the microbiome in terms of oral hygiene. As currently within each group there is a mixture of healthy and DS patients, which may account for the lack of difference seen. However, further separating the groups into healthy and DS with good or poor oral hygiene would allow us to rule out any changes that were a result of disease rather than poor hygiene. In terms of this study, having DS indicated that any changes induced by poor oral hygiene were not affected by disease, however, we remain cautious before making a definite conclusion, given the complexity of the relationship and strong correlation between disease and poor oral hygiene.

In terms of denture cleaning frequency, only a few notable changes were observed on the denture. Those that cleaned twice a day had increased levels of *Bacilli* in comparison to those that cleaned only once. This is likely due to colonization of streptococcal species, which are the main primary colonizers in the oral cavity (Moore et al., 1982, Syed and Loesche, 1978), and thus would be more apparent

on the denture surface of those that clean twice per day as they will likely have fewer microbes already on the denture surface. However, a recent study suggests that it is the method of cleaning employed which is a more important factor in altering microbial abundance and composition, rather than the frequency (Duyck et al., 2016). Therefore, given these findings, it begs the question, is maintaining a strict hygiene regimen as important as we have been led to believe?

Sleeping with a denture *in situ* poses several risks, including choking and developing aspiration pneumonia (Iinuma et al., 2015). Removing dentures at night provides the opportunity for cleaning, e.g. soaking dentures overnight in an anti-microbial solution, whereas sleeping with them in simply allows for further accumulation of plaque (Duyck et al., 2016). Given the unique site of dentures, in direct contact with the mucosa, the accumulated plaque on the denture surface has greater opportunity to colonise and subsequently infect the mucosa if not removed. In this study, a number of notable changes in the abundance of several bacteria were found across both the denture and mucosa. Common to both were increases in *Bacteroidia, Epsilonproteobacteria* and *Fusobacteria*. The role of *Bacteroidia* in the oral cavity is well documented as *Porphyromonas, Prevotella* and *Tannerella* genera are all included within this class. In terms of periodontal disease, the most pathogenic organisms involved are the red complex bacteria, *P. gingivalis, P. intermedia* and *T. forsythia (Socransky et al., 1998)*. Therefore, sleeping with a denture *in situ* may be causing an increase in these pathogenic bacteria and may contribute towards the increased diversity seen at the mucosa of DS individuals. Genera that have the potential to be pathogenic and fall under the *Epsilonproteobacteria* class include *Helicobacter* and *Campylobacter*. Within our samples no Helicobacter was detected, however Campylobater was detected at the denture, mucosal and dental sites. Campylobacter species are classed into two groups, zoonotic and human oral species (Lee et al., 2016). The zoonotic pathogens i*nclude C. jejuni* and *C. coli* which primarily causes gastroenteritis in humans. The human oral species include *C. curvus, C. rectus, C. gracilis, C. concisus, C. showae* and *C. ureolyticus* which naturally colonise oral cavity as commensals. However, these oral species have been linked with periodontal disease and have more recently been associated with intestinal disease (Kaakoush and Mitchell, 2012, Macuch and Tanner, 2000). Thus, there is potential that these normally harmless commensal species could play a role in DS, and requires further

investigation. However, the role of *Fusobacteria* within the oral cavity is well documented (Aas et al., 2005), which is most commonly the PD pathogen *F. nucleatum* (Dzink et al., 1988). However, whether the increasing levels of this microorganism can be associated with the development of DS disease remains unknown. Moreover, upon the denture surface the most notable change was the increase in *Clostridia* on those sleeping with their denture *in situ*, this pattern was also apparent even when the groups were separated into healthy and DS. Nonetheless, the DS 'no' group levels, although still lower in comparison to the 'yes' groups, were slightly elevated, suggesting that disease is also contributing towards increasing *Clostridia*. Therefore, this further supports the theory that changes in the microbiome cannot be pinpointed to a single cause, and that various factors are responsible for shaping the microbiome.

16S genomics is not without its limitations and can present numerous challenges (Sharpton, 2014). Amplicon sequencing does not always produce accurate estimates of the sample diversity, as the genomic loci sequenced may represent some taxa better than others(Human Microbiome Project, 2012). Furthermore, sequencing errors that are commonly introduced during PCR, can lead to incorrect sequences that may be rejected, therefore potentially losing important taxa (Wylie et al., 2012). The conserved nature of the 16S locus means that sequences can be shared between distantly related taxa, and could result in the overestimation of community diversity (Acinas et al., 2004). Moreover, amplicon sequencing is limited to identifying taxa for which the genetic sequences are known, but also by the sequences which are available in the genome databases, which affects how well the diversity of a community is interpreted (Langille et al., 2013). One of the main limitations of this study is that the majority of OTUs detected have only been identified up to genus level. Therefore, this will make it more difficult to decide on the correct species to include in devising an appropriate biofilm model of DS. However, given the vast number of studies regarding the composition of the oral microbiome, there is already an excellent understanding of the bacterial species normally found in the oral cavity based on the genera we have identified (Wade, 2013, Zaura et al., 2009a). Nonetheless, 16S rRNA sequencing essentially only gives an insight into the composition of the community, yet fails to give us an understanding of the biological functions and interactions associated with the microbes that are present. Shotgun

metagenomics is an emerging NGS technique which avoids the limitations found with standard 16S sequencing whilst providing an insight into the composition as well as the biological functions of a community (Sharpton, 2014). With this technique all DNA within the sample is degraded into tiny fragments and sequenced, reads are then sampled from the 16S taxonomically informative genomic loci, and from coding sequences that deliver insight into the biological functions of the sequenced genome. Although shotgun metagenomics is limited in its sequencing depths, and this sis where 16S genomics has an advantage. However, with the ever adapting NGS technologies it is likely vast improvements in the read depth of this technology will improve. Thus, the progression towards sequencing methods which provides a more in depth interpretation of the microbial environment seems the most probable and logical outcome for future sequencing studies.

In conclusion, given the complexity of the oral environment, comprehending the intricate interactions of this ecosystem is a difficult task, and the addition of a denture further adds to the complexity. However, this study has taken a significant step forward towards understanding this environment. With a detailed knowledge of the microbial composition, research in this area can progress further, with a more in depth focus on how these organisms interact with one another as well as the host, allowing us to develop meaningful biofilm models of denture plaque. Moreover, going forward it would be useful to look at longitudinal dynamic changes of the denture microbiome and its effect on oral health.

From this study we can conclude that the bacterial microbiome composition of denture wearers is not consistent throughout the mouth and varies depending on sample site. Moreover, dental plaque is unsurprisingly more diverse than denture plaque and the mucosal microbiome, and with this we have shown that the presence of natural teeth has a significant impact on the overall microbial composition. Furthermore, the findings from this study do suggest that maintaining good oral hygiene and hygiene practices do not appear to have as strong an influence on altering the microbial composition as we had previously assumed. Yet, the lack of differences are not necessarily absence of evidence, it may simply be due to the fact the this was originally designed as a pilot study and not designed to take into account additional factors such as hygiene.

We have deduced that there are a vast number of environmental variables with the potential to alter this environment, which ultimately means that the oral microbiome of each denture wearer is unique to the individual. Nonetheless, with the detailed knowledge we have gained from this study we can achieve a better understanding of the pathogenesis of denture related disease and which will aid towards the development of potential therapeutics.

CHAPTER FINDINGS

The bacterial microbiome composition of denture wearers is not consistent throughout the mouth and varies depending on sample site.

The dental microbiome is more diverse than the denture and mucosal microbiome.

The presence of natural teeth has a significant impact on the overall microbial composition.

Evidence suggests that the compositional changes responsible for disease progression are occurring at the mucosa.

Individual AMPs do not appear to have a direct implication on specific bacteria the microbiome composition. It is likely their antimicrobial effects are broader spectrum.

Sleeping with a denture *in situ* leads to a shift in the microbial composition of denture plaque.

4 Developing a Denture Biofilm Model

4.1 Introduction

The role and clinical significance of fungal-bacterial interkingdom interactions within the oral cavity is of growing interest, with particular emphasis on how they impact disease (Sumi et al., 2002, Ealla et al., 2013). In the context of DS the majority of research has to date focused on how *C. albicans* is the causative microbial agent (Budtz-Jorgensen, 1970, Gendreau and Loewy, 2011, Johnson et al., 2012, Pereira-Cenci et al., 2008). Despite this, recent data indicates that approximately 10-fold more bacteria than yeasts colonise the surface of dentures (Chapter 2). This has led to the development of denture biofilm models of polymicrobial composition, though these tend to either be limited to 2-3 organisms or rely on clinical samples of unknown inocula (Yassin et al., 2016, Cavalcanti et al., 2015). Therefore, to fully understand the impact of multi-species biofilm consortia on denture wearers and their impact on denture cleansing (Li et al., 2010, Coulthwaite and Verran, 2008, Urushibara et al., 2014), then there is a clear requirement to develop clinically relevant *in vitro* defined multi-species denture biofilm.

Poly(methyl) methacrylate (PMMA) is the primary denture material of choice, though this has an uneven surface that results in a heterogeneous topography in which yeasts and bacteria can co-colonise, forming biofilms and escape from denture cleansing therapies (Li et al., 2010, Mendonca e Bertolini et al., 2014, Ramage et al., 2012). Investigations to determine the optimal method for cleaning dentures have focussed on the various physical and chemical cleansing techniques, both individually and in combination. Yet, most of these techniques evaluate treatment over a short period of time and therefore do not accurately simulate an optimal denture routine clinically (Felton et al., 2011, Pavarina et al., 2003, Pellizzaro et al., 2012). Daily denture cleansing treatment of *Candida* biofilms has been previously investigated and found that despite a significant reduction in viable *C. albicans* cells, a residual reservoir of yeast cells remained, resulting in ineffective cleansing (Faot et al., 2014, Freitas-Fernandes et al., 2014, Ramage et al., 2012). A limitation to these studies was using a single species biofilm model, as this is unreflective of the denture polymicrobial environment. Therefore, the development of a multispecies denture model that will give a more accurate

reflection of the impact of daily denture cleansing is paramount. Microbiome analysis of denture plaque, described in chapter 3, has shown that there are several key bacterial taxa represented in both health and disease. Therefore, denture plaque biofilms containing these taxonomic groups would provide a clinically relevant model for research into health, disease and potential therapeutics.

4.2 Aims

We hypothesised that the composition of the denture biofilm changes over time and as a result of denture cleansing treatments. Therefore, the aims of this chapter were to develop a multispecies biofilm model that was representative of a denture environment using the microbiome compositional data, and assess the effectiveness of various denture cleansing treatments. The following key questions were investigated:

- Is there a difference in model denture biofilm viability and biomass under different atmospheric conditions?
- What proportion/composition of the overall biofilm does each individual species contribute?
- Is combinational cleansing and brushing more effective than intermittent cleansing over a time course?
- Is there a difference in viable biofilm composition post-treatment?

4.3 Materials and Methods

4.3.1 Growth and standardisation of denture biofilm microorganisms

Laboratory strains were selected to be included within this denture biofilm model based on microbiome data from this chapter 3. The organisms selected for the model were therefore *Streptococcus mitis* NCTC 12261, *Streptococcus intermedius* ATCC 27335 and *Streptococcus oralis* ATCC 35037, which were grown and maintained at 37 ^{o}C on colombia blood agar (CBA [Oxoid, Hampshire, UK]) in 5 % CO_2. *C. albicans* 3153A was maintained on Sabouraud's dextrose agar (Oxoid) at 30 ^{o}C for 48 h. *Actinomyces naeslundii* ATCC 19039, *Veillonella dispar* ATCC 27335, were maintained at 37 ^{o}C on fastidious anaerobic agar (FAA [Lab M, Lancashire, UK]) under anaerobic conditions (85% N_2, 10% CO_2 and 5% H_2, [Don Whitley Scientific Limited, Shipley, UK]). *Rothia dentocariosa* DSM 43762 was maintained on CBA (Oxoid) at 37 ^{o}C under aerobic conditions. *Lactobacillus casei* DSM 20011 and *Lactobacillus zeae* DSM 20178 were both grown and maintained on MRS agar (Oxoid) in 5% CO_2.

An overnight broth of *C. albicans* was grown in yeast peptone dextrose (YPD [Sigma-Aldrich]) for 18 h at 30 ^{o}C. *S. mitis, S. intermedius* and *S. oralis* were grown in tryptic soy broth (TSB [Sigma-Aldrich, Dorset, UK]) supplemented with 0.6% w/v yeast extract (Formedium, Hunstanton, UK) and 0.8 % w/v glucose (Sigma-Aldrich). *V. dispar, A. naeslundii* and *R. dentocariosa* were grown in 10 mL of brain heart infusion (BHI [Sigma-Aldrich]) broth. *L. casei* and *L. zeae* were grown overnight in MRS broth (Oxoid). Cultures were grown for 24 to 48 h at 37 ^{o}C as necessary, washed by centrifugation and resuspended in phosphate buffered saline (PBS [Sigma-Aldrich]). All cultures were standardised and adjusted to a final working concentration of 1 x 10^7 cells/mL, in artificial saliva (AS), for downstream biofilm studies.

4.3.2 Development and standardisation of a denture biofilm model

In order to assess the optimum developmental conditions, biofilms were grown under both 5% CO_2 and anaerobic conditions. In order to standardise the biofilms, cell viability, biomass and composition were assessed via alamar blue, crystal

violet (CV) and qPCR, respectively. Microbes were grown in AS, of which components included, porcine stomach mucins (0.25% w/v), sodium chloride (0.35% w/v), potassium chloride (0.02 w/v), calcium chloride dihydrate (0.02% w/v), yeast extract (0.2% w/v), lab lemco powder (0.1% w/v), proteose peptone (0.5% w/v) in ddH_2O (Sigma-Aldrich). Urea was then added independently to a final concentration of 0.05% (v/v).

4.3.2.1 Developing the denture biofilm

S. mitis, S. intermedius, S. oralis and *C. albicans* were standardised in AS to 1 x 10^7 cells/mL and 200 µL was added to each well on a 96 well plate (Corning, NY, USA) The plate was then incubated at 37 ^oC in 5% CO_2 or anaerobic conditions for 24 h. Following incubation, the supernatant was removed. Standardised *A. naeslundii*, *V. dispar*, *R. dentocariosa*, *L. casei* and *L. zeae* were added to the plate containing the previous four organisms and incubated at 37^oC in 5% CO_2 or anaerobic conditions for 24 h. Biofilms were incubated for a further 4 days at 37^oC in 5% CO_2, or under anaerobic conditions, with spent supernatants removed and replaced with fresh AS daily.

4.3.2.2 Denture biofilm cell viability

Following biofilm development, biofilms were washed in PBS to remove any non-adherent cells. Biofilm viability was then assessed using the AlamarBlue® viability assay. 10% alamarBlue® (Invitrogen, Paisley, UK) was added to the biofilms prior to incubation for 4 h in the dark at 37^oC in 5% CO_2 (Kirchner et al., 2012). The alamarBlue® is a colorimetric assay whereby an oxidation/reduction reaction occurs resulting in a colour change based upon cellular metabolic activity. Resazurin is the active ingredient of alamarBlue® that is reduced in the mitochondria of viable cells to produce resorufin, observed by a blue to pink colour change. Following incubation, the absorbance was read at 570 nm and the reference wavelength at 600 nm. The percentage reduction in viability was calculated according to the manufacturer's instructions. To calculate the percentage viability the following calculation was used:

$[(\varepsilon_{OX})\lambda_2 A\lambda_1 - (\varepsilon_{OX})\lambda_1 A\lambda_2 / (\varepsilon_{RED})\lambda_1 A'\lambda_2 - (\varepsilon_{RED})\lambda_2 A'\lambda_1] \times 100$

λ_1 = 570 nm λ_2 = 600 mn

$(\varepsilon_{OX})\lambda_2$ = 117,216, $(\varepsilon_{OX})\lambda_1$ = 80,586, $(\varepsilon_{RED})\lambda_1$ = 155,677, $(\varepsilon_{RED})\lambda_2$ = 14,652

$A\lambda_1$ = OD reading for test well, $A\lambda_2$ = OD reading for test well

$A'\lambda_1$ = OD reading for negative control, $A'\lambda_2$ = OD reading for negative control

For each experiment this was performed in triplicate on three separate occasions.

4.3.2.3 Denture biofilm biomass quantification using crystal violet

Once the alamar blue assay was completed, biofilms were washed twice with PBS to remove any residual AlamarBlue® and left overnight to dry. Biomass was quantified with 0.05% w/v CV solution was added to each biofilm and incubated at room temperature for 20 min to allow uptake of the dye. Following incubation, the CV solution was discarded and the biofilms were washed with running tap water to remove any unbound dye, and 100 µL of 100% ethanol applied to destain each biofilm. The contents of the wells were mixed thoroughly by pipetting and transferred to a new 96 well flat-bottom microtitre plate for measurement. The biomass was quantified spectrophotometrically by reading absorbance at 570 nm in a microtitre plate reader (FluoStar Omega, BMG Labtech International, Ringmer, East Sussex, UK). All absorbance values were blank corrected based upon the negative control where no biofilms were formed. Each isolate had four replicates and biomass measured on three separate occasions.

4.3.2.4 Quantitative analysis of denture biofilm formation

For quantitative analysis of denture biofilms, the microbes were grown and standardised in AS, as previously described in section 4.3.1. 1 mL of AS containing

1 x 10^7 cells/mL of each microorganism was added to each well of a 24 well plate (Corning, NY, USA) containing polymethylmethacrylate (PMMA) 13 mm^2 discs (Chaperlin and Jacobs Ltd, Surrey, UK]. The plate was then incubated at 37°C in 5% CO_2, or under anaerobic conditions, as previously described. The 9 species denture biofilms were then stored at -80°C until required.

4.3.2.4.1 DNA extraction of denture biofilms

Following biofilm development discs were gently washed with PBS to remove any non-adherent cells. Biofilms grown on PMMA discs were sonicated at 35 kHz (Ultrawave) for 10 min in 1 mL PBS to remove the biomass (Ramage et al., 2012). DNA was extracted from the biofilms using the QIAamp DNA mini kit, as per manufacturer's instructions (Qiagen, Crawley, UK). The biofilms in PBS were centrifuged for 10 min at 10,000 *g*, then the supernatant discarded and the pellet was re-suspended in 180 µl of ATL buffer supplemented with 20 µL of proteinase K. Samples were incubated at 55°C for 20 minutes, and the mixture was transferred to a new tube containing 250 mg of sterile acid-washed glass beads of 0.5 mm and 0.1 mm diameter (Thistle Scientific, Glasgow, UK). The tubes were vortexed for 3 × 30 s on a Mini-BeadBeater, (Sigma-Aldrich, Gillingham, UK), at the maximal speed, while intermittently being placed on ice. Samples were centrifuged for 10 min at 5 000 *g* and the supernatant was transferred to a fresh microcentrifuge tube. 200 µL of buffer AL was added to each sample and mixed by pulse-vortexing for 15 s, and then incubated at 70°C for 10 min. 200µL of 100% ethanol was added to the sample, and mixed by pulse-vortexing for 15 s. It is essential that the sample, Buffer AL, and the ethanol are mixed thoroughly to yield a homogeneous solution. The mixture was then transferred to the QIAamp Mini spin column and centrifuged at 6000 *g* for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube and the filtrate was discarded, then 500 µL of buffer AW1 was added to the spin column and centrifuged at 6000 *g* for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube and the filtrate was discarded, then 500 µL buffer AW2 was added to the spin column and centrifuged at full speed (20,000 x *g*) for 4 min. The QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube tube and 50 µL buffer AE was added to the column. The samples were incubated at room temperature for 1 min, and centrifuged at 6000 *g* (8000 rpm) for 1 min. DNA was quantified using the NanoDrop™ ND-1000 spectrophotometer (Labtech International).

4.3.2.4.2 Nucleic acid quantification

DNA was quantified using the NanoDrop™ ND-1000 spectrophotometer (Labtech). AE buffer was used as a reference before the DNA was quantified and concentrations were recorded in ng/µL. Samples with a 260/280 nm ratio of 1.8 to 2.2 were deemed to be of high quality and were used for subsequent PCR reactions. DNA was stored at -20°C until required.

4.3.2.4.3 Preparation of DNA for denture biofilm standard curves

Serial 10-fold dilutions of the 1 x 10^8 cells/mL cultures were prepared ranging from 1 x 10^8 cells/mL to 1 x 10^3 cells/mL. Each dilution was centrifuged for 10 min a 10,000 g. The supernatant was removed, making sure to avoid disturbing the pellet. DNA was extracted using the QIAmp mini DNA extraction kit (Qiagen, Manchester,UK) as previously described. DNA quality and quantity was then quantified by NanoDrop® (ThermoScientific, Loughborough, UK), as previously described (section 4.3.2.4.2).

4.3.2.4.4 qPCR of denture biofilms

Real-time quantitative PCR (qPCR) was then performed to determine the total number of cells for each species found in the biofilm. Briefly, 1 µL of extracted DNA was added to a mastermix containing 10 µL SYBR® GreenER™ (Life Technologies, Paisley, UK), 7 µL UV-treated RNase-free water and 1 µL of 10 µM forward/reverse primers for each bacterial/fungal species. Primers were either taken from published literature or designed using the web-based GenScript real-time PCR primer design software (https://www.genscript.com/ssl-bin/app/primer). Primer sequences were checked for specificity to each target organism using the NIH-BLAST database. PCR amplification efficiencies of all primer sets were optimised prior to gene expression analysis, with efficiencies ranging from 90-110%. Details of the oligonucleotide primers (Eurogentec, Southampton, UK) used in this study are listed in Table 4.1. The thermal profile used consisted of 50°C for 2 min, 95°C for 2 min followed by 40 cycles of 95°C for 3 s and 60°C for 30 s using the step one plus real-time PCR unit (Applied Biosciences, UK). Biological replicates were analysed in duplicate. Melting curve

analysis was performed for all primer sets to ensure a single peak, which was indicative of primer specificity. A standard curve was run on each qPCR plate alongside the samples. Ct values were then used to approximate the number of corresponding colony forming equivalents (CFE's).

Table 4.1: Primer sequences for denture biofilm species.

Target	Primer sequence (5'-3')	Reference
16S	**F** – CGCTAGTAATCGTGGATCAGAATG **R** – TGTGACGGGCGGTGTGTA	
18S	**F** – CTCGTAGTTGAACCTTGGGC **R** – GGCCTGCTTTGAACACTCTA	
Streptococcus spp.	**F** – GATACATAGCCGACCTGAG **R** – CCATTGCCGAAGATTCC	(Periasamy et al., 2009)
A. naeslundii	**F** – GGCTGCGATACCGTGAGG **R** – TCTGCGATTACTAGCGACTCC	(Periasamy et al., 2009)
R. denticariosa	**F** –GGGTTGTAAACCTCTGTTAGCATC **R** – CGTACCCACTGCAAAACCAG	(Uchibori et al., 2012)
V. dispar	**F** – CCGTGATGGGATGGAAACTGC **R** – CCTTCGCCACTGGTGTTCTTC	(Periasamy and Kolenbrander, 2009)
L. casei	**F** –TGCACTGAGATTCGACTTAA **R** – CCCACTGCTGCCTCCCGTAGGAGT	(Desai et al., 2006)
L. zeae	**F** – TGCATCGTGATTCAACTTAA **R** – CCCACTGCTGCCTCCCGTAGGAGT	(Desai et al., 2006)

4.3.3 Sequential treatment of complex biofilms

For sequential treatment of complex denture biofilms, growth in 5% CO_2 was selected as the optimal growth conditions. Biofilms were prepared in a 24 well plate containing PMMA discs, as previously described. Following biofilm development, each disc was gently washed with 1 mL of PBS to remove any non-adherent cells. To demonstrate sequential combinational denture cleansing techniques were more advantageous than intermittent treatment, multispecies biofilms were treated daily over the course of 5 days, as illustrated in Figure 4.1. Treatments were either combinational therapy of brushing with hard water (HW) followed by a 3 min denture cleanser (DC) for 5 consecutive days or daily brushing with intermittent DC on day 1 and day 5 only.

For brushing treatments, toothbrushes containing HW were brushed 5 times across the surface of the PMMA disc containing the complex biofilm. This was based on the surface area and average time of denture brushing, as previously described (Ramage et al., 2012). For combinational treatment, brushing with HW was carried out before denture cleanser (DC) treatment (3 min). For treatment with the DC polident, PMMA discs containing multispecies biofilms were placed in a sterile beaker containing 150 mL of 375 ppm HW before the denture tablet was added, initiating treatment. PMMA discs were removed from the beaker and placed in a 24 well plate containing 1 mL of Dey-Engley neutralising broth (Sigma-Aldrich) and incubated for 15 min anaerobically. This ensured complete inactivation of the compound before microbiological analysis. Untreated controls were maintained in 1 mL HW during the treatment stage and served as a positive control. Blanks containing no inoculum were also included. Experiments were carried out in triplicate and on three separate occasions.

4.3.3.1 Biofilm viability and analysis by colony forming units (CFU)

CFU analysis was performed as a measure of how active each treatment was against the complex denture biofilms. Following treatment and neutralisation, PMMA discs were sonicated at 35 kHz for 10 min to remove the biomass, as previously described in section 4.3.2.4.1 (Ramage et al., 2012). 200 µl of sonicate was then transferred to a fresh microcentrifuge tube and ten-fold serial dilution in PBS were prepared from 10^0 to 10^{-6}. The Miles and Misra technique was employed in which 20 µL of each serial dilutions were plated in triplicate on BHI + 10% blood plates and incubated aerobically and anaerobically at 37^{o}C for 48 h (Miles et al., 1938). In addition, samples were also plated on SAB agar and incubated at 30^{o}C for 48 h. The number of colonies were counted and represented as total aerobes, total anaerobes and total yeasts.

4.3.3.2 Differentiation of total and live cells within biofilms

Viability of the treated biofilms was also assessed using live dead PCR in order to enumerate the definitive and relative composition of the biofilms, a technique that has been shown to differentiate viable and dead cells from various oral bacteria biofilms (Alvarez et al., 2013, Sanchez et al., 2014, Sanchez et al., 2013, Sherry et al., 2016). This method is based upon propidium monoazide (PMA), a

DNA-intercalating dye that is able to bind to DNA following exposure to a halogen light source (Nocker et al., 2006). Binding can only occur in dead cells or those with compromised membrane integrity, as PMA is unable to permeablise cell membranes (Sanchez et al., 2014). This covalent bonding prevents downstream amplification in quantitative PCR (qPCR) and therefore only live cells can be detected.

Samples were prepared as previously described by Sanchez et al (2014), with some modifications (Sanchez et al., 2014). In brief, the sonicated biofilm samples had 50 µM of PMA added to each sample and were incubated in the dark for 10 min to allow uptake of the dye. All samples, PMA positive and negative, were then exposed to a 650 W halogen light for 5 min. Samples were centrifuged at 10000 *g* for 10 min and the supernatants discarded. DNA was extracted using the QIAamp DNA mini kit (Qiagen), as previously described in section 4.3.2.4.1. No PMA controls were also included for each sample to determine total biomass. The extracted DNA underwent quality checks using the NanoDrop spectrophotometer (Fisher Scientific) as previously described in 4.3.2.4.2. Quantitative PCR was then carried out and standard curves were prepared as previously described in section 4.3.2.4.3, to quantify total and live bacteria and fungi within the biofilm. Primer sequences are shown in table 4.1.

4.3.3.3 Scanning electron microscopy

Scanning electron microscopy (SEM) was performed on the 9 species biofilms grown on PMMA discs. Following maturation, biofilms were carefully washed with PBS and their respective treatments were employed, as previously described (section 4.3.3) Biofilms were carefully washed with PBS and then fixed with a fixative solution containing 2% para-formaldehyde, 2% gluteraldehyde and 0.15 M sodium cacodylate, and 0.15% w/v alcian blue (pH 7.4) and left in solution for 18 h. The fixative was carefully discarded and replaced with 0.15 M sodium cacodylate buffer and stored at 4ºC until processing. Samples were then prepared for SEM as previously described (Erlandsen et al., 2004). Samples were washed 3 × 5 min with 0.15 M cacodylate to ensure all gluteraldehyde had been removed. Samples were then treated with 1% osmium tetroxide solution containing 0.15 M sodium cacodylate (1:1) and incubated in the fume hood for 1 h. Samples were rinsed 3 × 10 min with distilled water and then treated with 0.5% uranyl acetate

and incubated in the dark for 1 h. Uranyl acetate was removed from the samples and quickly rinsed with water before a series of dehydration steps were carried out. Two 5 min rinses of 30, 50, 70 and 90% alcohol were followed by 4 × 10 min rinses of absolute and dried absolute alcohol. Hexamethyldisilazane (HMDS) was used to dry the specimens by soaking the samples for 5 min before transferring to a plate containing fresh HMDS. All samples were then placed in a dessicator overnight to allow evaporation of any residue and drying. The specimens were then mounted and sputter-coated with gold in an argon filled chamber, and then viewed under a JEOL JSM-6400 scanning electron microscope. Images were assembled using Photoshop software (Adobe, San Jose, CA, USA).

4.3.4 Statistical Analysis

Data distribution, graph production and statistical analysis were performed using GraphPad Prism (version 5; La Jolla, CA, USA). After assessing whether data conformed to a normal distribution, One-way Analysis of Variance (ANOVA) and *t* tests were used to investigate significant differences between independent groups of data that approximated to a Gaussian distribution. A Bonferroni correction was applied to the p value to account for multiple comparisons of the data. Any non-parametric data was analysed using the Mann-Whitney U-test or the Kruskal-Wallis test with a Dunn's post-test to assess differences between independent sample groups. Statistical significance was achieved if $P<0.05$.

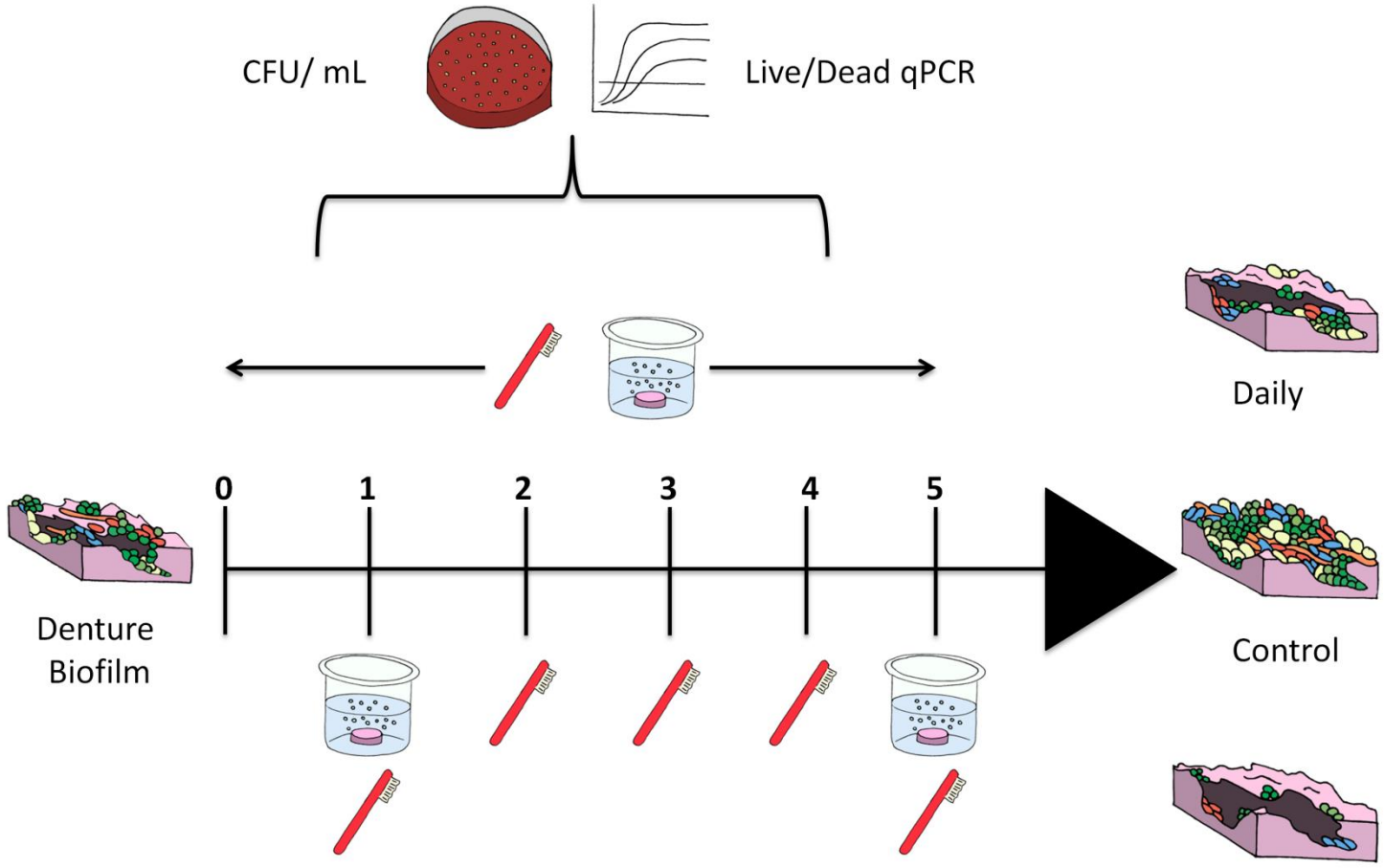


Figure 4.1: Sequential treatment of denture biofilm protocol.
PMMA discs were placed in 24 well plates for biofilm culture. Biofilms were treated daily with brushing and denture cleansing for 5 days or were brushed every day with denture cleansing on day 1 and day 5. Untreated controls were maintained in artificial saliva during treatments.

4.4 Results

4.4.1 Development and standardisation of a denture biofilm model

Using data from the microbiome analysis of denture plaque (chapter 3), the contribution of each genera was determined and averaged across the 108 patient samples that were sequenced. The ten most abundant genera found within denture plaque were calculated (Table 4.2). *Actinomyces* was most predominant, contributing to 26.97% of the overall biomass, followed by *Streptococcus* (23.5%), *Rothia* (10.09%), *Lactobacillus* (7.31%), *Veillonella* (6.85%), *Prevotella* (3.62%), *Haemophilus* (2.38%), *Scardovia* (2.12%), *Bradyrhizobium* (1.39%) and *Bifidobacterium* (1.09%). Based on these data, species that contributed to less than 5% of the overall biomass were not selected to be included in the denture biofilm model. Nine species were selected to be included in the denture biofilm model, *C. albicans, S. mitis, S. oralis, S. intermedius, A. naeslundii, V. dispar, R. dentocariosa, L. casei and L. zeae*. These particular species were selected based on literature and previous work by our lab, as they are commonly found species within the oral cavity for each genera (Chen et al., 2010, Millhouse et al., 2014).

Table 4.2: Most abundant genera found within denture plaque from the microbiome analysis.

Most abundant genus		
Genera	**Total Reads**	**%**
Actinomyces	22431	26.97
Streptococcus	19543	23.50
Rothia	8388	10.09
Lactobacillus	6080	7.31
Veillonella	5696	6.85
Prevotella	3012	3.62
Haemophilus	1977	2.38
Scardovia	1766	2.12
Bradyrhizobium	1152	1.39
Bifidobacterium	905	1.09

The denture biofilms were tested under different growth conditions, either anaerobic or 5% CO_2 in order to determine under which conditions there was optimal biofilm biomass and viability. In terms of biomass, there were no significant difference between 5% CO_2 or anaerobic growth (Mean: 0.53 v 0.44, $p>0.05$) (Fig 4.2A). Under both growth conditions there was high biofilm viability, and no significant differences observed (Mean: 78.33 v 87.18, $p>0.05$) (Fig 4.2B). Therefore, given the similarity in biomass and viability, growing biofilms in 5% CO_2 was chosen as the preferred growth conditions when preparing biofilms hereafter.

The composition of the denture biofilm was assessed to determine the individual species contribution to the biofilm using qPCR. Figure 4.3A shows the number of bacterial cells detected for each species, regarding CFE. Therefore, in terms of the overall proportion each species contributes to the biofilm, *Streptococcus* species were by far the most predominant, comprising up 49.2% of the biofilm (Fig 4.3B). This is then followed by *A. naesundii* (33.6%), *V. dispar* (11.9%), *L. zeae* (2.9%), *R. dentocariosa* (1.9%), *C. albicans* (0.98%) and *L. casei* (0.39%).

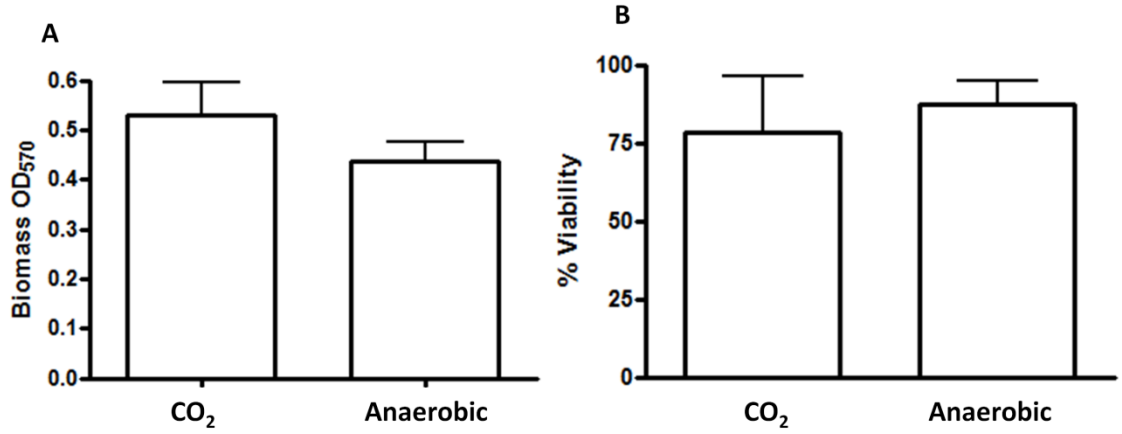


Figure 4.2: Biofilm formation and viability of multi-species denture biofilms under alternate growth conditions.
Multi-species denture biofilms were grown on Thermanox™ coverslips within 24 well plates with AS media changed daily for 4 days. Upon biofilm maturation and development, biofilm formation was measured using a crystal violet assay **(A)** and metabolic activity was measured using the alamarBlue® assay **(B).** All samples were assayed in triplicate, on three separate occasions. Data represents the mean. Statistical analysis was performed using a Mann Whitney test as data did not conform to a normal distribution.

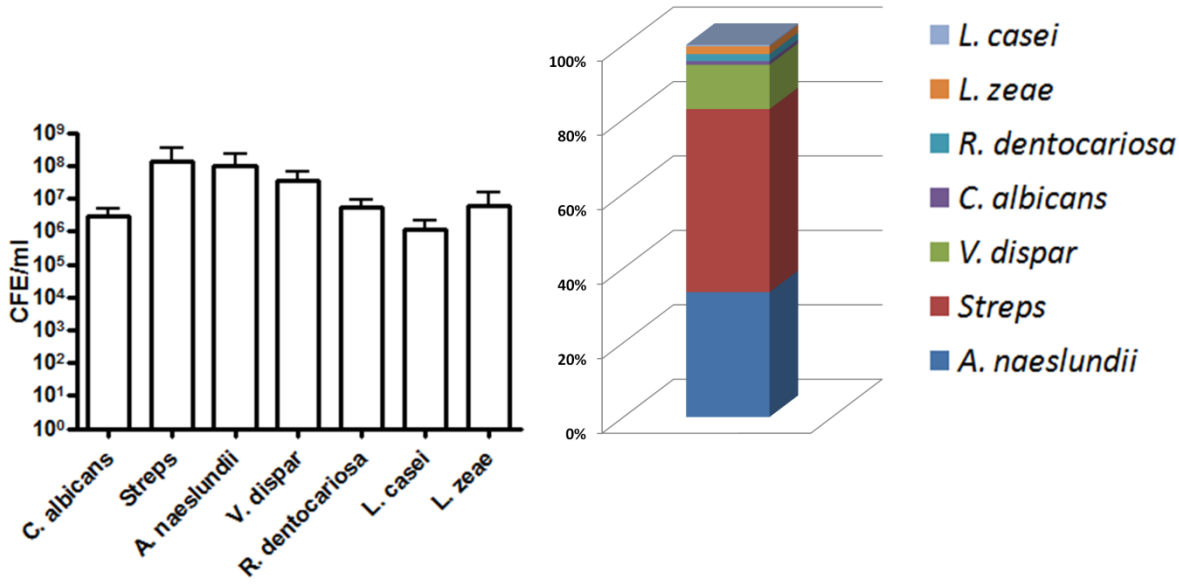


Figure 4.3: Individual species contribution to the denture biofilm.
DNA was extracted from mature biofilms for quantification of each species using SYBR® GreenER™ based qPCR. Standard curves were prepared for each species and values were extracted for each sample, based on Ct values. The contribution of each individual species is shown as the number of cells detected for each species **(A)** and as a proportion of the biofilm (**B).** All samples were assayed in triplicate, on three separate occasions. Data represents mean ± SD.

4.4.2 Quantitative analysis of a denture biofilm after daily combinational treatment

Longitudinal analysis of the effect of daily denture cleansing treatments on denture biofilms was carried out over the course of 5 days. Three groups were included, untreated biofilms as a positive control (UN), daily brushing followed by denture cleansing (BR+DC) and intermittent denture cleansing whereby brushing was carried out each day with the denture cleanser only used on day 1 and day 5 (BR) (Figure 4.1). These treatments were selected based on patient denture cleaning habits and previous studies, however, the majority only looked at the effects of these denture cleaning treatments over a short period of time, and thus a more longitudinal study was required to investigate the long term effects (Felton et al., 2011, Pavarina et al., 2003, Pellizzaro et al., 2012).

Total aerobic and anaerobic bacteria, and total yeasts, were initially quantified using CFU analysis. Both total aerobes (Fig 4.4A) and anaerobes (Fig 4.4B) remained relatively stable across the 5 days in terms of CFU/ml for the untreated group, with aerobes showing only a small decrease from 1.52×10^8 to 1.02×10^8 CFU/mL and anaerobes a slight increase from 1.72×10^8 to 1.85×10^8 CFU/mL. For the intermittent brushing treatment (BR), bacteria were undetectable on day 1, but saw a steady increase from day 2 to day 4, before declining again on day 5; a pattern also observed for both aerobes and anaerobes. As for the daily cleansing treatment, no CFUs were detectable on any day for either aerobes or anaerobes. Total yeast levels, in the UN group saw a decline over the five day time course, going from 5.43×10^5 to 2.63×10^5 CFU/mL (Fig 4.4C). For the BR group, no yeasts were detectable on day 1, but there was a sharp increase to 5.01×10^4 CFU/mL on day 2, this remained steady until day 4 before becoming undetectable again on day 5.

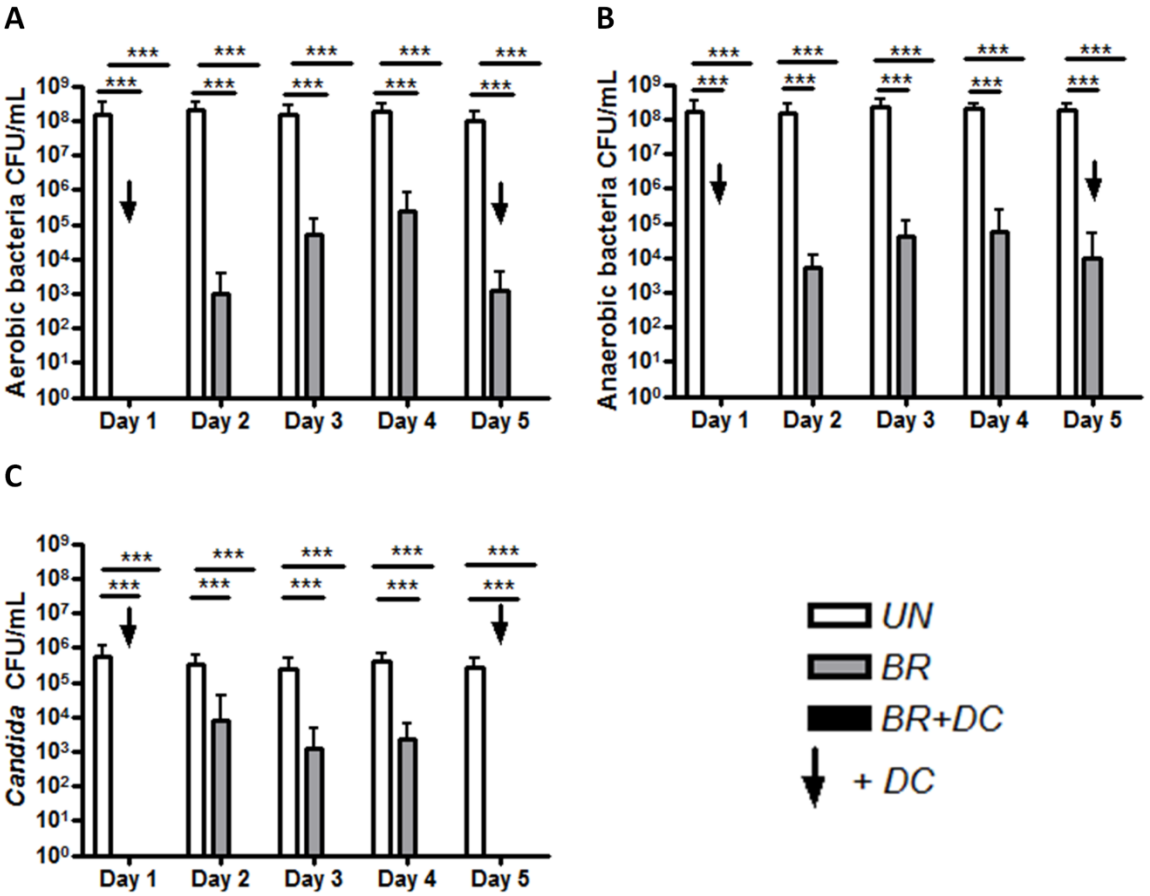


Figure 4.4: Daily CFU counts of bacteria and yeasts post treatment.
Multispecies complex biofilms were grown on PMMA for 7 days, as previously described. Biomass and viability of total aerobes **(A)**, anaerobes **(B)** and *Candida* **(C)** was assessed by CFU. All testing was carried out in triplicate and on three independent occasions. Data represents mean ± SD (***p<0.0001). Statistical analysis was performed using a one-way ANOVA with Bonferonni post-test to compare all groups to each other.

Quantitative PCR was employed as a more sensitive assay, which would provide the capacity to distinguish between total and live cells. The premise of this approach was to take away the inherent bias of colony counting as a means of viable cell counting. The improved sensitivity of qPCR is demonstrated here, due to the ability to detect the presence of bacteria and yeast within the DC group (Fig 4.5), which previously remained undetected by CFU analysis.

For both total bacteria (Fig 4.5A) and live bacteria (Fig 4.5B), the number of bacteria was significantly lower in both the BR and DC groups when compared to the untreated control, a pattern that was observed on all 5 days. However, no significant differences were seen between the BR and DC groups on any of the days, for either total or live cells. This indicated that both treatments are equally effective at reducing cell numbers, as the total cells remaining by day 5 for BR and DC were 2.07 x 10^6 and 3.48 x 10^6 CFE/mL, respectively, and live cells were 1.25 x 10^5 and 2.39 x 10^5 CFE/mL respectively.

The number of yeasts isolated for the discs shows a similar pattern to that of the bacteria, in that in comparison to the untreated control there was significantly less yeast in both the BR and DC groups. This result was true for day 1 to day 5 and could be observed for both total yeasts (Fig 4.5C) and live yeasts (Fig 4.5D). Again, as with the bacteria, no significant differences were found between the two treatment groups for either total of live cells. The intermittent BR treatment and daily DC treatment reduced the total yeast numbers, by 2 log10, to 3.62 x 10^4 and 5.67 x 10^4 CFE/mL, respectively, by day 5, and from 2.8 x 10^6 CFE/ml on the untreated control on day 1. Both treatments were even more effective at reducing the number of live cells which saw a 3 log10 reduction for both groups with the BR group, reduced to 8.89 x 10^2 CFE/mL, and the DC group to 3.48 x10^2 CFE/ml, when compared to the untreated control on day 1, 1.89 x 10^5 CFE/mL. Thus, although a considerable number of microbes, both live and dead remain on the discs post cleansing, the treatments employed are significantly effective given the extensive reduction in the overall microbial burden.

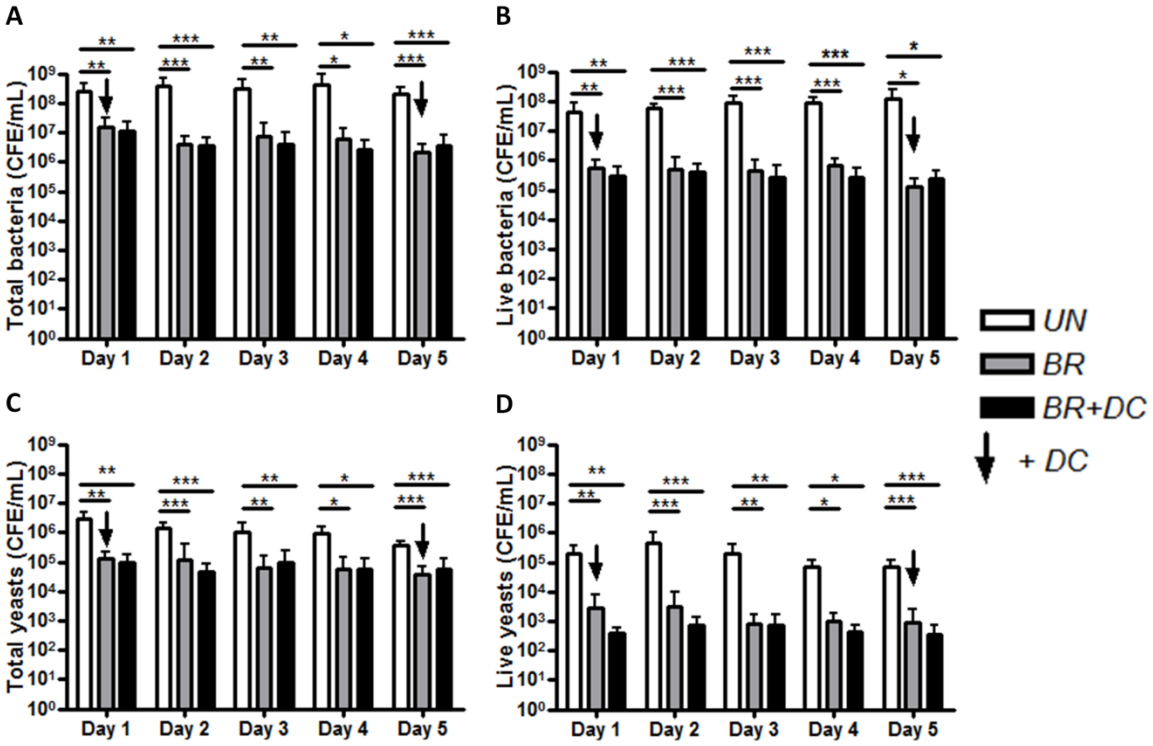
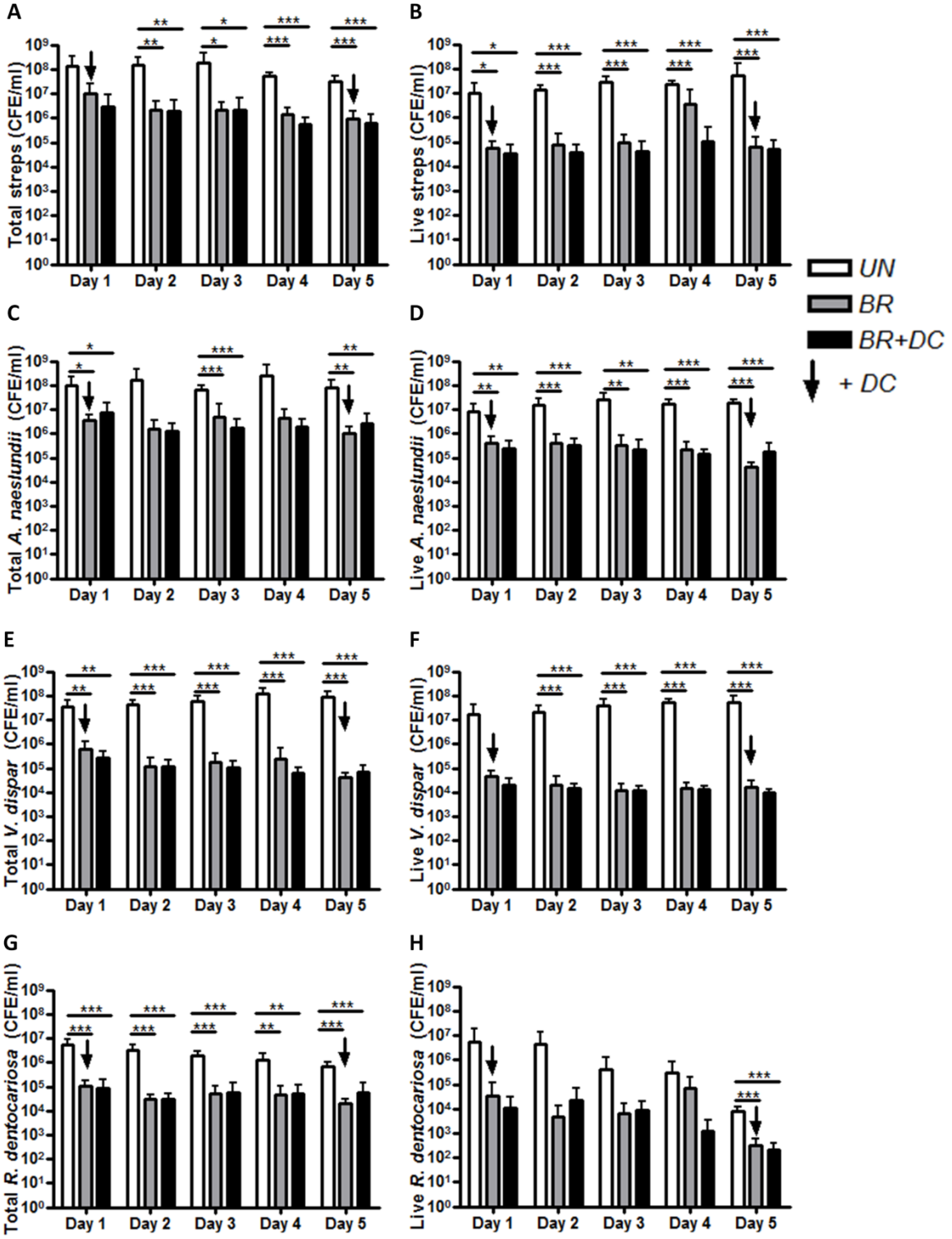


Figure 4.5: Daily CFE counts of total and live bacteria and yeasts post treatment.
Following treatment, each PMMA disc was sonicated before 50 µM of PMA was added and exposed to a 650 W halogen light source for 5 min to allow photo activation. Samples containing no PMA were also included to account for total biomass. DNA was extracted from each sample using the Qiagen DNA extraction kit, for quantification of each species using SYBR® GreenER™ based qPCR to determine the number of total and live cells remaining following treatment for quantification of total **(A)** and live **(B)** bacteria and total **(C)** and live yeast **(D)**. All testing was carried out in triplicate and on three independent occasions. Data represents mean ± SD (*p<0.05, **p<0.01, ***p<0.0001). Statistical analysis was performed using a one-way ANOVA with Bonferonni post-test to compare all groups to each other.

4.4.3 Individual species contribution to denture biofilms during combinational treatment

The number of total and live cells were quantified for each individual species and compared across treatment groups (Fig 4.6). Any changes in the individual species contribution to each biofilm were investigated over the 5 day time course, for both treatments and untreated controls. Initially the total cell count was quantified and converted into a percentage of the overall biofilm, to determine the contribution of each species. Interestingly the untreated control saw a number of changes over the 5 days. *Streptococcus* species began as the most predominant species on day 1 (49.2%) which peaked at day 3 (59.9%), before taking a dramatic decline, contributing only 15.96% by day 5 (Fig 4.7Ai-ii). *A. naeslundii*, tended to fluctuate more, beginning at 35.6% on day 1, but returning to a similar level by day 5 (39.6%). *V. dispar*, however, saw a steady increase from 11.9% to 40.9% on day 5. As for *C. albicans* (0.18%), *R. dentocariosa* (0.33%), and *L. zeae* (1.76%), they all declined by day 5, yet *L. casei* saw a small increase in its overall contribution (1.21%). Moreover, in terms of the treatments, the BR group, as with the UN group, saw *streptococcus* species remain as the majority species (68.4%), with levels declining steadily until day 4 (22.7%), before taking a sharp increase on day 5 (46.5%). *A. naeslundii* increased, overtaking streptococci as the most predominant species, which peaked on day 4 (70.7%) before settling at 47.9% on day 5. *V. dispar* levels remained low in comparison to the control, decreasing from 4.2% to 1.9% by day 5. The remaining species saw dynamic fluctuations, but levels remained relatively steady overall.


A
Total streps (CFE/ml)
B
Live streps (CFE/ml)
C
Total A. naeslundii (CFE/ml)
D
Live A. naeslundii (CFE/ml)
E
Total V. dispar (CFE/ml)
F
Live V. dispar (CFE/ml)
G
Total R. dentocariosa (CFE/ml)
H
Live R. dentocariosa (CFE/ml)
Day 1
Day 2
Day 3
Day 4
Day 5
UN
BR
BR+DC
+ DC

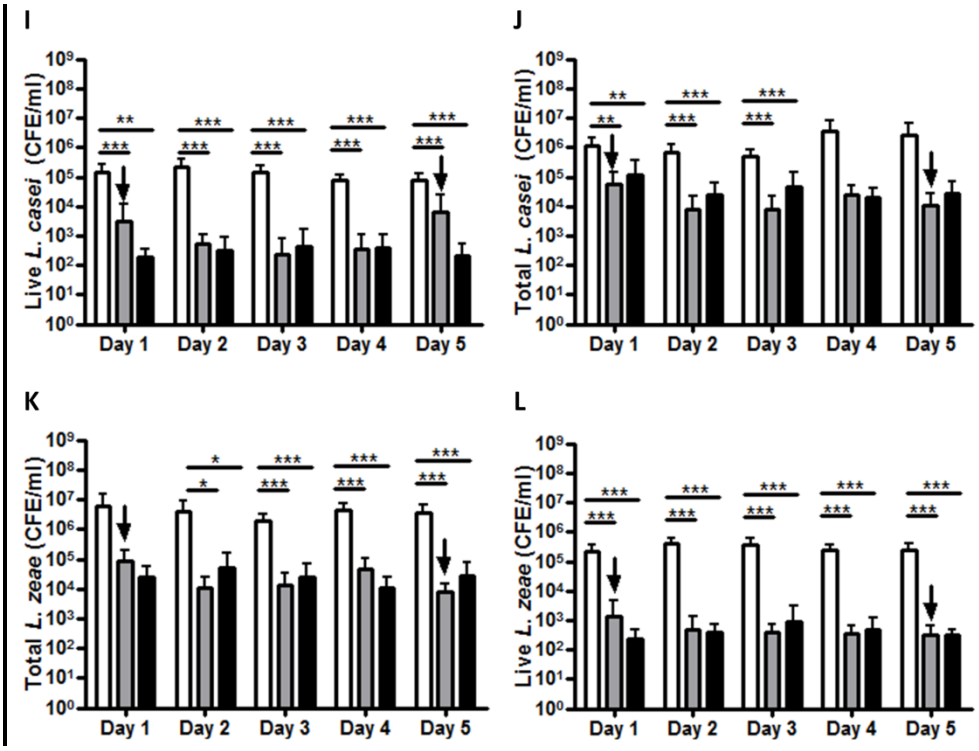
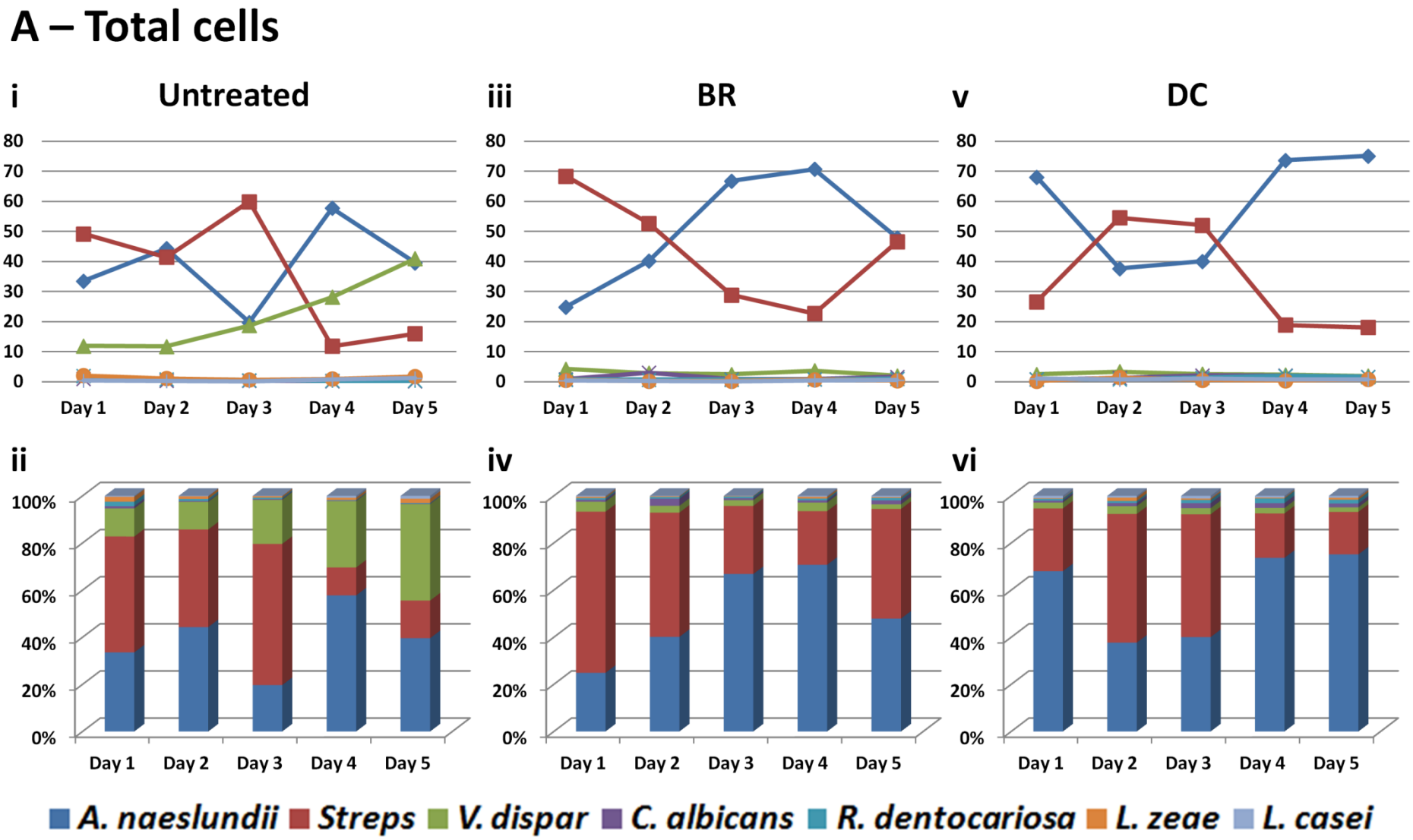


Figure 4.6: Impact of denture cleansing regimens on individual bacterial species within denture biofilms.
Complex 9 species biofilms were grown on PMMA for 7 days and were treated as described previously. Biofilms were treated with PMA and DNA extracted as previously described, for quantification of each species using SYBR® GreenER™ based qPCR to determine the number of total and live cells remaining following treatment. CFE counts were enumerated for *A. naeslundii* total **(A)** and live **(B)** cells, *Streptococcus* total **(C)** and live **(D)** cells, *V. dispar* total **(E)** and live **(F)** cells, *R. dentocariosa* total **(G)** and live **(H)** cells, *L. casei* total **(I)** and live **(J)** cells and *L. zeae* total **(K)** and live **(L)** cells. Untreated controls were also included. All testing was carried out in triplicate and on three independent occasions. Data represents mean ± SD (*p<0.05, **p<0.01, ***p<0.0001). Statistical analysis was performed using a one-way ANOVA with Bonferonni post-test to compare all groups to each other.

Daily combinational treatments demonstrated similar levels and pattern to the intermittent treatment, in terms of *C. albicans, V. dispar, R. dentocariosa, L.zeae* and *L. casei*, all showing small fluctuations across the 5 day treatment, but remaining relatively stable (Fig 4.7Av-vi). Again, as previous streptococcal species and *A. naeslundii* are the primary components of the DC biofilm, however this time *A. naeslundii* begins (68%) and ends (75.2%) as the largest contributor, but not before declining on day 2 (37.7%). Streptococcal species on the other hand saw a fluctuation on days 2 and 3, before gradually declining by day 5 (18.1%).

When focusing solely on the live cells, the denture biofilm composition was significantly altered from that of the total groups, for both treatments and the untreated control. For the untreated control (Fig 4.7Bi-ii), *V. dispar* made up the majority of the live cells on day 1 (42.8%), and was at its highest on day 4 (55.4%). The streptococcal species were the second most abundant on day 1 (24.4%), which seemed to plateau until day 4, before increasing considerably at day 5 (43.4%). *A. naeslundii* showed an initial increase, peaking at day 3 (27.3%), but gradually declined by day 5 (14.7%). Interestingly, *R. dentocariosa* which initially comprised 11.9% of the biofilm, dramatically declined to 0.01% by day 5. *C. albicans, L. casei* and *L. zeae*, all contributed less that 1% each to the biofilm, and all three gradually declined further over the 5 days. As for intermittent cleansing (Fig 4.7Biii-iv), *A. naeslundii* was the most abundant on day 1 (73.9%), before falling to 32.8% by day 5. As *A. naeslundii* gradually fell the streptococcal species overtook, showing a considerable increase from 10.7% to 48% from day 1 to day 5, respectively. 8.1% of the biofilm was composed of *V. dispar*, falling to 2.2% by day 4 but climbing back up to 13% at day 5. As with the untreated control, *R. dentocariosa* fell noticeably, yet *L. casei* had a sizable increase going from 0.6% to 5.1%. The BR+DC group had a similar composition to that of the BR group on day 1 in terms of *A. naeslundii* (77.6%) and *streptococcus* (11.6%), yet despite some fluctuations *A. naeslundii* remained the dominant species by day 5 (74.2%), whilst streptococcus species comprised 21.1% (Fig 4.7Bv-vi)


A – Total cells
i
Untreated
iii
BR
v
DC
80
70
60
50
40
30
20
10
0
Day 1
Day 2
Day 3
Day 4
Day 5
ii
iv
vi
100%
80%
60%
40%
20%
0%
A. naeslundii
Streps
V. dispar
C. albicans
R. dentocariosa
L. zeae
L. casei

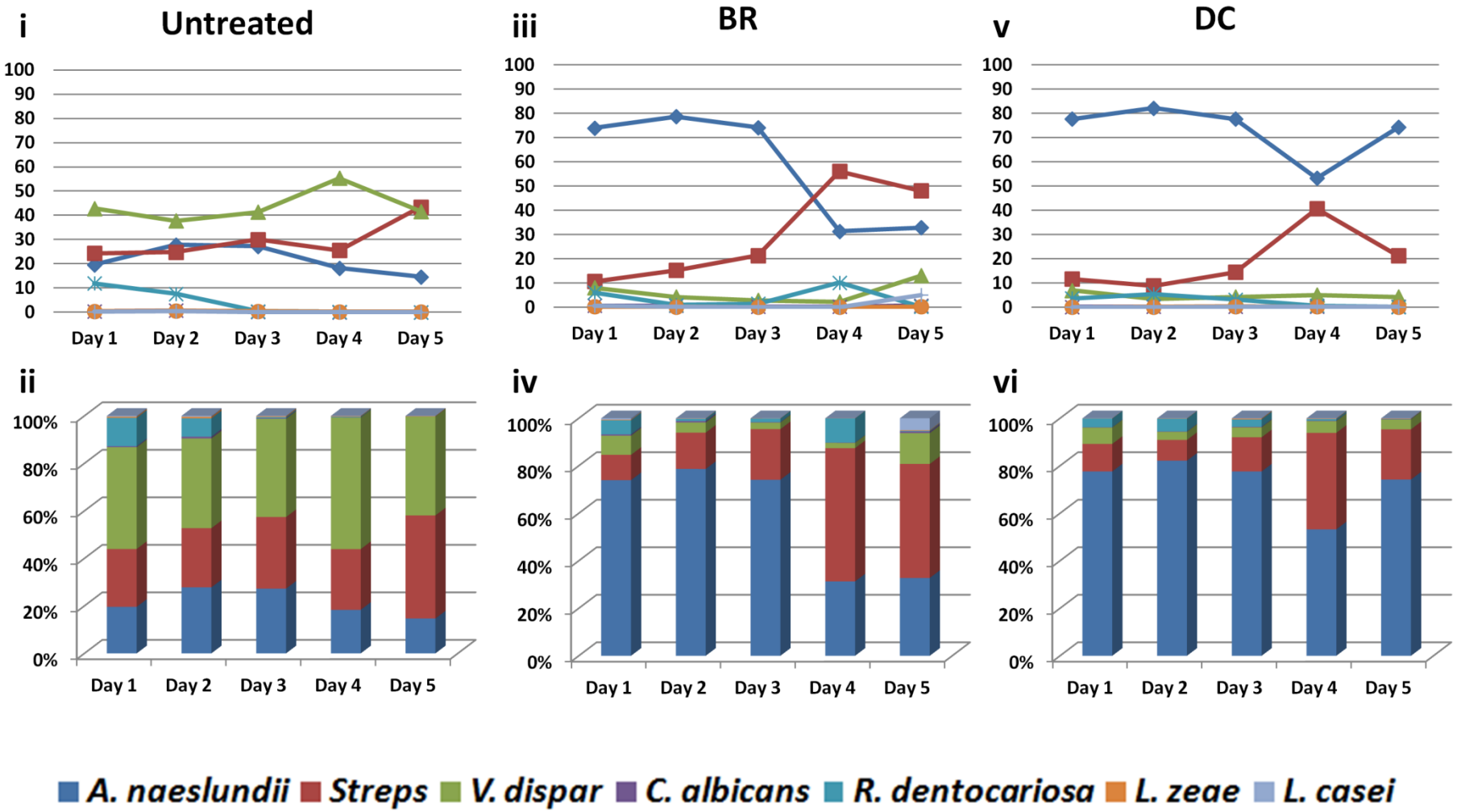

B – Live cells
i
Untreated
iii
BR
v
DC
ii
iv
vi
100
90
80
70
60
50
40
30
20
10
0
100%
80%
60%
40%
20%
0%
Day 1
Day 2
Day 3
Day 4
Day 5
A. naeslundii
Streps
V. dispar
C. albicans
R. dentocariosa
L. zeae
L. casei

Figure 4.7: Compositional analysis of denture biofilms post treatment regimens for total and live cells.
Multispecies complex biofilms were grown on PMMA for 7 days, as previously described. Following maturation, biofilms were washed and either treated daily with brushing and denture cleansing (BR + DC) or brushed daily with the addition of a DC on day 1 and 5 only (BR). Biofilms were treated with PMA and DNA extracted as previously described, for quantification of each species using SYBR® GreenER™ based qPCR to determine the number of total and live cells remaining following treatment. The composition of the biofilms following combinational treatment was determined using species-specific primers for the **total** number of cells for untreated biofilms **(Ai-ii)**, (BR) biofilms **(Aiii-iv)** and (BR + DC) biofilms **(Av-vi)**. The composition of **live** cells within each biofilm were also determined for the untreated biofilms **(Bi-ii)**, (BR) biofilms **(Biii-iv)** and (BR + DC) biofilms **(Bv-vi).** All testing was carried out in triplicate and on three independent occasions.

4.4.4 Effect of combinational treatment on denture biofilm architecture

In order to visualise changes in the biofilms growing on PMMA discs, scanning electron microscopy (SEM) was carried out. Images were taken of UN, BR and BR+DC biofilms on day 1, 3 and 5 DC using x 2500 magnification (Fig 4.8). On day 1, the untreated controls have a thick biofilm with only small patches of PMMA visible. The biofilms are complex, with many *C. albicans* yeast cells visible, which are surrounded by cocci shaped bacteria, which will be primarily streptococci and *V. dispar*, based on the CFE data. Many rod shaped bacteria can be observed, which based on their abundance, will primarily be *A. naeslundii*, chains of *Lactobacillus* are also present. For both treatments, the biomass is visibly reduced, as well as fewer yeast cells, yet some candidal hyphae are present. *Streptococci* are the predominant bacteria, with some rods interspersed.

By day 3, the biomass of the untreated control had increased considerably, with virtually none of the PMMA disc surface visible. As on day 1, streptococcal species are the most predominant, with the classic streptococcus chains present. Furthermore the increase in *V. dispar* by day 3 is likely to also be contributing towards the cocci shaped bacteria. Many rods are visible, however they seem to be located deeper within the biofilm but are being masked by the more abundant cocci adhering to them at the surface. Nonetheless, both the BR and BR+DC treatments removed the majority of the biofilm, with only a few clusters of *streptococci* dispersed across the surface.

On day 5, the final treatment day, the untreated control had abundant biomass, but shows an increased number of rods which reflects the large increase in *Actinomyces* from the quantitative data. However, the thickness and complexity of the biofilm makes it more difficult to differentiate between individual species as mainly only the microbes on the surface are visible. Again, both treatments effectively reduce the biomass, leaving very few cells on the surface. However, bacteria can be seen colonising deep within cracks and crevices on the PMMA discs which have had treatment, which may not be accessible during treatment.

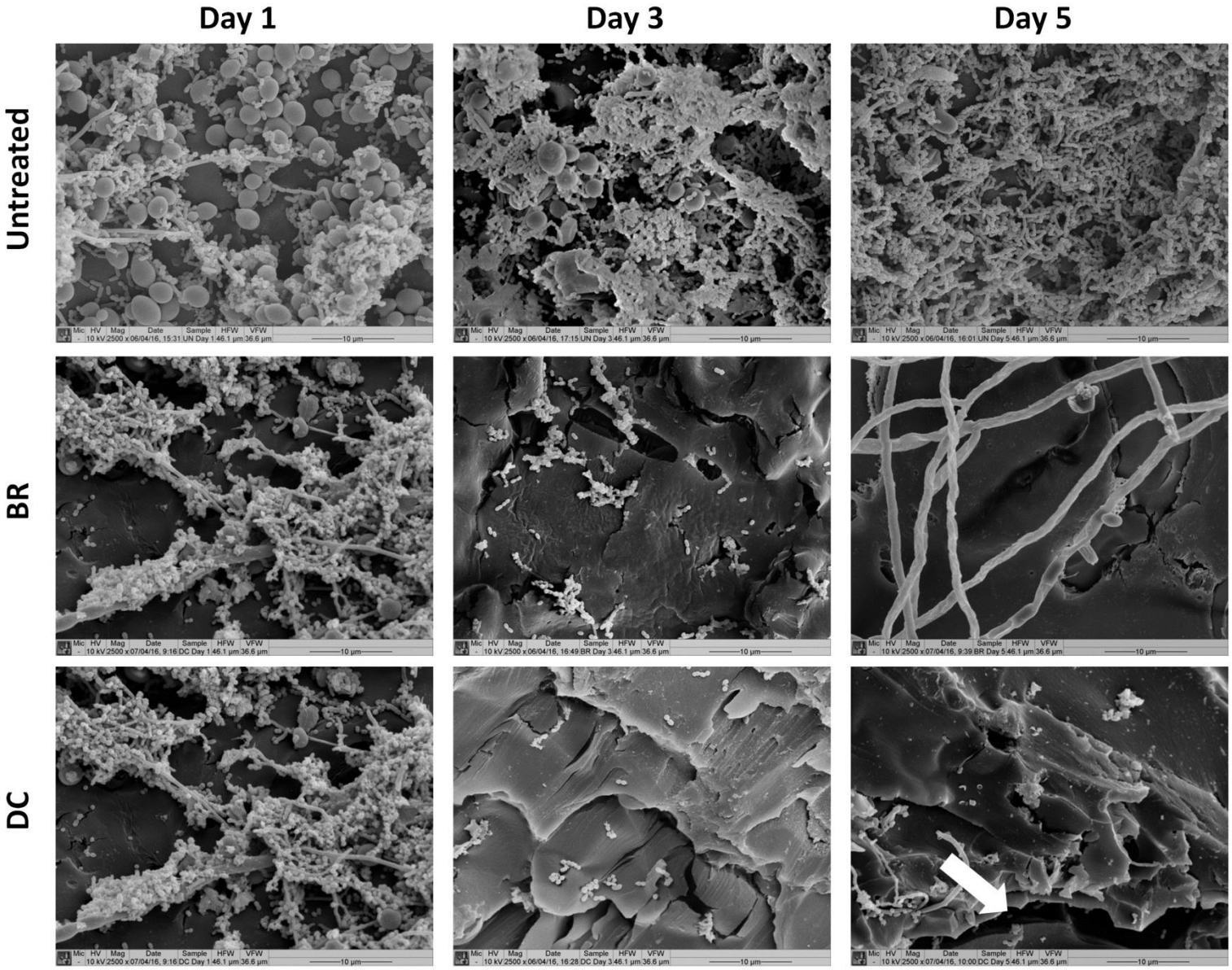

Day 1
Day 3
Day 5
Untreated
BR
DC

Figure 4.8: Daily combinational treatment impacts biofilm architecture by reducing total biomass and live cells.
Multispecies biofilms were grown on PMMA for 7 days, and treated as previously described. Biofilms were then processed and viewed on a JEOL-JSM 6400 scanning electron microscope and images assembled using Photoshop software. All images are shown at 2500× magnifications and are representative of the sample. Scale bars represent 10 μm. Note the pores within the PMMA as denoted by the arrows.

4.5 Discussion

Biofilm models of disease are becoming more utilised in terms of research as they are an effective way to model disease and investigate pathogenesis and potential treatments, when *in vivo* experiments are not possible (Millhouse et al., 2014, Camelo-Castillo et al., 2015, van der Waal et al., 2016). Oral disease is an area of research in which biofilm models have played a key role, particularly in understanding the pathogenesis of periodontal disease (Millhouse et al., 2014, Bao et al., 2014). Disease of the oral cavity is more often than not associated with teeth, however, approximately one fifth of the population are edentulous, yet are still affected (Shulman et al., 2004). Wearing dentures is predominantly the main cause of oral disease in the edentulous (Gendreau and Loewy, 2011), nevertheless, there is a distinct lack of research into the microbiology behind denture related disease. Therefore, using data from chapter 2 which investigated the microbiome of dentures, an *in vitro* biofilm model was developed, which truly reflected the microbial composition of denture plaque. With this model, using sensitive molecular techniques, we have been able to model and monitor the microbial composition of denture biofilms and how they change over time and adapt following therapeutic treatments, based on recent work from our laboratory (Sherry et al., 2016). Furthermore, newer techniques such as live/dead PCR have enabled us to gain a more detailed insight into the individual species susceptibility to antimicrobials.

Given that over 700 bacterial species have been identified with in the oral cavity (Chen et al., 2010), selecting the species to include in the model proved challenging. However, the advancement of NGS has allowed us to gain a more in depth understanding of microbiomes. The previous chapters provided us with the information of the most predominant genus present within denture plaque; this however meant that we were faced with the problem of which species from each genus to include in the model. Based on previous oral biofilm models from our laboratory (Millhouse et al., 2014, Shahzad et al., 2015, Sherry et al., 2013), and previous literature into human oral microbiome studies (Chen et al., 2010), we selected eight bacterial species to be cultured alongside *C. albicans*. Only species from the top 5 most abundant genus were included as a cut off of 5% was instated, otherwise too many species would be included in model, which could quickly become unmanageable. Having to limit the number of microbes which we include

within the model is indeed a caveat, as it may be implied that the model has too many species missing to truly reflect the denture microbiome. However, in order to maintain manageability and accuracy of experiments, a limit had to set; particularly given that over 700 OTU's were identified in some samples.

Unlike previous biofilm models prepared by our group, this model was not grown under anaerobic conditions, as our biofilms were still able to grow effectively with high biomass and viability in 5% CO_2. This is likely because the anaerobic bacteria included were A. *naeslundii* and *V. dispar*, both of which are facultative anaerobes, and the model included no strict anaerobes such as *Prevotella intermedia* or *Porphyromonas gingivalis*, in contrast to models of dental plaque developed by our group. Furthermore, based on this study, both *A. naeslundii* and *V. dispar* did not appear to be restricted in their growth, despite not growing under their optimal conditions, as they were frequently the most predominant species within the biofilms.

Previous work by our lab investigated the effectiveness of different denture cleansing regimens on reducing biofilm biomass and viability, and discovered that a daily combinational treatment of both brushing and using a denture cleanser was most effective (Sherry et al., 2016). However, the biofilm model used in this study was based on an 11 species biofilm with various orally important microorganisms, including *C. albicans*. Whilst the data from this study is extremely useful, the biofilm model is not truly reflective of denture plaque, as it includes bacterial species that are pathogenic in nature such as, *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans*. Thus, there is a need for a more accurate denture biofilm model when testing treatments directed specifically at denture plaque.

A 5 day treatment regimen looking at the effectiveness of daily combinational treatment with brushing and cleansing compared to intermittent cleansing was carried out. The initial CFU analysis indicated that daily cleansing completely removed all the aerobes, anaerobes and yeasts residing on the discs after daily cleansing, yet the intermittent cleansing saw re-growth on days 2-5, these findings are in agreement with previous studies carried out by our group (Ramage et al., 2012, Sherry et al., 2016). Furthermore, qPCR analysis of the same samples highlighted the inaccuracy of CFU analysis. The more sensitive qPCR assay was

able to detect bacterial DNA for daily and intermittent cleansing treatments where CFU analysis indicated there were no cells remaining. This emphasises the need for molecular analysis to become the gold standard technique used in microbiological analysis.

The accuracy of the molecular CFE data allows us to gain a clearer understanding of the efficiency of the treatments used. In comparison to the untreated control, both treatments significantly reduced the number of microbes on the denture. However, determining which treatment is more effective is problematic as they appear to be equally successful. Nonetheless, the data indicates that many microbes still remain on the denture surface, a large proportion of which are still alive, and therefore they have the potential to proliferate and form a biofilm. Based on the SEM images for the treatments very few microbes can be observed on the denture surface, however, the denture surface is uneven and covered in cracks and crevices which both *C. albicans* and bacteria have been shown to colonise. These crevices therefore may provide protection during the treatments and are likely to be the source of a large proportion of the remaining viable cells.

Using species-specific primers, we were able to investigate how each species responded to each of the treatments. For the untreated control there was an interesting shift in the composition across the 5 days, despite being left untouched. In terms of the total cells, the streptococci and *A. naeslundii* remained the predominant species. Of particular interest was the significant increase in *V. dispar* from day 1 to 5, this in combination with the *A. naeslundii* meant that by day 5 the total cell count was made up of 80.56% of anaerobic bacteria. This was also reflected in the live cell analysis, whereby *V. dispar* comprises the majority of the biofilm. Although *V. dispar* and *A. naeslundii* are generally associated with being commensals (Avila et al., 2009), they have the potential to create an anaerobic environment which may encourage the growth of other more pathogenic anaerobes, such as *P. gingivalis or A. actinomycetemcomitans* (Darveau et al., 2012, Henderson et al., 2003). Some may argue that this will not happen as many of these pathogenic bacteria are associated with dentate individuals, and that they disappear from the oral cavity after the removal of teeth (Danser et al., 1995, Danser et al., 1997). However, studies by Sachedo *et al* (2008) have demonstrated the presence of such bacteria in edentulous individuals (Sachdeo et

al., 2008). In addition to this, large proportions of denture wearers have partial dentures and thus will have natural teeth remaining (GlaxoSmithKline, 2010).

The biofilm composition after both intermittent and daily cleansing treatments looks quite different to the untreated control. Unsurprisingly streptococci and *A. anaeslundii* comprise a minimum of 90% of the total cells and at least 80% of the live cells for every day of treatment. This finding is predictable given that streptococci and *Actinomyces* species are attributed to being amongst the primary colonisers of the oral cavity (Nyvad and Kilian, 1987, Sbordone and Bortolaia, 2003, Whittaker et al., 1996). Therefore, after the treatments remove the majority of cells, the biofilm will have to re-establish itself again.

Given that denture related microbiological studies predominantly focus on the role of *Candida* species (Coco et al., 2008a, Pereira-Cenci et al., 2008, Gendreau and Loewy, 2011), a surprising outcome from this study is how little *C. albicans* actually contributes to the overall biofilm. In the untreated biofilm, *C. albicans* comprises less than 1% of the biofilm and gradually contributes less and less as the biofilm matures over the 5 days, suggesting that if left for longer, it may disappear completely. Similarly low levels are also found for both treatments. This therefore begs the question, has the role of *Candida* in denture plaque been overestimated? Nevertheless, just because it only contributes to a small proportion of the biofilm, is not to say that it has no effect. For example *P. gingivalis* comprises less than 1% of a periodontal biofilm model yet is considered the keystone pathogen in the disease (Malcolm et al., 2016). Furthermore, looking at the SEM images, the biofilm would appear to be abundant in *Candida* yeast cells. However, in terms of scale they may appear more abundant than they are, but not when accounted for as individual cells. Moreover, there is a distinct lack of visible hyphae in the biofilm, as *C. albicans* cells can be seen principally in its yeast form. In its hyphal form *Candida* has been known to form a synergistic relationship with certain bacteria, particularly *Streptococci* and *Actinomyces* (Diaz et al., 2012b, Grimaudo et al., 1996, Arzmi et al., 2015). These bacteria can adhere to the hyphae, which provides biofilm stability by forming a scaffold and also aids their ability to invade cells (Schlecht et al., 2015). However, the lack of hyphae may be explained by the presence of *Lactobacillus*, as these microbes are noted for having a strong antagonistic relationship, inhibiting *C. albicans* adherence, proliferation and hyphal formation (Manzoni et al., 2006, Morales and Hogan, 2010, Krasner et al.,

1956, Boris and Barbes, 2000). Interestingly though, in our denture microbiome study we showed a positive correlation between the abundance of *Candida* and *Lactobacillus* within denture plaque (Bilhan et al., 2009) (Chapter 2 section 3.4.5). Therefore, the relationship between these microbes is clearly complex, and their interactions with one another perhaps vary depending on the circumstances. Most studies investigating the bi-direction relationship between these tend to focus on their interactions in the vagina, which may be completely different to how they interact on the denture.

Based on the evidence from this study, the advantages of using molecular methods over standard culture methods in terms of microbial detection and quantification are apparent. Nonetheless, despite the numerous advantages of molecular technologies such as qPCR, it does have its limitations. The primary reason being cost, as molecular qPCR is an expensive process to carry out, also it requires expert handling and involves a more complex data analysis. In terms of live/dead PCR propidium monoazide (PMA) is extremely toxic and has to be handled with care. Furthermore, as it relies on penetration of the cell membrane of dead cells to function, and therefore there is the argument that microbial cells that died for reasons other than a disrupted cell membrane are not accounted for. Moreover, the process can be more laborious and time consuming than standard cell viability assays such as 2,3 bis(2-methoxy-4-nitro-5sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) and alamar blue. Nevertheless, with every technique there are caveats, however the significant improvements in accuracy provided by these techniques surpasses their disadvantages.

In this study we have been able to develop a biofilm model that truly represents denture plaque using data from a previous study looking at the microbiome of dentures. One of the main findings from this study is the inaccuracy of culture-based methods when quantifying biofilms, which emphasises the need for molecular based methods to be used in its place. From this research, we concluded that both combinational treatments employed in this study significantly reduced both the biomass and viability of these biofilms, with both treatments just as proficient as one another. Therefore, given the lack of differences between treatments, this suggests that intermittent cleansing may be the most economical choice. Nonetheless, even with treatments, there were some microbes left residing on the PMMA disc surface and within the cracks and crevices.

Furthermore, the accuracy of this model has allowed us to see how the biofilm composition changes over time when left untreated, and it appears that as time passes the biofilm becomes more anaerobic. However, whether more anaerobic means more pathogenic requires a longitudinal study of increasing length. Of note, the contribution of *Candida* to the overall biofilm is very small, raising the question: 'has the role of this microbe been over estimated in denture plaque?' Therefore, this study emphasises the importance of placing more focus on the role of bacteria and bacterial/*candida* interactions in denture related disease.

CHAPTER FINDINGS

CFU analysis is considerably less effective at quantifying biofilms than molecular CFE analysis using qPCR.

Intermittent cleansing and daily cleansing both significantly reduce the biomass and viability of denture biofilms.

Both treatments were found to be equally effective.

When left untreated the denture biofilm takes on a predominantly anaerobic composition

5 Dentures act as a reservoir for respiratory pathogens

5.1 Introduction

Internationally, more than 810 million people are aged 60 years or over, which is expected to reach two billion by 2050 (22% of the entire global population) (Guzmán et al., 2012). Delivery of systemic and oral healthcare will face greater challenges with this ever-increasing elderly population. Obvious links between oral and systemic disease have been described, for example with rheumatoid arthritis, cardiovascular disease and respiratory infection (Farquharson et al., 2012, Pizzo et al., 2010). Several of these elderly individuals will experience a general decline in oral health, which for some may translate to complete or partial edentulousness. Currently it is estimated that approximately 20% of the US and UK population wear some form of removable denture (Coulthwaite and Verran, 2007, Shulman et al., 2004). Dentures can encourage the growth of denture plaque in the form of biofilms, which are complex polymicrobial group comprised of bacteria and yeasts. The unique architecture of the biofilm and the rough surface topography of the denture, with its cracks and crevices, act as a physical barrier to provide protection for the microorganisms against the effects of the host immune system and chemotherapeutic agents (Verran et al., 2014). The close proximity of the denture plaque biofilm to the respiratory tract represents a reservoir of potential opportunistic respiratory pathogens. Consequently, the risk of denture wearers aspirating opportunistic pathogens from the denture into their lungs is increased.

Pneumonia is the principal cause of death associated with infection in patients older than 65, costing the national health service (NHS) in excess of £440 million annually (Guest and Morris, 1997). Aspiration pneumonia is a form of respiratory infection with potentially life threatening consequences associated with entry of contaminated foreign material such as gastric or oropharyngeal contents, including food debris, dental and/or denture plaque, and saliva into the lungs. Poor oral hygiene may further contribute to the development of AP (Quagliarello et al., 2005). What is more, it has been discovered that aspiration of oropharyngeal contents is a common occurrence in healthy individuals, as approximately 45% aspirate material into the lungs during sleep, but in whom normal immune functions provide protection (Gleeson et al., 1997). The

deterioration of the immune system with age, known as immunosenescence, increases their risk of developing pneumonia as the elderly are likely to be less effective at clearing the invading pathogen. In addition, other risk factors that may contribute towards developing AP, such as dysphagia and chronic obstructive pulmonary disease (COPD), are more common in the elderly, thus putting this sub-population at an increased risk of infection (El-Solh, 2011a). Failure to clean a denture adequately leads to an accumulation of plaque, with the denture surface capable of carrying up to 10^{11} microbes per milligram (Nikawa et al., 1998). Dental plaque composition has been well characterised and many of the oral bacteria identified have been linked with systemic infections (Inaba and Amano, 2010). There is adequate evidence to substantiate a relationship between dental plaque and respiratory infection, principally amongst the dependant elderly and hospitalised patients (Muller, 2014, Russell et al., 1999). Nevertheless, there still remains a gap in our understanding of the direct implications of denture plaque in the development of respiratory disease.

5.2 Aims

The aims of this chapter were to undertake a comprehensive targeted evaluation of putative respiratory pathogens residing upon dentures using a targeted quantitative molecular approach. The following key questions were investigated:

- Do respiratory pathogens colonise dentures?
- If respiratory pathogens do colonise dentures, how abundant are they within the denture plaque?
- Is there a relationship between denture stomatitis and prevalence of respiratory pathogens found on dentures?

The data represented in this chapter has been published in:

The data presented in this chapter has been presented at the following conferences:

L. O'Donnell, C. Nile, K. Smith, V. Hannah, L. Cross, D. Robertson, G. Ramage. The Oral Cavity acts as a Reservoir for *Staphylococcus aureus*: Implications for Systemic Infections from Prosthetic Dental Appliances, ECCMID, Barcelona, Spain, May, 2014.

5.3 Materials and Methods

5.3.1 DNA extraction of denture sonicate

Denture sonicate samples were collected and processed as previously described (section 2.3.2). One ml of the denture sonicate, which had been stored in RNAlater™ was removed and used for DNA extraction. The denture sonicates were centrifuged for 10 minutes at 10,000 g. DNA was extracted from individual denture sonicate samples using the QIAmp mini DNA extraction kit (QIAgen). The supernatant was discarded and the pellet was re-suspended in 180 µl of ATL buffer supplemented with 20 µl of proteinase K. From this point onwards the DNA extraction process was carried out as previously described (section 4.3.2.4.1). DNA was quantified using the NanoDrop™ ND-1000 spectrophotometer (Labtech) as described in (section 4.3.2.4.2).

5.3.2 Identification of respiratory pathogens on dentures

DNA samples extracted from the denture sonicate were screened using qPCR for the presence of nine of well know respiratory pathogens, which included: *Staphylococcus aureus, Moraxella catarrhalis, Streptococcus pneumoniae, Streptococcus pyogenes, Haemophilus influenzae B, Pseudomonas aeruginosa, Legionella pneumophila, Klebsiella pneumoniae and Chlamydophila pneumoniae*.

5.3.3 Microbial culture of respiratory pathogens

Laboratory strains of the respiratory pathogens were selected for use in this study. *Staphylococcus aureus* ATCC 25923 and *Moraxella catarrhalis* ATCC 43617 were grown and maintained at 37°C on CBA plates (Oxoid) in aerobic conditions. *Streptococus pneumoniae* ATCC 6303 and *Streptococcus pyogenes* ATCC 12344 were maintained at 37°C in 5% CO_2 on CBA plates. *Haemophilus influenzae B* ATCC 10211, *Pseudomonas aeruginosa* PA14 and *Legionella pneumophila* ATCC 33154 were grown on chocolate agar (E and O labs), LB agar and charcoal agar (E and O labs), respectively, in 5% CO_2 at 37 °C. *Klebsiella pneumoniae* DSM 12059 was maintained on MacConkey agar (E and O labs) under anaerobic conditions (85% N_2, 10% CO_2 and 5% H_2, [Don Whitley Scientific Limited, Shipley, UK]).

Overnight broths were prepared for each bacterial species. A single colony of *S. aureus*, *S. pneumoniae*, *S. pyogenes* and *Moraxella catarrhalis* was inoculated into 10 mL of BHI broth (Sigma-Aldrich). *H. infulenzae B* and *K. pneumoniae* were grown in tryptic soy broth (TSB) (Sigma-Aldrich) and *P.* aeruginosa was maintained in lysogeny broth (LB) (Sigma-Aldrich). *L. pneumophila* was grown in *Legionella* BYCE growth supplemented broth (Oxoid). Cultures were grown for 24 to 48 h at 37°C as necessary, washed by centrifugation and resuspended in PBS (Sigma-Aldrich). All cultures were standardised and adjusted to a final working concentration of 1 x 10^8 cells/mL in PBS.

5.3.4 Preparation of respiratory pathogen standard curves

Preparations of standard curves used within this chapter were carried out with the help and support of Dr Karen Smith. Serial two-fold dilutions of the 1 x 10^8 cells/mL cultures were prepared. Each dilution was centrifuged for 10 min a 10,000 g. The supernatant was removed, making sure to avoid disturbing the pellet. Pellets were re-suspended in proteinase K extraction buffer and incubated at 55°C for 20 min. DNA was extracted using the QIAmp mini DNA extraction kit (Qiagen,) as previously described (section 4.3.2.4.1). DNA quality and quantity was then quantified by NanoDrop® (ThermoScientific).

5.3.5 Detection of respiratory pathogens in denture plaque using qPCR

Primers were either taken from published literature or designed using the web-based GenScript real-time PCR primer design software (https://www.genscript.com/ssl-bin/app/primer). Primer sequences were checked for specificity to each target organism using the NIH-BLAST database. PCR amplification efficiencies of all primer sets were optimised prior to gene expression analysis, with efficiencies ranging from 90-110%. Details of the oligonucleotide primers (Eurogentec, Southampton, UK) used in this study are listed in Table 5.1. 200 ng of DNA was used in a mastermix containing SYBR® GreenER™ (Life Technologies), UV-treated RNase-free water and forward/reverse primers (10 µM). qPCR was carried out using the step one plus real-time PCR unit (Applied Biosciences), as previously described (section 4.3.2.4.4), under the following conditions; 50°C for 2 min, 95°C for 2 min followed by 40 cycles of 95°C

for 3s and 60ºC for 30 s. Data analysis was carried out using StepOne software V2.3, (Life Technologies). Baseline threshold values of the samples were adjusted to correspond with the equivalent standard curve; Ct values were then used to approximate the number of corresponding CFE's based on standard curves created from serial two-fold dilutions of each bacterial species.

5.3.6 IL-8 ELISA

Clarified saliva samples were diluted 1:5 in assay buffer (PBS, 0.5% BSA, 0.1% Tween20). IL-8 [Invitrogen, Paisley, UK], was used according to manufacturer's instructions. Capture antibody (1 µg/mL) was prepared in NA_2HCO_3 and 100 µL added to each well of a Nunc™ Maxisorp® flat bottomed microtitre plate [Fisher, Loughborough, UK]. Plates were sealed and incubated overnight at 4ºC. Contents were then discarded and washed with 300 µL of wash buffer of PBS containing 500 µL Tween 20 /L. Plates were then blocked with 300 µL of assay buffer containing 0.5 % bovine serum albumin (BSA) for 1 hour at room temperature to block non-specific binding. After incubation, contents were discarded and 100 µL of each sample loaded in duplicate as well as standards of known concentrations ranging from 2000 – 31.25 pg/mL. At this time detection antibody was diluted to 0.04 µg/mL in assay buffer and added to each well containing sample or standard. Plates were then sealed and incubated for 2 h at room temperature on a shaking platform at 700 rpm. Following incubation the contents of the plate were discarded and 100 µL of detection antibody diluted to 0.04 µg/mL in assay buffer added to each well before a further incubation of 2 h at room temperature at 700 rpm. Next, the plate was washed and 50 µL of a 1/2500 dilution of streptavidin-HRP in assay buffer was added to each well for a further 30 min incubation shaking at 700 rpm. Finally, the supernatants were discarded and 100 µL of TMB [R&D Systems, Abingdon, UK] was added to each well and incubated in the dark for 30 min before addition of 100 µL 1 mM hydrochloric acid (HCL) to stop the reaction. Absorbance was read using a plate reader [FLUOstar Omega BMG Labtech, VA, USA] at 405 nm with a 650 nm wavelength correction. A standard curve was constructed by plotting the mean absorbance for each standard against the appropriate protein concentration and the R-squared calculated using a computer program [Omega analysis software, VA, USA]. Results were calculated using a 4-parameter curve fit to determine the concentration of protein release in samples tested. All samples were tested in duplicate.

5.3.7 Statistical analysis

Graph production, data distribution and statistical analysis were performed using GraphPad Prism (version 4; La Jolla, CA, USA) or IBM SPSS statistics (version 21; Chicago, Il, USA). Since continuous data including real-time qPCR determination of bacterial cell numbers on dentures did not conform to a normal distribution the Man-Whitney U-test was used for comparisons between the different denture wearer subsets. The Spearman bivariate correlation analysis was used to determine relationships between the Newton Grade of inflammation and bacterial numbers.

Table 5.1: Respiratory pathogen primers used for qPCR.

Species	Type strain	Media/Conditions	Gene	Primer sequence	Amplicon Size
S. aureus	DSMZ 1104	BHI, aerobic	*SAR0134*	**F** – ATTTGGTCCCAGTGGTGTGGGTAT **R** – GCTGTGACAATTGCCGTTTGTCGT	143
MRSA		BHI, aerobic	*MecA*	**F** – AACCACCCAATTTGTCTGCC **R** – TGATGGTATGCAACAAGTCGTAAA	135
H. influenzae B	DSMZ 11969	MHB + 0.4% haemophilus test medium, 5% CO_2	*GryB*	**F**–CTTACGCTTCTATCTCGGTGATTAATAA **R** – TGTTCGCCATAACTTCATCTTAGC	138
P. aeruginosa	PA14	LB, aerobic	*RpoD*	**F** – GGGCGAAGAAGGAAATGGTC **R** – CAGGTGGCGTAGGTGGAGAA	178
S. pneumoniae	DSMZ 14377	BHI, 5% CO_2	*CspA*	**F** – ACGCAACTGACGAGTGTGAC **R** – GATCGCGACACCGAACTAAT	352
M. catarrhalis	DSMZ 11994	BHI, aerobic	*OmpCD*	**F** – ACACGCAACTCTTGACGAAG **R** – CTGAGCCTGTCATTGAGGAA	180
S. pyogenes	DSMZ 20565	BHI, 5% CO_2	*SpeB*	**F** – TGC TAAAGTCGCTACGGTTG **R** – GAATTGATGGCTGATGTTGG	148
C. pneumoniae	ATCC VR-1360D DNA	NA	*MomP*	**F** – TTACTTAAAGAAACGTTTGGTAGTTCATTT **R** – TAAACATTTGGGATCGCTTTGAT	154
K. pneumoniae	DSMZ 12059	TSB, anaerobic	*PhoE*	**F** – AGAATTCAGATTCCCAACGG **R** – ACAAGAACGCGAACAAACTG	167
L. pneumophila	DSMZ 25038	YEB with BCYE supplement, 5% CO_2	*Mip*	**F** – CAATGTCAACAGCAATGGCTGCAAC **R** – CTCATAGCGTCTTGCATGCCTTTAGCC	160
16S			*16S*	**F** – ACTCCTACGGGAGGCAGCAGT **R**- TATTACCGCGGCTGCTGGC	198

MRSA: Methicillin –resistant Staphylococcus aureus, BHI: Brain heart infusion, MHB: Mueller Hinton broth, NA: Not applicable,TSB: Tryptic soy broth, YEB:Yeast extract broth, BCYE: Buffered charcoal yeast extract.

5.4 Results

5.4.1 Detection of respiratory pathogens

In order to determine if dentures could act as a reservoir for respiratory pathogens, DNA was extracted from the denture sonicate, and assessed for the presence of nine pathogens associated with respiratory infection. The dentures of 84 patients (64.6%) carried potential respiratory pathogens. The following six were identified by qPCR using species specific primers (Table 5.1): *S. aureus, S. pneumoniae, P. aeruginosa, H. influenzae B, S. pyogenes* and *M. catarrhalis.*

S. aureus was the most prevalent, with 67 patients (51.2%) being shown to carry this pathogen. Of these 67 patients, re-testing using primers specific for the *mecA* gene demonstrated that two (3%) dentures were colonised with MRSA. *H. influenzae B* (15.3%), *P. aeruginosa* (11.5%), *S. pneumoniae* (6.9%), *S. pyogenes* (0.8%) and *M. catarrhalis* (0.8%) were also detected within denture plaque of our patients (Table 5.2). Fifty-eight patients (44.6%) had dentures colonised by a single pathogen, 24 patients (18.5%) were colonised by two pathogens, and two patients (1.5%) were colonised by three. None of the samples were shown to be positive for *L. pneumophila, C. pneumoniae* or *K. pneumoniae*.

5.4.2 Quantifying respiratory pathogens

Standard curves of the six respiratory pathogens detected were prepared for each bacteria (Figure 5.1); R^2 values ranged from 0.91 to 0.99. Table 5.2 shows the mean CFE counts of each organism detected on dentures. In terms of overall proportion of denture plaque, *P. aeruginosa* was found to be the most abundant respiratory pathogen, with a mean count of 4.3×10^6 CFE when present. This was followed by *S. pneumoniae* and *S. aureus*, which were detected at relatively high levels of 2.5×10^5 CFE/denture and 1.3×10^5 CFE/denture, respectively. Conversely, *H. influenzae B, S. pyogenes* and *M. catarrhalis*, where present, were all detected at levels lower than 10^5 CFE/denture. The total number of bacteria residing on dentures shown to harbour respiratory pathogens was calculated using the 16S gene, with mean quantities ranging between 7.97×10^9 – 4.5×10^{10}

CFE/denture. As a proportion of the overall plaque, *P. aeruginosa* was most abundant contributing to 0.015% of the total. The remaining pathogens all contributed to less than 0.001% of the complete plaque sample.

Table 5.2: Prevalence and quantity of respiratory pathogens colonising dentures.

Species	Positive Samples	Prevalence (%)	Average CFE	Average Total 16S CFE	Proportion of 16S (%)
*S. aureus**	67	51.2	$1.3x10^5$	$4.5x10^{10}$	$2.8x10^{-4}$
H. influenzae B	20	15.3	$2.4x10^4$	$1.7x10^{10}$	$1.4x10^{-4}$
P. aeruginosa	15	11.5	$4.3x10^6$	$3.34x10^{10}$	$1.5x10^{-2}$
S. pneumoniae	9	6.9	$2.5x10^5$	$4.4x10^{10}$	$6.3x10^{-4}$
M. catarrhalis	1	0.8	$2x10^3$	$7.97x10^9$	$2.5x10^{-5}$
S. pyogenes	1	0.8	$3.7x10^4$	$3.2x10^{10}$	$1.2x10^{-4}$
C. pneumoniae	ND**	ND	ND	ND	ND
K. pneumoniae	ND	ND	ND	ND	ND
L. pneumophila	ND	ND	ND	ND	ND

* Two *S. aureus* samples were found to be MRSA positive.
**ND = Not detected
CFE: colony forming equivalent

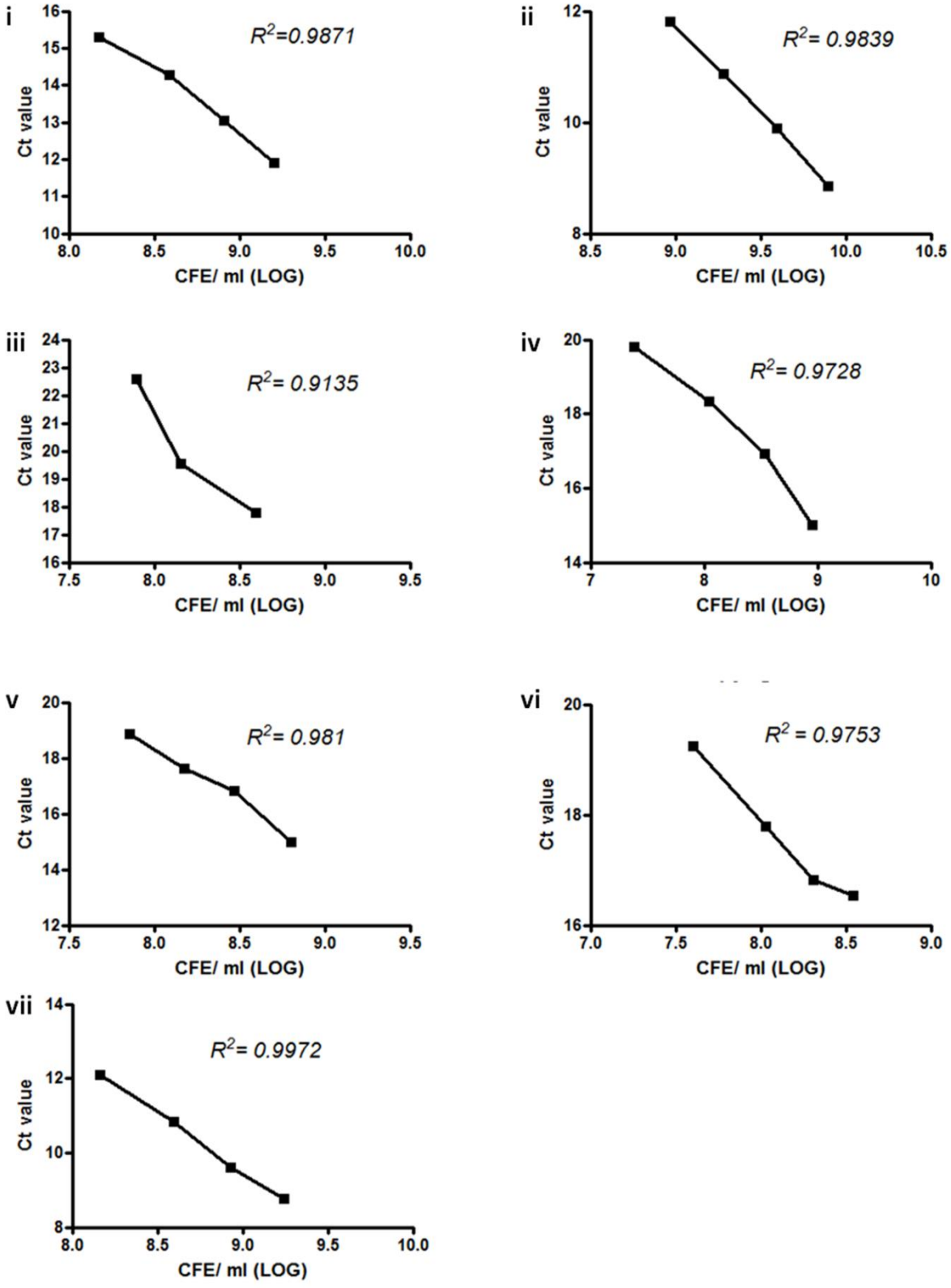


Figure 5.5.1: Standard curves of each of the respiratory pathogens detected on dentures.
DNA from known concentrations of *S. aureus* **(i)**, *H. influenzae* B **(ii)**, *P. aeruginosa* **(iii)**, *S. pneumoniae* **(iv)**, *M. catarrhalis* **(v)**, *S. pyogenes* **(vi)** and 16S **(vii)** were used for qPCR and their equivalent Ct used for the preparation of a standard curve. Unknown bacterial counts could then be quantified by extrapolating from the appropriate curve. Each DNA dilution was assessed in duplicate for each of the primer sets.

5.4.3 Oral inflammation, hygiene and respiratory pathogens

The relationship between DS and presence of respiratory pathogens was investigated. In relation to DS, we determined the prevalence of respiratory pathogens amongst healthy and diseased patients, based on clinical presentation using Newton's classification, as previously classified (section 2.3.1) (Table 5.3). The overall prevalence of these was similar between the healthy (85.2%) and diseased (89.8%) groups. However, individual species variation was more evident, as upon the dentures of patients with oral inflammation there appeared to be greater numbers of *S. pneumoniae* ($p=0.060$), though no statistically significant differences were observed.

Therefore, the relationship between oral inflammation and salivary Interleukin-8 levels were investigated as a means to determine whether salivary IL-8 was a potential biomarker of inflammation grade in DS patients. IL-8 levels were found to be significantly higher in denture wearers with inflammation [Median: 291.4 (min: 2.07,max: 2821) v 532.3 (min:94.68, max: 3301), $p<0.001$] (Fig 5.2) and positively correlated with the Newton's inflammation grade ($rho=0.315$, $p<0.01$). Therefore, henceforth, salivay IL-8 levels were employed as a surrogate marker of inflammation severity in DS patients. Taking this approach, no significant correlations were identified between salivary IL-8 levels and denture CFE levels of individual bacteria.

Data taken from patient clinical information sheets and a self-reported oral health questionnaire assessed the oral hygiene of patients with and without respiratory pathogens colonising their dentures. Interleukin-8 levels were significantly higher in denture wearers with poor oral hygiene ($p<0.01$) and were found to have a positive correlation with poor oral hygiene ($rho=325$, $p<0.001$). Whether the denture wearers left their dentures in overnight did not appear to alter salivary IL-8 concentrations. Interestingly, only 68% of patients with respiratory pathogens were classed as having good oral hygiene compared to 81% of those without respiratory pathogens. However, having better oral hygiene did not appear to alter significantly the proportion of dentures carrying particular bacteria or the numbers of those bacteria on dentures. Sleeping with the denture *in situ* was also more common amongst respiratory pathogen positive individuals (58%), when

compared to those without (49%). A greater proportion of dentures that were left in patients' mouths overnight were found to carry *S. pneumoniae*, and an increased median number of *S. pneumoniae* were detected on those dentures (p=0.041 and p=0.038, respectively). In contrast, the proportion of dentures positive for *P. aeruginosa* and quantities of *P. aeruginosa* found on dentures that were left in overnight was lower than dentures that were removed (p=0.038 and p=0.04, respectively).

Table 5.3: Prevalence of respiratory pathogens in plaque of patients with healthy palatal mucosa and those with denture stomatitis.

	Healthy		Denture stomatitis	
Species	**Patients**	**Prevalence (%)**	**Patients**	**Prevalence (%)**
S. aureus	40	49.4	27	55.1
H. influenzae B	16	19.8	4	8.2
P. aeruginosa	10	12.3	5	10.2
S. pneumoniae	3	3.7	6	12.2
M. catarrhalis	ND*	ND	1	2.0
S. pyogenes	ND	ND	1	2.0
C. pneumoniae	ND	ND	ND	ND
K. pneumoniae	ND	ND	ND	ND
L. pneumophila	ND	ND	ND	ND
MRSA	ND	ND	2	4.1
OVERALL	69	85.2	44	89.8

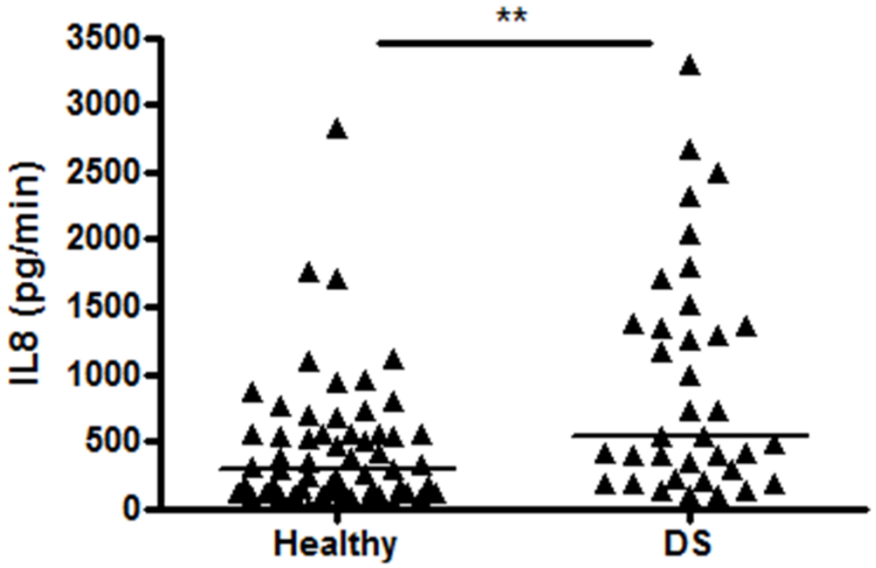


Figure 5.5.2: Salivary IL-8 levels are elevated in denture stomatitis sufferers. Salivary levels of IL-8 were compared between healthy and diseased individuals. Data represents median (** $p<0.01$). Statistical analysis was performed using a Mann Whitney test as data did not conform to a normal distribution.

5.5 Discussion

Advancements in healthcare in the last 100 years have led to an increasing population of elderly individuals. Pneumonia is the 4th leading cause of death globally, accounting and thus this ageing population, particularly those aged 65 and over, and are at a greater risk of developing the disease (Guest and Morris, 1997). A large proportion of these cases of pneumonia can be attributed to oral bacteria emanating from the oropharynx (Andrews and Steen, 2013). Approximately 70% of UK adults older than 75 years old wear dentures, which are colonised by complex biofilm consortia. These individuals are at an increased risk of developing a life-threatening infection as a result of putative respiratory pathogens residing upon their denture (Iinuma et al., 2014). Moreover, a recent study showed that patients who wear their denture overnight double their risk of pneumonia. In this chapter, for the first time, qPCR has been employed to assess the pathogenic potential of denture plaque in terms of respiratory infection from a large cohort of denture wearers. This study reports a 65% prevalence rate of individuals carrying significant quantities of respiratory pathogens on their dentures. This was independent from existing oral disease status, indicating that even denture wearers with good oral hygiene and healthy palatal mucosa, may harbour respiratory pathogens on their dentures.

Biofilms, by their nature, are complex aggregates of microorganisms, and difficult to remove homogenously from the denture. To this end we used a mild sonication technique previously used in our studies of indwelling prostheses (Coco et al., 2008a, Tunney et al., 1998). This method was shown to adequately remove the biofilm from the surface of the denture. Given the dense and complex microbial population of the oral cavity, the presence of similar genera and species is problematic for accurate quantitative microbial counts on selective agars (Sumi et al., 2002). Therefore, we adopted an alternative specific non-culture based method of a qPCR-based approach to identify a panel of defined respiratory pathogens. This technique enabled us to rapidly and specifically assess the presence and quantity of defined respiratory pathogens. Moreover, this targeted molecular approach provided the ability to screen accurately for specific microorganisms in complex samples. Similar technology has been employed in studies of dental plaque, but has been restricted to conventional PCR only (Abe

et al., 2001, Zuanazzi et al., 2010). While there are numerous putative respiratory pathogens, our study focussed on nine key bacterial pathogens widely implicated in respiratory infections. We found that almost 65% of patients' dentures carried one or more of the pathogens from our panel. This finding is similar to a recent study reporting that 64% of the healthy patient group contained respiratory pathogens, this was however was a culture based approach, and only nine patients were screened in this study (Przybylowska et al., 2014). We are limited in our comparisons as, to our knowledge; this is the first study to use a quantitative molecular based approach to investigate the presence of respiratory pathogens on dentures.

qPCR has proven to be an vital tool, as this study demonstrated a high prevalence of *S. aureus* (51.2%), yet in another study, which was primarily culture based, of the 50 patients only 10% carried *S. aureus* on their dentures (Sumi et al., 2002). This inconsistency can be attributed to the improved detection abilities of qPCR as in our parallel study using standard microbial culture *S. aureus* was detected in only 20% of samples (data not shown). *H. influenza* was also detected in 15.3%, whereas Sumi and colleagues (2002) were unable to detect this organism at all. Dentures from 6.9% of our patients carried *S. pneumoniae*, which is considerably lower than the 63% prevalence reported by Abe and colleagues (2001). Nevertheless, this discrepancy is likely to be caused by the non-specific nature of the primers in Abe and colleagues study (Abe et al., 2001). As based on the findings from our denture microbome data we showed that streptococcal species make up on average 23.5% of the overall denture plaque composition, thus using non-specific primers will likely detect other streptococci given their high abundance. We used the *cspA* gene specifically, instead of the generic primers, to minimise detection of closely related streptococcal species. Together, these organisms were detected in relatively low abundance in relation to total bacterial flora ($<6 \times 10^{-4}$ %), suggesting that these bacteria occupy limited niches upon the denture surface. *P. aeruginosa* however, comprised 0.015% of the total denture microbiome, which may be associated with its resistance to standard mechanical and chemical cleaning methods when grown on denture acrylic (Paranhos et al., 2009).

We were interested to know if having DS put the individual at greater risk of respiratory pathogen colonisation of denture plaque. Previous studies have shown that DS sufferers have poorer denture hygiene with higher microbial colonisation (Budtz-Jorgensen et al., 1975, Coco et al., 2008a), which supports our finding of higher inflammation levels in those with poor hygiene. However, our results revealed a similar prevalence of respiratory pathogens between healthy and diseased individuals (89.2% and 85.8% respectively), suggesting that DS does not increase the likelihood of respiratory pathogen colonisation. In spite of previous studies showing higher prevalence of AP in patients who do not follow adequate dental hygiene measures (Bassim et al., 2008), and that targeted oral hygiene measures can reduce the incidence of AP in at risk groups (van der Maarel-Wierink et al., 2013). This study detected no difference in the prevalence of respiratory pathogen carriage in those with and without signs of DS, signifying that the presence of the denture was enough risk in itself.

The high detection rate of *S. aureus* residing on dentures is concerning, given the emergence of drug resistant strains. Two patients in this study were found to be positive for the MRSA *mecA* gene (1.5%). Our prevalence rate is low, yet is comparable to a previous study conducted in Scotland in which found that 5% of denture wearers carried MRSA in their oral cavity (Kulak et al., 1997). However, the prevalence of MRSA in the general population is on average lower than that of the institutionalised or hospitalised elderly (14.8%) (Abe et al., 2001). This may be cause for concern as many over-the-counter oral hygiene antimicrobials are ineffective against MRSA biofilms (Smith et al., 2013). Sleeping with a denture *in situ* is a habit practised by a large proportion of the patients in this study (55%). *S. pneumoniae* in particular was shown to be significantly more abundant on dentures, which were kept in their owner's mouths overnight. Aspiration of oropharyngeal contents most commonly occurs when the individual is sleeping, thus these patients are putting themselves at an increased risk of developing infection. This is particularly important given that currently 20-30% of *S. pneumoniae* worldwide are multidrug resistant (Lynch and Zhanel, 2005). Furthermore, 11.5% of patients carried *P. aeruginosa,* a disreputable cause of life-threatening pneumonia for intensive care patients with, a mortality rate of 44.5% (Tumbarello et al., 2013). Oropharyngeal *P. aeruginosa* isolates have also been shown to have a high rate of antibiotic resistance (Oostdijk et al., 2010).

Moreover, soaking dentures in water overnight is commonplace, thus this may be an unintentional source of contamination, so some form of disinfectant such as hypochlorite should be included such as hypochlorite alongside for non-metal containing prostheses. This is important given that studies have indicated the presence of *P. aeruginosa* in water and its capacity to form biofilms in plumbing systems (Loveday et al., 2014).

In summary, this study has shown that dentures are a reservoir for respiratory pathogens in the oral cavity, thus increasing the risk of developing AP. This study has utilised a robust qPCR based method of sampling the denture microflora which has been characterised by comparison of the data to date derived from culture independent techniques. We have shown that there is a high prevalence of putative respiratory pathogens on the dentures of ambulatory adults, a finding that could explain the source of infection in some cases of AP. Adoption of routine, oral hygiene practices including mechanical cleaning, and the use of antiseptic cleansing agents could help to reduce the risk of respiratory infection among the elderly population (Ramage et al., 2012). However, ultimately what is required to definitively associate dentures with increased risk of pneumonia are more longitudinal studies reporting the clinical outcomes of the patients being followed, but with a specific focus on the role of dentures. Although other studies have demonstrated the presence of respiratory pathogens on dentures, these studies focus on a single time point, and fail to follow up on the patient.

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Dentures act as a reservoir for respiratory pathogens, increasing the risk of developing aspiration pneumonia.

S. aureus was found to be the most prevalent pathogen colonising dentures, and where detected, *P. aeruginosa* the most abundant.

No association between the presence of respiratory pathogens on dentures and denture stomatitis was determined.

6 Discussion

6.1 Introduction

DS is a disease for which there is currently a limited understanding of the aeitiopathogenesis. This is primarily because studies investigating this disease focus heavily on fungal infection by the pathogenic *Candida* spp. of yeasts, consequently neglecting the role of bacteria also residing on dentures. In addition, arguably there has been a disproportionate level of investigation focussed on periodontal microbiology. Despite this, and perhaps as a catalyst, there been a recent realisation that DS is in fact a polymicrobial disease. Here fungi and bacteria interact synergistically to survive in complex biofilms, with the inadvertent consequence of enhanced fungal pathogenicity. Understanding a disease of this nature is complex, and has to be investigated methodically, beginning with the aetiological agent(s). 'What are the aetiological agent(s) of DS?' is a fundamental question, because if we do not know the cause then we cannot begin to understand the pathogenic mechanisms behind it, and in turn begin to manage it clinically.

The central problem associated with DS research is the microbial composition of denture plaque has yet to be elucidated in depth. The novelty of the work described herein lies in the fact that it was the first to use advanced next generation sequencing methods to examine the composition of denture plaque in both healthy and diseased individuals. This has allowed us to begin to understand the aeitiopathogenesis of DS in greater detail and provides a platform for further research into the development of ways of improving clinical management of DS patients.

6.2 Is the denture microbiome relevant?

The human microbiome refers to the collection of genes and genomes of members of the microbiota found in the human body (Turnbaugh et al., 2007). The microbiome at specific locations within the body are generally studied opposed to looking at the human microbiome as a complete entity, such as the oral or gut microbiome *per se* (Chen et al., 2010). Microbiome analysis is able to give a global representation of the microbial communities at the site of investigation due to

advances in sequencing technology. Other technologies, including microbial culturing, standard qPCR, DNA-DNA hybridisation, all provide valuable information, yet really only offer a biased snapshot of the microbial communities present. Uncultivable bacteria are recognised as one of the key problems associated with less advanced sequencing techniques, as these bacteria are generally not detected during conventional screening (Diaz-Sanchez et al., 2013). This means that microbes that may play a potentially significant role in the pathogenesis of a particular disease are missed. Next generation, high throughput, sequencing on the other hand is able to detect these bacteria, and depending on the sequencing platform used, can identify them down to species level. The importance of microbiome studies cannot be underestimated as recent evidence strongly indicates that, in the near future, we will be able to use individual microbiota profiles in clinical practice as a biomarker of the patients' health (Cenit et al., 2014). Moreover, there is potential to use this technology in order to predict if an individual is more at risk of developing a certain disease, though clearly further pioneer studies are required to take this forward.

In terms of the oral cavity this study has addressed the point that whilst the dental microbiome has been well characterised, in comparison the denture microbiome has been relatively unexplored. However, the relevance of studying the denture microbiome comes from the vast number of people that wear some form of denture prosthesis. With approximately one fifth of adults wearing a denture, there is an elevated likelihood that they will experience denture-related disease at some point. Some individuals will be fortunate and have no issues with their denture, yet others will continue to be plagued by recurrent problems of a chronic, and often debilitating nature. DS is the primary disease associated with denture microbiology, with prevalence rates of up to 70% (section 1.2.3). However, other diseases are associated with denture microbiology, including angular chelitis, a common inflammatory condition affecting the corners of the mouth (Skinner et al., 2005). Denture wearers are within the high risk group for this disease, with approximately 7 per 1000 people affected; a figure which increases 3-fold in denture wearers (Shulman et al., 2004, MacEntee et al., 1998). Samples taken from infected areas principally isolate *C. albicans, S. aureus* and herpes simplex virus (Skinner et al., 2005).

These are just some examples of the local implications that can be caused by denture microbiology, yet what is now becoming a more heavily focused area of research are the potential systemic implications. The links between denture plaque and respiratory infection has been a primary focus within this body of research (chapter 5). The successful identification of numerous well-known respiratory pathogens colonising dentures was reported herein, and this combined with the high number of people that sleep overnight wearing their dentures and the rate of aspiration of oropharyngeal contents, significantly increases their risk of developing serious pulmonary infection. Nonetheless, the potential systemic implications of dentures are more than just respiratory infection, as oral bacteria have been implicated in other diseases such as endocarditis (Carmona et al., 2002). Furthermore, a knock on effect of denture related disease is malnutrition, a consequence of inflamed oral mucosa, resulting in an inability to comfortably wear dentures, and thus the individual cannot properly consume foods (Prakash et al., 2012).

Given the numerous local and systemic consequences that could be related back to the microbiology of a denture, the relevance of understanding the denture microbiome and the pathogenesis behind disease cannot be overstated. However, what these implications ultimately translate to a high cost to the NHS. For that reason, more time and money should be invested in denture research to find a more targeted treatment for denture related disease, which consequently should have a knock on effect in terms of local and systemic consequences, ultimately reducing the cost and burden to the NHS.

6.3 Current and potential treatments

Disappointingly, it has become apparent that there still remains a clear lack of evidence for the most appropriate denture cleansing strategy. The most recent guidelines for care and maintenance of complete dentures suggested that removal of the “bacterial biofilm” is of the upmost importance in order to sustain good oral and systemic health and prevent DS (Felton et al., 2011). In addition to removal of the denture biofilm, the guidelines also advocate reduction and maintenance of low microbial levels on the denture, either with daily soaking and brushing with an effective, non-abrasive cleanser, which should only be performed extra-orally and thoroughly rinsed thereafter, following manufacturers instructions (Felton et al.,

2011). The type of brush and cleanser used are of critical importance, as mechanical cleansing augmented with dentifrices have been shown to induce abrasions causing physical defects on the denture acrylic, leading to enhanced bacterial adhesion through altered surface topography (Charman et al., 2009, Mainieri et al., 2011, Sorgini et al., 2012, Verran et al., 2014). Moreover, the frequency of cleansing has an important bearing (Apratim et al., 2013), as *in vitro* and *in vivo* studies have reported that the sporadic use of denture cleansers allows build up of mature denture plaque (Apratim et al., 2013, Lucena-Ferreira et al., 2014, Ramage et al., 2012), which while responsive to a range of treatment options still leaves behind residual live cells, even with agents such as sodium hypochlorite (Jose et al., 2010). In fact, some studies have suggested that *C. albicans* may be favoured for selection due to its thick cell wall and hardy protective chitin layer (Lucena-Ferreira et al., 2014). However, in contrast, this study demonstrated that daily cleansing may not be critical, as there were no significant differences in the quantity of bacteria or fungi colonising denture discs when a denture cleanser was used daily or intermittently. Nonetheless, daily brushing was required to dislodge the biofilm mass. Collectively, these studies suggest a drive towards a consensus approach to managing denture cleansing, which has still to be forthcoming.

Although current cleansing methods are effective in killing cells and disrupting the denture biofilm structure, the biofilm quickly reforms as live cells still remain and the process begins all over again. As demonstrated in this study, significant number of live cells remained even after daily cleansing and brushing. Thus, an alternative approach to denture cleansing is required; perhaps a more targeted solution. Biofilm formation in itself is an effective protective mechanism from antimicrobials, protecting cells within by blocking access of antimicrobial drugs. The ECM produced by the biofilm has been shown to play an important role in the protection against antimicrobials (Rajendran et al., 2013), therefore, targeting a key component of the ECM involved in protection could result in a more effective treatment. For example, extracellular DNA (eDNA) is a known component of bacterial and fungal biofilms and the use of DNAse in conjunction with antifungals, such as amphoterecin B, has been shown to effectively enhance biofilm disruption (Rajendran et al., 2014). This presents a possible option for the use of DNAse in combination with standard denture cleansers, which may target other cells buried deep within the denture biofilm being protected by the ECM. Nonetheless, whilst

this treatment approach shows potential, it should be kept in mind that *C. albicans* species are heterogenic by nature in terms of eDNA release, thus the effectiveness of this treatment is strain dependant (Rajendran et al., 2014). Moreover, given that *Candida* spp. are outnumbered by approximately 10 fold by bacteria, then their role also needs to be considered and factored into new therapeutics.

This in turn raises another problem regarding treatment methods, as the microbiome of each individual is unique, and whilst the majority of species may be similar, the strains are not necessarily. This indicates that the most logical solution is patient specific treatment. With the advances in NGS technology, a patient's microbiome can be sequenced within hours. However, most of these platforms have not yet advanced to a level in which chair-side sequencing is possible. One of the more recent platforms to be introduced is the Ion Torrent™ system. Ion Torrent™ technology directly translates chemically encoded information (DNA bases [A, C, G, T]) into digital information on a semiconductor chip (Merriman and Rothberg, 2012). The result is a sequencing technology that is simpler, faster, more cost effective and scalable than any other technology available, putting it within the reach of any lab or clinic, however remote. Currently, using this technology the process from sample collection to data takes around 4 hours, and no doubt as NGS technologies continue to advance this time will decrease. Obviously further research is required, however, chair/bed-side diagnosis with targeted patient specific treatment, would appear to be the direction of the future (Quick et al., 2016).

6.4 The Relevant Use of a Model

The term model is used rather liberally in science, though their use in the study of disease is without question if fit for purpose. Oral biofilm models have been used by several groups to study microbial interactions as well as biofilm-host interactions (Millhouse et al., 2014, Park et al., 2014, Guggenheim et al., 2001, Periasamy and Kolenbrander, 2009). The majority of these biofilm models are based around periodontal disease. These models have included both defined and undefined consortia of bacteria. Undefined consortia are considered *in vivo* models as samples are taken directly from the patient and the biofilms are grown directly from these samples. Whilst these types of models have the advantage of

accurately representing the microbiome of periodontal plaque, the investigator remains blind to the microbes that are present in the biofilm, thus there are usually problems with reproducibility of results. In addition, a sample taken directly from the patient is likely to contain numerous bacterial genera, making studying the intricate microbial interactions extremely complex. Therefore, using NGS methods to identify the microbes found within the biofilm allows for the selection of the key microbes to include within an *in vitro* biofilm model. These offer many advantages over *in vivo* biofilm models, most notably being ethical and cost considerations. Furthermore, they also offer the advantage of being a controlled reproducible environment, which allows for detailed studies of real time changes in both the biofilm and host responses, and how they interact. The sequential addition of bacteria, and indeed fungi, during the growth of multi-species biofilms allows their development in a manner that reflects that of natural denture plaque.

Biofilm model studies are necessary for research into health, disease and therapeutics both in the oral cavity and beyond (Millhouse et al., 2014). The initial study highlighting the important role that biofilm models could play in future studies of antimicrobial resistance and chemotherapeutics was carried out by Nickel and colleagues (1985) when investigating *P. aeruginosa* biofilms, where they found that in its planktonic form the cells were 1000-fold more susceptible to the antibiotic tobramycin than in its biofilm form (Nickel et al., 1985). As for *Candida* spp., countless studies have demonstrated the enhanced resistance of these fungi to several of the 'gold standard' antifungal agents including: amphoterecin B, fluconazole, caspofungin and nystatin in their biofilm form (Hawser and Douglas, 1995, Chandra et al., 2001). Furthermore, in terms of oral bacteria, these findings also apply *to S. gordonii, P. gingivalis, F. nucleatum and A. actinomycetemcomitans*, where antibacterial resistance increased between 100-1000 fold upon treatment with doxycycline or chlorohexadine (Park et al., 2014). Moreover, they also emphasised the importance of interspecies interactions on influencing antimicrobial sensitivity, by showing that multispecies biofilms were 100-1000 fold more resistant to the antimicrobials than single species. The overuse of antibiotics has led to the emergence of resistant organisms, which means alternate treatments need to be developed (Gillam and Turner, 2014). Biofilm models therefore provide a relatively inexpensive platform upon which

potential new treatments can be tested. Moreover, these models can give an insight into more than just the microbial interactions. They can also be adapted to give insight into the host response. These biofilm models are also easily adaptable in that the components can easily be swapped to create a model most relevant to a particular disease of interest.

6.5 Clinical implications and future work

This body of work has investigated the composition of the denture, dental and mucosal microbiome of a denture wearer using NGS. The data obtained has been used to investigate differences in the microbiome composition between health and disease and subsequently develop a biofilm model representative of denture plaque. Although differences were detected in terms of diversity between the healthy and DS denture microbiome, minimal changes were detected in terms of composition. However, it was the presence of natural dentition that had the most significant impact on altering the microbiome.

The clinical implications of this research have the potential to be vast. As what this study has done is given an in depth insight into the microbiome of dentures and the oral cavity of denture wearers by showing us 'what is there', which is the first key step in allowing us to begin to understand the complex microbe-microbe interactions; as well as host-microbe interactions. The subsequent development of the denture biofilm model has further provided us with the platform whereby we can begin to investigate these intricate interactions. With such a model we are better equip to begin to understand this disease at both the host and microbial level. With this, there should hopefully come the development of potential therapeutics so that DS can be classed as an easily treatable and manageable disease. Furthermore, the identification of numerous respiratory pathogens as a component of denture plaque, regardless of health or disease should serve as a warning for the potential systemic implications. The aim of this investigation was not to scaremonger and say that patients will develop pneumonia if they have these pathogens on their dentures. However, what would ideally result from this study is increased awareness of the importance of good denture and oral hygiene, and the implementation of strict oral hygiene regimes in hospitals and long term care facilities nationally. The benefits of oral hygiene regimens have already been shown to reduce incidences of pneumonia, thus given that the NHS currently

spends £400 million annually treating patients with pneumonia, the potential impact in terms of alleviating burden and cost to the NHS could be massive.

Going forward it would be particularly interesting to investigate how the host responds to the presence of these biofilms by setting up a co-culture model with the biofilm and host cells, as described previously (Millhouse et al., 2014). The biofilm could be cultured alongside different cell types including host tissue epithelial cells and immune cells to investigate if, and how, the biofilm is able to modulate the host response. This setup would allow us to begin to assess the mechanisms by which biofilms differentially modulate host immune responses, focusing separately on either the biofilm or host cells. For example, RNA extraction from either microbial or host cells could allow for gene expression analysis by which significant genes being up or down regulated can be identified. Mutant strains deficient in these key genes could then either be included within the model to assess the role and importance of the gene. Furthermore, the inclusion or removal of certain species of bacteria or fungi from the biofilm would help evaluate their contribution to the modulation of host responses and help understand the role of health and disease-associated bacteria in oral biofilms.

This thesis has provided an in depth analysis of denture plaque, whilst providing important data to the evidence base. However, it only represents a snapshot of the microbiome from a single point in time. This work could, and will, be taken further with a more longitudinal study in which biofilm development and the associated compositional changes can be observed over time. Moreover, this kind of study would also present the opportunity to test new antimicrobial treatments, and assess their effectiveness and the impact on microbiome composition and viability over time *in vivo*.

The next logical step would therefore involve a clinical trial to test these hypotheses. Currently, a clinical trial which will test the antimicrobial efficacy of a new denture cleanser will shortly be undertaken within our clinics. Patients will be given either daily or weekly treatments and the microbiome composition assessed at several time points over the course of three weeks, using a variety of methods including NGS, qPCR and CFU analysis. This work should hopefully reveal important information, regarding the species that are more resistant to treatment, thus revealing new targets for more targeted chemotherapeutics.

6.6 Summary

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The key findings presented in this chapter are as follows:

The bacterial microbiome composition of denture wearers is not consistent throughout the mouth and varies depending on sample site.

The presence of natural teeth has a significant impact on the overall microbial composition.

Evidence suggests that the compositional changes responsible for disease progression are occurring at the mucosa.

CFU analysis is considerably less effective at quantifying biofilms than molecular CFE analysis using qPCR.

When left untreated the denture biofilm takes on a predominantly anaerobic composition

Dentures act as a reservoir for respiratory pathogens, increasing the risk of developing aspiration pneumonia.

Appendix I: Patient information sheet

University of Glasgow

NHS Greater Glasgow and Clyde

Information Sheet

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An investigation of the microbiology of denture plaque.

- You are being invited to take part in a study to investigate the plaque that grow on dentures and their association with oral and general health. This study is being run by the University of Glasgow and is funded by a grant from the Biotechnology and Biological Sciences Research Council (BBSRC) supported by Glaxosmithkline (GSK).
- This sheet provides you with the information about the study and how it involves you.
- Before you decide whether to take part it is important for you to understand why the research is being done and what it will involve.
- Please take time to read the following information carefully **before** deciding whether or not to take part.
- Please feel free to ask us if there is anything that is not clear or if you would like more information.

What is this study?

What is the purpose of the study?

There is some evidence to suggest that many patients with dentures have difficult cleaning them resulting in oral infections including thrush and other microbial disease. In this study which is part of a PhD project we want to study the health of the mouths of patients wearing dentures who have signs of inflammation and compare them with patients who do not have any signs of oral infection. We also want to determine whether any of these microorganisms are associated with diseases outwith the mouth.

How does it involve you?

Why have I been chosen?

We aim to recruit patients who are generally fit and well who are wearing either a complete or partial denture. Recruitment is taking place within the Restorative Dentistry department at Glasgow Dental Hospital and School where the research team is based. We aim to recruit 158 patients in the course of the study. Your dentist has identified that you meet the criteria required to participate in the study and you have not been identified as someone who has is or has been involved in research in the dental school recently.

None of the doctors are being paid to include you in the study.

Do I have to take part in the study?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time or a decision not to take part, will not affect the standard of care you receive either now or in the future.

Where can I get independent advice about this study?

We will provide your General Dentist with the information about the study should you wish to discuss it with him/her.

What does the study involve for me?

This type of study only involves no extra visits for you to Glasgow Dental Hospital and School.

Today you will be asked to:

- Complete a standard medical history questionnaire with the assistance of the dental research assistant.
- You will be asked to complete an oral health questionnaire with the assistance of the dental team should you require it.
- You will be examined clinically to assess your gums, the other soft tissues in your mouth and your teeth.
- You will be asked to donate: A swab from the mouth, a scraping of dental plaque, and a sample taken from your denture through cleaning it in an ultrasonic bath of sterile water.
- We will collect a sample of saliva to assess your salivary flow. This will involve saliva draining from your mouth into a container for five minutes without swallowing.

What are the possible disadvantages, side effects or risks of taking part?

None. All sampling is non invasive and are standard clinical practice. The only possible consideration is that your appointment time will be increased slightly by the extra time taken for sampling.

What are the possible benefits of taking part?

You will be provided with a very thorough check of your mouth and teeth as part of your normal care. The ultrasonic treatment of the denture will result in an extremely thorough clean.

The findings of this study will help to inform the planning of targeted health care resources for patients with dentures, especially those patients who experience oral infections as a result of their dentures.

What will happen to my clinical and personal information?

The findings of the medical history questionnaire and the check-up of your mouth and teeth will be recorded in the confidential record we prepare for you at Glasgow Dental Hospital and School. Your clinical records will be reviewed by the research team.

All the personal, clinical and questionnaire data that you provide for research purposes will be encoded (so that your personal details such as name and address are not stated) and stored securely. This information will not be revealed to anyone other than the researchers.

We would like permission to contact you again if funding becomes available to follow up any changes in the samples as part of a future study.
The information will be used for research purposes only.

What will happen to my samples of dental plaque and saliva?

In this study:

Your samples will be encoded so that your personal details are not stated on them. The samples of swab, dental plaque, saliva and the denture cleaning fluid will be used to examine differences between the microorganisms which grow in the mouths of patients with dentures who have disease and those who do not. We will also examine the microorganisms for different characteristics that might explain how oral disease happens. The saliva will be used to measure components of your immune system that might protect from disease. The samples and micro-organisms isolated from the plaque may be used in future laboratory studies relating to denture research.

General Note:

We would also like to point out that there might be a commercial gain from future research to the University of Glasgow and their research partners.

As is normal in such studies, your samples are considered as a donation to the University of Glasgow who will 'own' and be responsible for its storage. This means that *you are waiving any commercial rights to the samples and will not have a share of profits* that may ensue from the commercial exploitation of the findings of this study or future studies using the sample.

What will happen to the results?

The results will be published in medical journals. However, no individual taking part will be identifiable from these.

Who has approved this study?

The study has been reviewed and approved by the West of Scotland Research Ethics Committee.

Who is organising and funding the study?

This study is being conducted by researchers at Glasgow Dental Hospital and School. Your dentist at Glasgow Dental Hospital is a members of the team doing this study. For further information please contact:

Name: Dr Douglas Robertson and Miss Lindsay O'Donnell
Address: Department of Restorative dentistry, Glasgow Dental Hospital and School, 378 Sauchiehall Street, Glasgow, G2 3JZ
Tel: 0141 211 9624
Email: douglas.robertson.2@glasgow.ac.uk

This study is being funded by a research grant from the BBSRC

Thank you for taking the time to read this. If you have any queries please contact the research team. His/her details are shown above.

Appendix II: Consent form

University of Glasgow

NHS Greater Glasgow and Clyde

The influence if denture microbiota on oral and systemic disease

CONSENT FORM

Local Project Leader: Dr Douglas Robertson

	Please Initial Box
1. I have read the attached information sheet on the above project and have had the opportunity to ask questions about the project.	
2. I understand that my participation in the study is voluntary and that I am free to withdraw at any time.	
3. I agree to take part in the study and consent to give a sample of dental and denture plaque to assess the level of microorganisms in my mouth and a saliva sample to measure antimicrobial factors that are present.	
4. I agree that the samples taken may be used for future studies for which separate ethical approval will be sought by the investigators. Please Circle Yes No	
5.I agree that my doctor and dentist may be informed that I am taking part in this study	
6. I agree that the research team can access my clinical records	
7. I agree to be contacted by the research team for inclusion in a follow up study should funding become available.	

Name of patient:	Date:	Signature:
Researcher:	Date:	Signature:

1 copy for patient, 1 copy to be placed in the clinical notes and 1 copy for researcher

Version 2 GDH 10/12/12/ DPR

Appendix III: Clinical Information Sheet

University of Glasgow

Denture Biofilm Study

Date___/___/___

Study Code

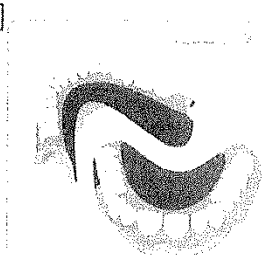
Summary Clinical Report

Gender Male ☐ Female ☐

Date of Birth ___/___/___

COMPLETE DENTURES

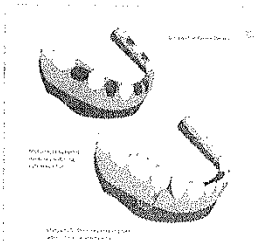
☐



MAXILLARY ☐ MANDIBULAR ☐ BOTH ☐

REMOVABLE PARTIAL DENTURE

☐



MAXILLARY ☐ MANDIBULAR ☐ BOTH ☐

IS THE DENTURE A GOOD FIT? YES☐ NO☐

If not, how is the denture deficient? Unretentive ☐ Unstable ☐ poor bite ☐ other_____

DENTURE MATERIAL:

ACRYLIC ☐ CoCr ☐ Other (please specify) ☐

TEETH PRESENT: Cross out missing teeth Tick if Not Applicable ☐

Hard Tissue: Please comment on any untreated decay or Non carious Tooth surface loss

TIME SINCE LAST TOOTHBRUSHED

(time in hours approximately)

ORAL HYGIENE (Please circle) : Excellent Good Poor

University of Glasgow

Denture Biofilm Study

Study Code

Date___/___/___

Denture stomatitis (see Photos):

No Inflammation ☐ Grade 1 ☐ Grade 2 ☐ Grade3 ☐

Comment:

Soft tissue: Please comment on any other soft tissue lesions including obvious periontal disease

Medical History:

Drug History:

ANTIFUNGAL TREATMENT DURING THE LAST 6 MONTHS YES ☐ NO ☐

ANTIBIOTIC TREATMENT DURING THE LAST 6 MONTHS YES ☐ NO ☐

CHLORHEXIDINE TREATMENT DURING THE LAST 6 MONTHS YES ☐ NO ☐

If the answer, last three questions, is YES, which one?

When? _____

For how long? _____

Please check all samples have been takemn:

A Denture swab ☐

B Sonicate ☐

C Intra oral swab ☐ Specify area_____

D Plaque sample ☐ tooth number_____

E Saliva ☐

Appendix IV: Oral Health Questionnaire

Denture Biofilm Study

Study Code

Oral Health Questionnaire

1. Are you registered with a general dental practitioner? Yes ☐ No ☐ Don't Know ☐

2. If the answer to question 1 is "Yes" how often do you visit your dentist?

At least once a year ☐ Occasionally ☐ Only when having trouble with teeth ☐ Never ☐

3. Do you sleep with your denture in? Yes ☐ No ☐ Sometimes ☐
4. How old is your denture?_____

5. How often do you clean your denture?

Never ☐ Less than once/day ☐ Once a day ☐ Twice a day ☐ More than twice a day ☐

6. How often do you clean your teeth (if applicable)?

Never ☐ Less than once/day ☐ Once a day ☐ Twice a day ☐ More than twice a day ☐

7. If you do clean your teeth, do you use a toothpaste containing fluoride?

Yes ☐ No ☐

8. Nowadays, there are more things available in supermarkets to help with dental hygiene. Do you use anything other than an ordinary toothbrush and toothpaste for dental hygiene purposes? If so, do you use?

Electric toothbrush ☐ Floss ☐ Interdental brushes ☐ Toothpicks/sticks ☐ Other ☐

9. Do you use a mouthwash? Yes ☐ No ☐

If the answer is "Yes" which one?

_____

10. What do you use to clean your denture?

Denture cleanser ☐ toothpaste ☐ Bleach/Milton ☐ Chlorhexidine ☐

Denture Biofilm Study

Study Code

11. What brand of denture cleanser do you use if any?_____

12. In the last 12 months have you had any painful aching or discomfort in your mouth?

Never ☐ Hardly ever ☐ Occasionally ☐ Fairly often ☐ Very often ☐

If you have experienced any pain or discomfort in the last 12 months please answer questions 13 and 14. Otherwise please move to question 15.

13. What type of pain or discomfort do/did you have?

Sharp ☐ Throbbing ☐ Dull ache ☐ Burning hot or scalded ☐ Tingling sensation ☐

14. How severe is the pain on a scale of 1 to 10? (please circle)

No Pain — Intolerable Pain

1 2 3 4 5 6 7 8 9 10

15. Does your mouth feel dry when eating a meal? Yes ☐ No ☐

16. Do you find it difficult to swallow dry foods? Yes ☐ No ☐

17. Do you sip liquids to help swallow dry foods? Yes ☐ No ☐

18. Do you wake during the night because your mouth is dry? Yes ☐ No ☐

19. Do you feel as if you have too little or too much saliva or, you are not aware of it?

Too little ☐ Too much ☐ Unaware of saliva ☐

Denture Biofilm Study

Study Code

20. In the last 12 months have you felt that your sense of taste has altered because of problems with your teeth, mouth or dentures?

Never ☐ Hardly ever ☐ Occasionally ☐ Fairly often ☐ Very often ☐

If you have noticed an alteration in your sense of taste:

21. Did/do you have an unpleasant taste? Yes ☐ No ☐

22. Did/do you have a metallic taste? Yes ☐ No ☐

23. In the last 12 months have you found it difficult to talk because of problems with your teeth mouth or dentures?

Never ☐ Hardly ever ☐ Occasionally ☐ Fairly often ☐ Very often ☐

24. In the last 12 months have you found it uncomfortable to eat any foods because of problems with your teeth mouth or dentures?

Never ☐ Hardly ever ☐ Occasionally ☐ Fairly often ☐ Very often ☐

25. In the last 12 months have you felt self-conscious because of problems with your teeth mouth or dentures?

Never ☐ Hardly ever ☐ Occasionally ☐ Fairly often ☐ Very often ☐

26. In the last 12 months have you felt tense because of problems with your teeth mouth or dentures?

Never ☐ Hardly ever ☐ Occasionally ☐ Fairly often ☐ Very often ☐

27. In the last 12 months has your diet been unsatisfactory because of problems with your teeth mouth or dentures?

Never ☐ Hardly ever ☐ Occasionally ☐ Fairly often ☐ Very often ☐

Denture Biofilm Study

Study Code

28. In the last 12 months have you had to interrupt meals because of problems with your teeth mouth or dentures?

Never ☐ Hardly ever ☐ Occasionally ☐ Fairly often ☐ Very often ☐

29. In the last 12 months have you found it difficult to relax because of problems with your teeth mouth or dentures?

Never ☐ Hardly ever ☐ Occasionally ☐ Fairly often ☐ Very often ☐

30. In the last 12 months have you been a bit embarrassed because of problems with your teeth mouth or dentures?

Never ☐ Hardly ever ☐ Occasionally ☐ Fairly often ☐ Very often ☐

31. In the last 12 months have you been a bit irritable with other people because of problems with your teeth mouth or dentures?

Never ☐ Hardly ever ☐ Occasionally ☐ Fairly often ☐ Very often ☐

32. In the last 12 months have you had difficulty doing your usual jobs because of problems with your teeth mouth or dentures?

Never ☐ Hardly ever ☐ Occasionally ☐ Fairly often ☐ Very often ☐

33. In the last 12 months have you felt that life in general was less satisfying because of problems with your teeth mouth or dentures?

Never ☐ Hardly ever ☐ Occasionally ☐ Fairly often ☐ Very often ☐

34. In the last 12 months have you been totally unable to function because of problems with your teeth mouth or dentures?

Never ☐ Hardly ever ☐ Occasionally ☐ Fairly often ☐ Very often ☐

Thank you for taking the time to complete this questionnaire.

Appendix V: Pairwise comparisons of Bacterial Classes of Healthy and DS Dental Microbiome

Dental												
	Healthy						DS					
Class	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinobacteria	17.25	16.04	64.16	1.04	>0.05	12.27	19.66	14.29	66.10	0.65	>0.05	16.71
Bacilli	12.83	7.01	76.23	0.26	>0.05	17.51	13.57	6.23	96.88	0.26	>0.05	18.60
Bacteroidia	12.63	5.97	48.44	0	>0.05	13.75	15.81	15.32	58.57	0	>0.05	14.84
Betaproteobacteria	5.04	1.38	36.36	0	>0.05	8.17	3.85	0.39	32.34	0	>0.05	7.68
Clostridia	23.69	21.17	51.43	0.65	>0.05	13.82	24.97	21.17	68.96	0.39	>0.05	12.61
Coriobacteriia	0.24	0	1.69	0	>0.05	0.40	0.64	0.13	6.10	0	>0.05	1.28
Epsilonproteobacteria	1.41	1.10	5.07	0	>0.05	1.22	1.85	1.43	5.84	0	>0.05	1.61
Flavobacteriia	6.24	2.73	42.60	0	>0.05	8.95	3.91	1.69	19.61	0	>0.05	4.82
Fusobacteriia	16.12	15.91	43.12	0.26	>0.05	11.63	12.33	10.78	33.12	0	>0.05	9.64
Gammaproteobacteria	0.22	0.19	1.41	0	>0.05	0.63	0.13	0.27	1.15	0	>0.05	0.65
Other	0.09	0	0.52	0	>0.05	0.15	0.28	0.26	0.65	0	>0.05	0.13
Spirochaetes	0.70	0	5.46	0	>0.05	1.18	0.85	0.13	4.16	0	>0.05	1.27
Synergistia	0.03	0	0.65	0	>0.05	0.11	0.22	0	3.77	0	>0.05	0.72

Appendix VI: Pairwise comparisons of Bacterial Genera of Healthy and DS Dental Microbiome

Dental												
	Healthy						DS					
Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinomyces	5.10	2.79	33.12	0	>0.05	6.77	8.89	3.64	57.79	0	>0.05	12.74
Bifidobacterium	0.16	0	3.64	0	>0.05	0.64	0.43	0	5.07	0	>0.05	1.25
Campnophaga	5.84	2.67	41.82	0	>0.05	8.44	3.67	1.69	16.49	0	>0.05	4.40
Campylobacter	1.41	1.10	5.07	0	>0.05	1.23	1.85	1.43	5.84	0	>0.05	1.61
Corynebacterium	7.69	4.74	32.08	0	>0.05	8.77	5.44	1.95	27.92	0	>0.05	6.84
Dialister	0.94	0	9.61	0	>0.05	2.07	0.86	0.39	6.23	0	>0.05	1.37
Fusobacterium	6.02	3.77	26.8	0	>0.05	6.04	6.97	5.97	24.29	0	>0.05	5.99
Halomonas	0.47	0	11.56	0	>0.05	2.03	0.07	0	0.65	0	>0.05	0.17
Heamophilus	1.98	0.26	26.10	0	>0.05	4.81	1.84	0.13	14.10	0	>0.05	3.51
Kingella	1.14	0.45	10.78	0	>0.05	2.15	2.07	0.13	31.30	0	>0.05	6.02
Lactobacillus	4.26	0	75.06	0	>0.05	17.01	4.21	0	94.68	0	>0.05	17.80
Leptotrichicia	10.11	0	1.04	0	>0.05	0.25	0.05	0	0.65	0	>0.05	3.44
Moryella	10.09	5.84	33.64	0	>0.05	10.07	5.43	0	20.39	0	>0.05	0.14
Nessieria	1.89	0.13	18.70	0	>0.05	4.12	0.97	2.99	18.44	0	>0.05	5.99
Porphyromonas	0.48	0	4.16	0	>0.05	0.97	0.50	0	5.58	0	>0.05	0.12
Prevotella	11.13	5.07	45.97	0	>0.05	12.90	13.53	11.30	45.71	0	>0.05	12.92
Rothia	0.03	0	0.39	0	>0.05	0.08	0.31	0	3.51	0	<0.05	0.71
Selenomonas	6.59	5.52	21.43	0	>0.05	6.54	6.01	3.25	34.16	0	>0.05	8.44
Streptococcus	8.27	5.78	34.29	0	>0.05	9.15	8.36	4.54	37.27	0.26	>0.05	8.68
Tanerella	0.43	0.06	3.90	0	>0.05	0.85	0.48	0	5.07	0	>0.05	1.23
Treponema	0.70	0	5.46	0	>0.05	1.18	0.85	0.13	4.16	0	>0.05	1.27
Veillonella	12.69	9.42	48.44	0.26	>0.05	12.76	14.89	14.16	68.83	0.39	>0.05	14.07

Appendix VII: Pairwise comparisons of Bacterial Classes of Healthy and DS Denture Microbiome

Denture												
	Healthy						DS					
Class	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinobacteria	42.57	42.08	89.35	1.039	>0.05	24.73	42.76	38.96	88.57	16.88	>0.05	21.05
Alphaproteobacteria	2.73	0	50.52	0	>0.05	8.23	0.94	0	15.58	0	>0.05	2.850
Bacilli	32.44	28.96	94.49	0.39	>0.05	22.77	32.73	28.44	96.62	1.69	>0.05	25.01
Bacteroidia	2.97	1.17	40.65	0	<0.05	6.15	5.18	2.86	30.78	0	<0.05	1.06
Betaproteobacteria	2.44	1.04	18.05	0	>0.05	4.03	1.80	0.65	12.47	0	>0.05	3.02
Clostridia	8.65	4.42	41.17	0.26	<0.05	8.79	11.24	10.52	36.75	0.13	>0.05	8.04
Coriobacteriia	1.11	0.26	12.47	0	>0.05	2.26	1.28	0.52	14.55	0	>0.05	25.80
Epsilonproteobacteria	0.06	0	1.56	0	>0.05	0.21	0.1	0	0.52	0	<0.05	0.15
Flavobacteriia	0.14	0	0.78	0	>0.05	0.19	0.21	0	2.99	0	>0.05	0.56
Fusobacteriia	0.99	0.39	11.04	0	>0.05	1.91	1.11	0.52	5.46	0	>0.05	1.32
Gammaproteobacteria	5.71	0.13	70.52	0	>0.05	12.49	2.50	0.91	20.13	0	>0.05	4.50
Other	0.01	0	0.52	0	>0.05	0.07	0.02	0	0.26	0	>0.05	0.06
Sphingobacteriia	0.08	0	4.93	0	>0.05	0.61	0.02	0	0.65	0	>0.05	0.10
Spirochaetes	0.01	0	0.26	0	>0.05	0.04	0.03	0	0.52	0	>0.05	0.09
Synergistia	0.004	0	0.13	0	>0.05	0.02	0.04	0	0.91	0	>0.05	0.16

Appendix VIII: Pairwise comparisons of Bacterial Genera of Healthy and DS Denture Microbiome

Denture												
	Healthy						DS					
Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinomyces	26.49	18.31	86.88	0.26	p>0.05	25.16	27.71	21.30	83.25	0	p>0.05	22.32
Atopbium	1.02	0.26	12.34	0	p>0.05	2.08	1.17	0.39	14.03	0	p>0.05	2.43
Bifidobacterium	1.08	0	19.09	0	p>0.05	3.22	1.11	0.13	11.82	0	p>0.05	2.56
Bradyrhizobium	1.88	0	38.96	0	p>0.05	6.44	0.63	0	11.17	0	p>0.05	2.03
Campnophaga	0.06	0	0.52	0	p>0.05	0.14	0.18	0	2.58	0	p>0.05	0.50
Campylobacter	0.06	0	1.56	0	p>0.05	0.21	0.10	0	0.52	0	p>0.05	0.15
Cardiobacterium	0.02	0	0.26	0	p>0.05	0.06	0.02	0	0.26	0	p>0.05	0.06
Corynebacterium	0.64	0.13	10.52	0	p>0.05	1.63	1.37	0.26	18.44	0	p>0.05	3.42
Delftia	0.09	0	2.86	0	p>0.05	0.40	0.26	0	9.35	0	p>0.05	1.43
Dialister	0.04	0	0.78	0	p>0.05	0.12	0.12	0	1.95	0	p>0.05	0.35
Fusobacterium	0.14	0	1.82	0	p>0.05	0.31	0.25	0	1.30	0	p>0.05	0.37
Halomonas	0.36	0	2.73	0	p>0.05	0.72	0.32	0	4.16	0	p>0.05	0.72
Heamophilus	2.7	0.26	36.62	0	p>0.05	5.83	1.91	0.52	19.35	0	p>0.05	4.29
Kingella	0.35	0	4.16	0	p>0.05	0.73	0.47	0	4.81	0	p>0.05	1.02
Lactobacillus	7.28	0.91	95.19	0	p>0.05	16.31	7.36	1.69	84.94	0	p>0.05	15.46
Leptotrichicia	0.85	0.26	9.22	0	p>0.05	1.69	0.86	0.39	4.81	0	p>0.05	1.14
Moryella	0.72	0.26	8.18	0	p>0.05	1.46	0.71	0.26	5.58	0	p>0.05	1.17
Nessieria	1.27	0.13	16.62	0	p>0.05	3.32	0.53	0.13	8.70	0	p>0.05	1.44
Porphyromonas	0.08	0	0.91	0	p>0.05	0.19	0.16	0	3.38	0	p>0.05	0.54
Prevotella	2.81	1.17	39.61	0	P<0.01	5.97	4.85	2.86	29.87	0	p<0.01	6.75
Pseudomonas	0.24	0	14.42	0	p>0.05	0.23	0.01	0	0.12	0	p>0.05	0.01

Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Rothia	11.09	4.03	67.27	0	p>0.05	14.4	8.57	5.32	54.03	0	p>0.05	10.81
Scardovia	1.42	0	30.65	0	p>0.05	4.82	3.18	0.13	21.74	0	p>0.05	6.85
Schwartzia	0.02	0	1.30	0	p>0.05	0.16	0.02	0	0.39	0	p>0.05	0.07
Selenomonas	0.58	0	15.97	0	p>0.05	2.20	0.85	0.13	10.91	0	p>0.05	2.08
Staphylococcus	0.06	0	3.18	0	p>0.05	0.39	0.02	0	0.39	0	p>0.05	0.06
Streptococcus	23.19	19.48	80.91	0	p>0.05	19.42	23.97	15.58	86.62	0.65	p>0.05	22.25
Tanerella	0.01	0	0.39	0	p>0.05	0.05	0.03	0	0.78	0	p>0.05	0.12
Veillonella	6.40	3.12	41.17	0	P<0.05	7.69	7.53	6.62	27.40	0	P<0.05	5.32

Appendix IX: Pairwise comparisons of Bacterial Classes of Healthy and DS Mucosal Microbiome

Mucosal												
	Good						Poor					
Class	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinobacteria	17.56	13.13	51.30	0	<0.05	13.42	23.83	17.66	88.38	1.04	<0.05	16.43
Alphaproteobacteria	0.70	0.26	4.29	0	>0.05	2.12	0.53	0.26	5.84	0	>0.05	1.18
Bacilli	49.25	50.26	99.22	3.12	>0.05	26.12	40.24	40.00	89.00	4.03	>0.05	22.89
Bacteroidia	4.85	2.79	27.27	0	<0.05	5.79	8.64	6.88	37.92	0	<0.05	8.27
Betaproteobacteria	4.98	3.18	65.27	0	>0.05	12.71	4.48	2.60	14.29	0	>0.05	3.63
Clostridia	8.17	4.55	35.84	0.26	<0.05	8.52	10.07	8.44	26.10	0.13	<0.05	6.59
Coriobacteriia	0.57	0.13	4.68	0	>0.05	1.10	1.04	0.26	6.23	0	>0.05	1.58
Epsilonproteobacteria	0.18	0.13	1.82	0	>0.05	0.31	0.33	0.19	1.56	0	>0.05	0.39
Flavobacteriia	0.40	0.13	5.46	0	>0.05	0.90	0.45	0.13	3.34	0	>0.05	0.84
Fusobacteriia	1.87	0.91	7.27	0	>0.05	2.03	1.07	0.52	7.27	0	>0.05	1.44
Gammaproteobacteria	7.95	3.38	30.65	0.26	>0.05	8.17	8.45	4.94	59.22	0.30	>0.05	10.76
Other	0.02	0	0.39	0	>0.05	0.06	0.06	0	0.39	0	>0.05	0.09
Spirochaetes	0.03	0	0.65	0	>0.05	0.13	0.13	0	4.29	0	>0.05	0.85
Synergistia	0.02	0	0.52	0	>0.05	0.11	0.11	0	6.1	0	>0.05	1.21

Appendix X: Pairwise comparisons of Bacterial Genera of Healthy and DS Mucosal Microbiome

Mucosal												
	Healthy						DS					
Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinomyces	7.75	4.16	50.91	0	>0.05	9.95	8.78	5.46	72.99	0	>0.05	12.92
Atopbium	0.52	0.13	3.98	0	>0.05	0.97	0.58	0.26	3.64	0	>0.05	0.91
Bifidobacterium	0.64	0.13	15.54	0	>0.05	2.27	0.77	0.13	5.71	0	>0.05	1.42
Campnophaga	0.39	0.13	4.54	0	>0.05	0.88	0.33	0	3.51	0	>0.05	0.67
Campylobacter	0.18	0.13	1.82	0	>0.05	0.31	0.33	0.13	1.56	0	>0.05	0.40
Corynebacterium	0.63	0.13	8.70	0	>0.05	1.43	0.13	0.26	11.30	0	>0.05	2.57
Delftia	8.10	0	0.65	0	>0.05	0.15	0.11	0	1.69	0	>0.05	0.32
Dialister	0.05	0	1.04	0	>0.05	0.16	0.13	0	1.56	0	>0.05	0.32
Fusobacterium	0.25	0	2.47	0	>0.05	0.43	0.45	0.26	2.99	0	>0.05	0.65
Halomonas	1.28	0.39	13.90	0	>0.05	2.85	0.68	0.39	7.01	0	>0.05	1.27
Heamophilus	5.37	2.01	28.57	0	>0.05	7.29	5.71	5.59	26.01	0	>0.05	6.56
Jathingobacterium	1.82	0.45	17.40	0	>0.05	3.57	0.05	0	1.56	0	>0.05	0.26
Kingella	0.23	0	6.23	0	>0.05	0.88	0.13	0	1.43	0	>0.05	0.28
Lactobacillus	3.40	0	65.97	0	>0.05	12.09	3.39	0.13	88.57	0	>0.05	14.94
Leptotrichicia	0.96	0.32	7.27	0	>0.05	1.62	1.22	0.78	6.49	0	>0.05	1.39
Moryella	0.70	0.19	8.96	0	>0.05	1.40	0.78	0.13	11.43	0	>0.05	2.02
Nessieria	3.35	0.13	47.27	0	>0.05	7.92	3.48	0.78	35.71	0	>0.05	6.68
Porphyromonas	0.23	0	2.08	0	>0.05	0.49	1.01	0.13	13.38	0	>0.05	2.38
Prevotella	4.19	2.53	26.62	0	<0.05	5.82	6.85	5.84	23.12	0	<0.05	5.73
Rothia	11.15	7.27	50.52	0.26	>0.05	11.10	8.02	5.20	42.27	0	>0.05	9.27
Scardovia	0.15	0	5.07	0	>0.05	0.77	0.47	0	6.10	0	>0.05	0.14
Selenomonas	1.41	0.26	16.36	0	>0.05	2.90	1.11	0.13	10.13		>0.05	2.25
Staphylococcus	0.02	0	0.26	0	>0.05	0.06	0.02	0	0.39	0	>0.05	0.08

Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Streptococcus	39.14	35.78	87.66	2.73	>0.05	23.42	38.50	34.16	87.92	3.12	>0.05	24.62
Tanerella	0.02	0	0.26	0	>0.05	0.06	0.11	0	1.82	0	>0.05	0.32
Veillonella	4.79	3.44	25.58	0	>0.05	4.87	4.43	3.77	12.60	0	>0.05	3.16

Appendix XI: Pairwise comparisons of Bacterial Classes of Smokers and Non-smokers Dental Microbiome

Dental												
	Smoker						Non-smoker					
Class	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinobacteria	25.43	24.55	66.10	4.81	>0.05	19.24	17.03	15.19	64.16	0.65	>0.05	13.42
Bacilli	20.68	5.58	76.23	2.86	>0.05	2.86	11.75	6.62	96.88	0.26	>0.05	15.34
Bacteroidia	12.36	13.12	35.84	0.65	>0.05	11.58	14.42	9.35	58.57	0	>0.05	14.75
Betaproteobacteria	1.52	0.13	9.48	0	>0.05	2.93	5.05	1.17	36.36	0	>0.05	8.43
Clostridia	19.71	16.23	41.04	0.91	>0.05	11.76	25.14	22.08	68.96	0.39	>0.05	13.25
Coriobacteriia	0.52	0.06	2.72	0	>0.05	0.90	0.40	0	6.10	0	>0.05	0.94
Epsilonproteobacteria	1.34	0.91	4.03	0	>0.05	1.25	1.66	1.30	5.84	0	>0.05	1.46
Flavobacteriia	2.81	0.91	11.69	0	>0.05	3.99	5.61	2.86	42.60	0	>0.05	7.80
Fusobacteriia	12.92	10.52	34.03	0.26	>0.05	12.17	14.65	14.03	43.12	0	>0.05	10.68
Gammaproteobacteria	1.43	0.26	7.14	0	>0.05	2.32	3.21	0.91	26.10	0	>0.05	5.37
Other	0.21	0.26	0.39	0	>0.05	0.16	0.17	0.13	0.65	0	>0.05	0.17
Spirochaetes	0.68	0	4.16	0	>0.05	1.40	0.79	0.26	5.46	0	>0.05	1.19
Synergistia	0.40	0	3.77	0	>0.05	0.18	0.06	0	1/04	0	>0.05	0.19

Appendix XII: Pairwise comparisons of Bacterial Genera of Smokers and Non-smokers Dental Microbiome

Dental												
	Healthy						DS					
Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinomyces	12.16	4.47	57.79	0	>0.05	18.13	5.84	2.86	35.32	0	>0.05	7.58
Bifidobacterium	0.38	0	2.73	0	>0.05	0.87	0.27	0	5.07	0	>0.05	0.99
Campnophaga	2.60	0.91	11.04	0	>0.05	3.77	5.27	2.73	41.82	0	>0.05	7.31
Campylobacter	1.22	0.78	4.03	0	>0.05	1.25	1.69	1.30	5.84	0	>0.05	1.45
Cardiobacterium	0.14	0	0.78	0	>0.05	0.29	0.44	0	5.58	0	>0.05	0.89
Corynebacterium	6.49	2.73	21.82	0	>0.05	7.97	6.69	3.57	32.08	0	>0.05	8.03
Dialister	0.46	0.13	2.47	0	>0.05	0.75	1.01	0.06	9.61	0	>0.05	1.91
Fusobacterium	4.20	3.64	12.21	0	>0.05	4.27	6.84	5	26.88	0	>0.05	6.22
Halomonas	0.13	0	0.65	0	>0.05	0.23	0.32	0	11.56	0	>0.05	1.65
Heamophilus	1.32	0.13	7.14	0	>0.05	2.23	2.04	0.26	26.10	0	>0.05	4.54
Kingella	0.30	0.13	1.56	0	>0.05	0.48	1.84	0.26	31.30	0	>0.05	4.77
Lactobacillus	13.12	0	75.06	0	>0.05	28.76	2.36	0	94.68	0	>0.05	13.35
Leptotrichicia	9.09	1.18	33.64	0	>0.05	11.30	7.70	4.41	31.95	0	>0.05	8.16
Megasphera	0.18	0	0.78	0	>0.05	0.32	0.55	0	5.46	0	>0.05	1.14
Moryella	0.08	0	0.65	0	>0.05	0.20	0.09	0	1.04	0	>0.05	0.21
Nessieria	0.90	0	8.70	0	>0.05	2.74	1.57	0.13	18.70	0	>0.05	4.03
Porphyromonas	1.03	0	5.58	0	>0.05	2.06	0.39	0	3.51	0	>0.05	0.78
Prevotella	9.14	10.78	17.01	0.52	>0.05	7.43	12.82	7.79	45.97	0	>0.05	13.62
Rothia	0.13	0.06	0.52	0	>0.05	0.18	0.16	0	3.51	0	>0.05	0.54
Schwartzia	0.36	0.06	2.56	0	>0.05	0.61	0.53	0	3.56	0	>0.05	1.12
Selenomonas	4.67	1.30	24.29	0	>0.05	7.39	6.64	3.38	34.16	0	>0.05	7.45

Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Streptococcus	5.81	3.18	18.18	0	>0.05	6.38	8.78	5.46	37.27	0.26	>0.05	9.23
Tanerella	0.56	0	3.87	0	>0.05	1.27	0.43	0	5.07	0	>0.05	0.99
Treponema	0.68	0	4.16	0	>0.05	1.40	0.79	0.26	5.46	0	>0.05	1.19
Veillonella	12.32	12.27	29.74	0.39	>0.05	9.06	13.96	11.30	68.83	0.26	>0.05	14.02

Appendix XIII: Pairwise comparisons of Bacterial Classes of Smokers and Non-smokers Denture Microbiome

Denture												
	Smokers						Non-smokers					
Class	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinobacteria	46.30	44.16	88.57	6.36	>0.05	22.91	41.94	38.96	89.35	1.04	>0.05	23.61
Alphaproteobacteria	3.52	0	50.52	0	>0.05	11.54	1.74	0	33.25	0	>0.05	5.18
Bacilli	31.74	24.29	81.95	6.23	>0.05	22.58	32.79	30.00	96.62	0.39	>0.05	24.16
Bacteroidia	3.90	1.17	40.65	0	>0.05	9.35	3.81	1.69	30.78	0	>0.05	5.86
Betaproteobacteria	2.08	0.65	0	15.71	>0.05	3.86	2.21	0.91	18.05	0	>0.05	3.68
Clostridia	8.54	4.16	23.25	0.65	>0.05	7.94	9.89	7.14	41.17	0.13	>0.05	8.66
Coriobacteriia	0.66	0	3.12	0	>0.05	1.03	1.32	0.39	14.55	0	>0.05	2.59
Epsilonproteobacteria	0.18	0.13	1.56	0	>0.05	0.36	0.05	0	0.52	0	>0.05	0.12
Flavobacteriia	0.12	0	0.52	0	>0.05	0.17	0.13	0	2.08	0	>0.05	0.28
Fusobacteriia	0.64	0.26	3.25	0	>0.05	0.86	0.51	0.13	7.14	0	>0.05	1.16
Gammaproteobacteria	1.70	1.04	4.49	0	>0.05	1.97	1.04	4.97	70.52	0	>0.05	11.21
Other	0.01	0	0.13	0	>0.05	0.03	0.01	0	0.52	0	>0.05	0.07
Sphingobacteriia	0.01	0	0.13	0	>0.05	0.03	0.06	0	4.94	0	>0.05	0.52
Spirochaetes	0.03	0	0.52	0	>0.05	0.03	0.01	0	0.26	0	>0.05	0.01
Synergistia	0.08	0	0.91	0	>0.05	0.23	0.01	0	0.13	0	>0.05	0.03

Appendix XIV: Pairwise comparisons of Bacterial Genera of Smokers and Non-smokers Denture Microbiome

Denture												
	Smoker						Non-smoker					
Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinomyces	26.73	18.70	83.25	1.68	>0.05	24.48	27.03	19.61	86.88	0	>0.05	24.02
Atopbium	27.03	19.61	86.88	0	<0.001	24.02	1.18	0.39	14.03	0	<0.001	2.39
Bifidobacterium	0.29	0	1.82	0	>0.05	0.55	1.26	0.13	19.09	0	>0.05	3.23
Bradyrhizobium	2.50	0	38.96	0	>0.05	8.90	1.15	0	25.97	0	>0.05	4.04
Campnophaga	0.08	0	0.52	0	>0.05	0.14	0.11	0	2.60	0	>0.05	0.36
Campylobacter	0.18	0.13	1.56	0	>0.05	0.36	0.05	0	0.52	0	>0.05	0.11
Cardiobacterium	0.01	0	0.13	0	>0.05	0.03	0.02	0	0.26	0	>0.05	0.06
Corynebacterium	0.21	0.13	0.91	0	>0.05	0.27	1.08	0.13	18.44	0	>0.05	2.74
Delftia	0.01	0	0.13	0	>0.05	0.03	0.19	0	9.35	0	>0.05	1.05
Dialister	0.01	0	0.13	0	>0.05	0.03	0.08	0	1.95	0	>0.05	0.26
Fusobacterium	0.25	0	1.30	0	>0.05	0.40	0.17	0	1.82	0	>0.05	0.32
Halomonas	0.35	0	2.73	0	>0.05	0.70	0	0.34	4.16	0	>0.05	0.72
Heamophilus	1.01	0.39	3.90	0	>0.05	1.27	2.67	0.52	36.62	0	>0.05	5.73
Jathingobacterium	0.11	0	0.52	0	>0.05	0.17	0.16	0	4.68	0	>0.05	0.65
Kingella	0.36	0	3.90	0	>0.05	0.97	0.40	0	4.81	0	>0.05	0.83
Lactobacillus	3.49	0.58	24.94	0	>0.05	7.25	8.07	1.56	95.19	0	>0.05	17.04
Leptotrichicia	0.83	0.32	3.90	0	>0.05	1.16	0.86	0.26	9.22	0	>0.05	1.55
Moryella	0.86	0.13	5.56	0	>0.05	1.51	0.68	0.26	8.18	0	>0.05	1.31
Nessieria	1.24	0.13	15.71	0	>0.05	3.61	0.92	0.13	16.62	0	>0.05	2.55
Prevotella	3.73	1.17	39.61	0	>0.05	9.09	36	1.69	29.87	0	>0.05	5.66
Rothia	18.13	12.21	67.27	0.26	>0.05	19.25	5.37	3.90	53.12	0	>0.05	10.76
Scardovia	0.37	0	2.86	0	>0.05	0.79	2.5	0.13	30.15	0	>0.05	6.27
Selenomonas	0.14	0	1.43	0	<0.01	0.36	0.81	0.13	15.97	0	<0.01	2.35

Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Streptococcus	26.98	21.43	65.71	4.35	>0.05	18.73	22.76	16.10	86.62	0	>0.05	20.87
Veillonella	6.73	4.16	20.39	0.39	>0.05	6.19	6.87	4.94	41.17	0	>0.05	6.99

Appendix XV: Pairwise comparisons of Bacterial Classes of Smokers and Non-smokers Mucosal Microbiome

Mucosal												
	Smoker						Non-smoker					
Class	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinobacteria	16.45	12.34	44.81	3.12	>0.05	11.01	21.74	17.66	83.38	0	>0.05	16.94
Alphaproteobacteria	1.41	0.52	5.84	0	>0.05	1.80	0.46	0.26	4.03	0	>0.05	0.71
Bacilli	47.09	42.99	81.43	10.39	>0.05	23.10	45.29	44.42	99.22	3.12	>0.05	25.70
Bacteroidia	8.18	5.33	27.27	0	>0.05	8.99	5.96	4.29	37.92	0	>0.05	6.61
Betaproteobacteria	6.83	4.09	22.60	0.13	>0.05	7.36	6.02	2.47	65.97	0	>0.05	10.88
Clostridia	8.81	5.52	30.65	1.17	<0.05	8.68	8.96	7.01	35.84	0	<0.05	7.67
Coriobacteriia	0.59	0.26	3.51	0	>0.05	0.91	0.80	0.13	6.23	0	>0.05	1.41
Epsilonproteobacteria	0.32	0.06	1.82	0	>0.05	0.54	0.22	0.13	1.56	0	>0.05	0.30
Flavobacteriia	1.68	0.84	6.49	0	>0.05	2.01	1.34	0.65	7.27	0	>0.05	1.69
Fusobacteriia	7.82	6.03	28.96	0.26	>0.05	7.44	8.23	4.55	59.22	0.26	>0.05	9.68
Gammaproteobacteria	7.82	6.03	28.96	0.26	>0.05	7.44	8.23	4.55	59.22	0.26	>0.05	9.68
Other	0.03	0	0.13	0	>0.05	0.06	0.02	0	0.39	0	>0.05	0.08
Spirochaetes	0.04	0	0.52	0	>0.05	0.13	0.16	0	4.29	0	>0.05	0.62
Synergistia	0.07	0	1.17	0	>0.05	0.29	0.21	0	6.1	0	>0.05	0.86

Appendix XVI: Pairwise comparisons of Bacterial Genera of Smokers and Non-smokers Mucosal Microbiome

Mucosal												
	Smoker						Non-smoker					
Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinomyces	4.23	3.12	16.62	0	>0.05	4.40	9.05	5.58	72.99	0	>0.05	12.04
Atopbium	0.39	0.26	1.82	0	>0.05	0.51	0.58	0.13	3.90	0	>0.05	1.01
Bifidobacterium	1.65	0.13	15.84	0	>0.05	3.97	0.48	0.13	5.70	0	>0.05	1.05
Campnophaga	0.32	0	3.51	0	>0.05	0.87	0.38	0.13	4.55	0	>0.05	0.79
Campylobacter	0.32	0.06	1.82	0	>0.05	0.54	0.22	0.13	1.56	0	>0.05	0.30
Corynebacterium	0.50	0.13	3.77	0	>0.05	1.03	1.02	0.13	11.30	0	>0.05	2.13
Delftia	0.16	0	0.91	0	>0.05	0.25	0.09	0	1.69	0	>0.05	0.23
Dialister	0.03	0	0.13	0	>0.05	0.06	0.09	0	1.56	0	>0.05	0.26
Fusobacterium	0.42	0.19	1.56	0	>0.05	0.54	0.30	0.13	2.98	0	>0.05	0.52
Halomonas	2.58	0.65	13.90	0	>0.05	4.53	0.69	0.39	8.05	0	>0.05	1.32
Heamophilus	3.95	0.52	28.57	0	>0.05	7.15	5.86	2.86	26.49	0	>0.05	6.91
Jathingobacterium	2.89	0.26	15.06	0	>0.05	4.78	0.50	0	4.29	0	>0.05	0.92
Kingella	0.11	0	1.43	0	>0.05	0.63	0.21	0	6.23	0	>0.05	0.75
Lactobacillus	5.37	0.26	65.97	0	>0.05	16.49	2.95	0	88.57	0	>0.05	12.47
Leptotrichicia	1.19	0.13	6.49	0	>0.05	1.86	1.04	0.52	7.27	0	>0.05	1.46
Moryella	0.75	0.13	8.96	0	>0.05	2.20	0.73	0.13	11.43	0	>0.05	1.53
Nessieria	2.18	0.13	20.65	0	>0.05	5.22	3.68	0.39	47.27	0	>0.05	7.82
Porphyromonas	1.35	0.13	13.38	0	>0.05	3.29	0.37	0	3.25	0	>0.05	0.77
Prevotella	6.34	4.03	26.62	0	>0.05	7.44	5.02	4.16	23.12	0	>0.05	5.31
Pseudomonas	0.32	0	3.38	0	>0.05	0.93	0.84	0	57.27	0	>0.05	6.79
Rothia	9.35	6.75	39.09	0.52	>0.05	9.56	10.01	6.62	30.52	0	>0.05	10.71
Scardovia	0.23	0	2.47	0	>0.05	0.61	0.37	0	6.10	0	>0.05	1.00
Selenomonas	0.89	0.06	7.67	0	>0.05	2.02	1.38	0.39	16.36	0	>0.05	2.78

Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Staphylococcus	0.03	0	0.26	0	>0.05	0.09	0.01	0	0.39	0	>0.05	0.06
Streptococcus	39.29	33.12	78.96	9.09	>0.05	22.56	38.79	35.97	87.92	2.73	>0.05	24.19
Tanerella	0.06	0	0.65	0	>0.05	0.16	0.06	0	1.82	0	>0.05	0.23
Veillonella	5.10	4.48	17.27	0	>0.05	4.84	4.54	3.64	25.58	0	>0.05	4.13

Appendix XVII: Pairwise comparisons of Bacterial Classes of Good and Poor Hygiene Denture Microbiome

Denture												
	Good						Poor					
Class	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinobacteria	43.49	42.47	81.35	1.04	>0.05	24.66	40.81	38.96	83.51	2.21	>0.05	19.99
Alphaproteobacteria	1.76	0	50.52	0	<0.05	7.19	2.57	0.13	27.27	0	<0.05	5.42
Bacilli	32.17	28.70	96.49	1.69	>0.05	28.37	33.38	29.48	96.62	0.39	>0.05	23.36
Bacteroidia	3.52	1.43	40.65	0	>0.05	6.69	4.57	2.60	26.23	0	>0.05	6.22
Betaproteobacteria	2.19	0.91	18.05	0	>0.05	3.66	2.17	0.65	15.71	0	>0.05	3.73
Clostridia	9.41	6.17	41.17	0.26	>0.05	8.81	10.25	11.36	28.57	0.13	>0.05	8.06
Coriobacteriia	1.10	0.39	12.47	0	>0.05	2.07	1.40	0.32	14.55	0	>0.05	2.96
Epsilonproteobacteria	0.09	0	1.56	0	>0.05	0.22	0.07	0	0.52	0	>0.05	0.15
Flavobacteriia	0.15	0	2.08	0	>0.05	0.30	0.17	0	2.99	0	>0.05	0.52
Fusobacteriia	0.97	0.45	5.84	0	>0.05	1.26	1.18	0.26	11.04	0	>0.05	2.40
Gammaproteobacteria	4.96	1.20	70.52	0	>0.05	11.54	3.29	0.84	26.10	0	>0.05	6.32
Other	0.01	0	0.26	0	>0.05	0.04	0.02	0	0.52	0	>0.05	0.09
Sphingobacteriia	0.07	0	4.94	0	>0.05	0.57	0.02	0	0.65	0	>0.05	0.11
Spirochaetes	0.02	0	0.52	0	>0.05	0.07	0.02	0	0.26	0	>0.05	0.05
Synergistia	0.02	0	0.91	0	>0.05	0.11	0.02	0	0.52	0	>0.05	0.02

Appendix XVIII: Pairwise comparisons of Bacterial Genera of Good and Poor Hygiene Denture Microbiome

Denture												
	Good						Poor					
Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinomyces	29.88	21.30	86.88	0.26	>0.05	25.31	20.66	17.01	69.74	0	>0.05	19.71
Atopbium	1.04	0.39	12.34	0	>0.05	1.99	1.15	0.26	14.03	0	>0.05	2.68
Bifidobacterium	0.83	0.13	13.12	0	>0.05	2.18	1.65	0.13	19.09	0	>0.05	4.18
Bradyrhizobium	1.21	0	38.96	0	>0.05	5.47	1.76	0	23.77	0	>0.05	4.51
Campnophaga	0.09	0	1.95	0	>0.05	0.27	0.13	0	2.60	0	>0.05	0.45
Campylobacter	0.08	0	1.56	0	>0.05	0.20	0.06	0	0.52	0	>0.05	0.14
Cardiobacterium	0.02	0	0.26	0	>0.05	0.06	0.02	0	0.26	0	>0.05	0.06
Corynebacterium	1.08	0.13	18.44	0	>0.05	2.76	0.59	0.13	10.78	0	>0.05	1.85
Delftia	0.06	0	2.86	0	>0.05	0.34	0.36	0	9.35	0	>0.05	1.62
Dialister	0.07	0	1.95	0	>0.05	0.27	0.06	0	0.78	0	>0.05	0.18
Fusobacterium	0.18	0	1.30	0	>0.05	0.31	0.20	0	1.82	0	>0.05	0.40
Halomonas	0.32	0	2.73	0	>0.05	0.63	0.37	0	4.16	0	>0.05	0.88
Heamophilus	2.66	0.58	36.62	0	>0.05	5.49	1.77	0.13	20.13	0	>0.05	4.77
Jathingobacterium	0.17	0	4.68	0	>0.05	0.59	0.09	0	0.91	0	>0.05	0.22
Kingella	0.46	0	4.82	0	>0.05	0.90	0.27	0	4.16	0	>0.05	0.76
Lactobacillus	6.18	0.91	95.19	0	>0.05	15.12	9.78	1.95	84.94	0	>0.05	17.47
Leptotrichicia	0.79	0.32	5.84	0	>0.05	1.15	0.98	0.26	9.22	0	>0.05	2.05
Moryella	0.75	0.26	8.18	0	>0.05	1.39	0.63	0.13	5.58	0	>0.05	1.23
Nessieria	1.01	0.13	16.62	0	>0.05	2.63	0.90	0	15.71	0	>0.05	3.02
Prevotella	3.34	1.43	39.61	0	>0.05	6.51	4.25	2.40	25.84	0	>0.05	5.99
Rothia	9.99	6.75	54.03	0	>0.05	11..95	10.29	3.57	67.27	0	>0.05	15.49
Scardovia	0.99	0	16.75	0	>0.05	2.6	4.59	0.32	30.65	0	>0.05	9.08
Selenomonas	0.71	0.13	15.97	0	>0.05	2.24	0.64	0.13	10.91	0	>0.05	1.96

Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Staphylococcus	0.01	0	0.39	0	>0.05	0.05	0.11	0	3.31	0	>0.05	0.53
Streptococcus	24.14	18.77	80.91	1.04	>0.05	21.27	23.10	19.29	86.62	0.39	>0.05	18.93
Tanerella	0.02	0	0.78	0	>0.05	0.10	0.01	0	0.13	0	>0.05	0.03
Veillonella	6.60	4.29	41.17	0	>0.05	6.81	7.40	5.26	27.40	0	>0.05	6.96

Appendix XIX: Pairwise comparisons of Bacterial Classes for Cleaning Frequency at the Denture Microbiome

Denture												
	Once						Twice					
Class	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinobacteria	47.50	48.83	89.35	1.04	>0.05	23.73	39.67	35.83	88.70	1.69	>0.05	22.59
Alphaproteobacteria	1.32	0	12.21	0	>0.05	2.97	2.45	0	50.52	0	>0.05	8.14
Bacilli	25.06	18.57	81.95	0.91	<0.01	20.19	37.14	32.34	96.62	0.91	<0.01	24.50
Bacteroidia	4.75	1.56	30.78	0	>0.05	7.09	3.3	1.69	40.65	0	>0.05	6.16
Betaproteobacteria	2.40	0.78	15.71	0	>0.05	3.54	2.05	0.91	18.05	0	>0.05	3.75
Clostridia	8.77	5.84	30.52	0.26	>0.05	7.70	10.24	7.14	41.17	0.13	>0.05	9.04
Coriobacteriia	1.19	0.39	7.53	0	>0.05	1.98	1.2	0.39	14.55	0	>0.05	2.6
Epsilonproteobacteria	0.08	0	0.52	0	>0.05	0.14	0.07	0	1.56	0	>0.05	0.21
Flavobacteriia	0.11	0	0.78	0	>0.05	0.16	0.18	0	2.99	0	>0.05	0.47
Fusobacteriia	1.24	0.39	11.04	0	>0.05	2.06	0.91	8.18	0.39	0	>0.05	1.42
Gammaproteobacteria	7.31	1.30	70.52	0	>0.05	15.42	2.67	1.04	20.13	0	>0.05	3.40
Other	0.02	0	0.52	0	>0.05	0.08	0.01	0	0.26	0	>0.05	0.05
Sphingobacteriia	1.58	0	38	0	>0.05	7.43	33.50	33.50	66	1	>0.05	45.95
Spirochaetes	0.04	0	0.91	0	>0.05	0.16	0.01	0	0.15	0	>0.05	0.02
Synergistia	0.04	0	0.67	0	>0.05	0.11	0.01	0	0.13	0	>0.05	0.03

Appendix XX: Pairwise comparisons of Bacterial Genera for Cleaning Frequency at the Denture Microbiome

Denture												
	Once						Twice					
Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinomyces	31.24	2.27	83.25	0.26	>0.05	25.51	24.36	17.53	86.88	0	>0.05	22.50
Atopbium	1.11	0.39	7.53	0	>0.05	1.85	1.06	0.26	14.03	0	>0.05	2.43
Bifidobacterium	1.03	0.13	11.69	0	>0.05	2.23	1.14	0.14	19.09	0	>0.05	3.35
Bradyrhizobium	0.48	0	7.27	0	>0.05	1.51	1.94	0	38.96	0	>0.05	6.42
Campnophaga	0.05	0	0.39	0	>0.05	0.09	0.14	0	2.60	0	>0.05	0.42
Campylobacter	0.08	0	0.59	0	>0.05	0.14	0.07	0	1.56	0	>0.05	0.21
Cardiobacterium	0.02	0	0.26	0	>0.05	0.06	0.02	0	0.26	0	>0.05	0.05
Corynebacterium	0.76	0.13	7.40	0	>0.05	1.42	1.03	0.13	18.44	0	>0.05	2.99
Delftia	0.37	0	9.35	0	>0.05	1.53	0.02	0	0.39	0	>0.05	0.07
Dialister	0.09	0	1.95	0	>0.05	0.34	0.06	0	0.78	0	>0.05	0.16
Fusobacterium	0.19	0	1.82	0	>0.05	0.41	0.18	0	1.17	0	>0.05	0.29
Halomonas	0.35	0	4.16	0	>0.05	0.80	0.33	0	2.73	0	>0.05	0.66
Heamophilus	2.88	0.39	36.62	0	>0.05	7.05	2.07	0.32	19.09	0	>0.05	3.82
Jathingobacterium	0.09	0	0.78	0	>0.05	0.19	0.19	0	4.68	0	>0.05	0.62
Kingella	0.46	0	4.16	0	>0.05	0.89	0.36	0	4.81	0	>0.05	0.84
Lactobacillus	5.01	0.65	37.40	0	>0.05	9.19	8.72	1.69	95.19	0	>0.05	18.80
Leptotrichicia	1.05	0.26	9.22	0	>0.05	1.81	0.73	0.26	7.14	0	>0.05	1.25
Moryella	0.83	0.26	6.23	0	>0.05	1.44	0.64	0.26	8.18	0	>0.05	1.28
Nessieria	1.20	0.13	15.71	0	>0.05	3.07	0.84	0.13	16.62	0	>0.05	2.54
Prevotella	4.50	1.56	29.87	0	>0.05	6.85	3.09	1.43	39.61	0	>0.05	6
Rothia	10.86	6.36	67.27	0.13	>0.05	14.33	9.62	3.90	53.12	0	>0.05	12.37
Scardovia	1.22	0	21.95	0	>0.05	3.63	2.68	0.13	30.65	0	>0.05	6.69
Selenomonas	0.77	0	15.97	0	>0.05	2.72	0.65	0.13	10.91	0	>0.05	1.73

Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Staphylococcus	0.02	0	0.39	0	>0.05	0.07	0.05	0	3.12	0	>0.05	0.38
Streptococcus	18.78	12.99	65.71	0.39	>0.05	17.48	26.36	21.43	86.62	0.39	>0.05	21.76
Tanerella	0.01	0	0.13	0	>0.05	0.03	0.03	0	0.78	0	>0.05	0.11
Veillonella	5.87	4.94	19.87	0.13	>0.05	7.45	7.45	4.63	41.17	0	>0.05	7.74

Appendix XXI: Pairwise comparisons of Bacterial Classes for Sleeping with or without a Denture *in situ* at the Denture Microbiome

Denture												
	Yes						No					
Class	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinobacteria	44.67	38.96	89.35	1.04	>0.05	26.12	40.57	42.08	88.57	2.21	>0.05	22.03
Alphaproteobacteria	1.52	0	33.25	0	>0.05	5.37	2.17	0	50.52	0	>0.05	7.58
Bacilli	24.31	21.30	86.88	0.91	<0.01	19.84	37.15	34.61	82.86	0.39	<0.01	22.01
Bacteroidia	6.25	2.6	40.65	0	<0.05	8.83	2.16	1.49	12.34	0	<0.05	2.51
Betaproteobacteria	1.47	0.39	18.05	0	<0.05	3.02	3.13	1.36	17.66	0	<0.05	4.37
Clostridia	14.88	14.94	9.14	0.26	<0.001	9.14	5.57	4.87	21.43	0.26	<0.001	4.88
Coriobacteriia	1.68	0.65	14.55	0	>0.05	2.80	0.91	0.26	12.47	0	>0.05	2.1
Epsilonproteobacteria	0.13	0	1.56	0	<0.05	0.26	0.03	0	0.26	0	<0.05	0.66
Flavobacteriia	0.17	0	2.99	0	>0.05	0.45	0.14	0	2.08	0	>0.05	0.34
Fusobacteriia	1.73	1.04	11.04	0	<0.001	2.21	0.46	0.26	4.42	0	<0.001	0.74
Gammaproteobacteria	3.46	1.78	59.48	0	>0.05	9.21	4.77	1.82	37.01	0	>0.05	7.36
Other	0.01	0	0.26	0	>0.05	0.04	0.02	0	0.52	0	>0.05	0.08
Sphingobacteriia	0.09	0	4.94	0	>0.05	0.63	0.01	0	0.13	0	>0.05	0.03
Spirochaetes	0.02	0	0.52	0	>0.05	0.08	0.01	0	0.13	0	>0.05	0.04
Synergistia	0.03	0	0.91	0	>0.05	0.11	0.01	0	0.13	0	>0.05	0.02

Appendix XXII: Pairwise comparisons of Bacterial Genera for Sleeping with or without a Denture *in situ* at the Denture Microbiome

Denture												
	Yes						No					
Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinomyces	31	23.44	83.25	0	<0.05	23.58	21.55	10.58	86.88	0.39	<0.05	23.70
Atopbium	1.23	0.39	14.03	0	>0.05	2.33	0.88	0.13	12.34	0	>0.05	2.07
Bifidobacterium	0.89	0.13	11.69	0	>0.05	2.16	1.36	0.13	19.09	0	>0.05	3.80
Bradyrhizobium	1.35	0	25.97	0	>0.05	4.69	1.43	0	38.96	0	>0.05	5.82
Campnophaga	0.12	0	2.60	0	>0.05	0.35	0.09	0	1.95	0	>0.05	0.31
Campylobacter	0.11	0	1.56	0	>0.05	0.23	0.03	0	0.26	0	>0.05	0.06
Cardiobacterium	0.03	0	0.26	0	>0.05	0.06	0.02	0	0.26	0	>0.05	0.05
Corynebacterium	1.01	0.13	10.78	0	>0.05	2.31	0.82	0.13	18.44	0	>0.05	2.78
Delftia	0.09	0	2.86	0	>0.05	0.39	0.25	0	9.35	0	>0.05	1.39
Dialister	0.08	0	1.95	0	>0.05	0.29	0.05	0	0.78	0	>0.05	0.16
Fusobacterium	0.22	0	1.82	0	>0.05	0.40	0.13	0	0.78	0	>0.05	0.22
Halomonas	0.34	0	4.16	0	>0.05	0.75	0.34	0.06	2.73	0	>0.05	0.69
Heamophilus	1.48	0.39	20.13	0	<0.05	3.78	3.59	0.78	36.62	0	<0.05	6.63
Jathingobacterium	0.12	0	1.56	0	>0.05	0.27	0.19	0	4.66	0	>0.05	0.70
Kingella	0.36	0	4.16	0	>0.05	0.85	0.45	0.13	4.81	0	>0.05	0.87
Lactobacillus	8.23	1.04	95.16	0	>0.05	19.15	6.07	0.19	42.34	0	>0.05	10.08
Leptotrichicia	1.24	0.58	9.22	0	<0.001	1.80	0.32	0.13	3.64	0	<0.001	0.60
Moryella	1.03	0.26	8.18	0	>0.05	1.69	0.32	0.13	1.30	0	>0.05	0.39
Nessieria	0.35	0.13	8.70	0	<0.01	1.19	1.82	0.26	16.62	0	<0.01	3.84
Prevotella	4.80	1.62	39.61	0	>0.05	7.92	2.03	1.43	12.21	0	>0.05	2.46
Rothia	6.08	2.21	40.26	0	<0.001	8.45	14.06	8.38	67.27	0.13	<0.001	15.46
Scardovia	2.19	0	30.65	0	>0.05	5.92	2.02	0	29.74	0.13	>0.05	5.57
Selenomonas	1.07	0.13	15.97	0	>0.05	2.78	0.19	0.06	1.16	0	>0.05	0.30

Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Staphylococcus	0.06	0	3.12	0	>0.05	0.40	0.02	0	0.39	0	>0.05	0.07
Streptococcus	19.57	13.64	86.62	0	<0.05	18.59	28.79	25.65	80.91	0	<0.05	21.92
Tanerella	0.04	0	0.78	0	>0.05	0.13	0.02	0	0.13	0	>0.05	0.02
Veillonella	8.67	6.36	41.17	0	<0.01	7.77	4.39	3.05	20.39	0	<0.01	4.30

Appendix XXIII: Pairwise comparisons of Bacterial Classes of Good and Poor Hygiene Mucosal Microbiome

Mucosal												
	Good						Poor					
Class	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinobacteria	20.60	14.42	83.38	0	>0.05	17.70	21.06	18.18	61.56	1.04	>0.05	13.14
Alphaproteobacteria	0.56	0.26	5.84	0	>0.05	1.11	0.75	0.39	3.24	0	>0.05	0.97
Bacilli	47.48	43.64	99.22	3.12	>0.05	27.20	42.44	44.74	89.09	9.48	>0.05	20. 50
Bacteroidia	6.14	3.90	37.92	0	>0.05	7.69	6.77	6.04	22.08	0	>0.05	6.06
Betaproteobacteria	5.74	2.08	47.92	0.13	>0.05	9.22	6.90	3.83	65.97	0.13	>0.05	12.04
Clostridia	8.21	6.36	30.65	1.17	<0.05	7.23	10.17	8.57	35.84	0.13	<0.05	8.71
Coriobacteriia	0.59	0.13	3.89	0	>0.05	1.03	1.06	0.26	6.23	0	>0.05	1.70
Epsilonproteobacteria	0.23	0.13	1.82	0	>0.05	0.38	0.26	0.19	1.30	0	>0.05	0.31
Flavobacteriia	0.38	0.13	5.46	0	>0.05	0.88	0.50	0.13	3.64	0	>0.05	0.86
Fusobacteriia	1.21	0.65	6.89	0	>0.05	1.55	1.73	0.91	7.27	0	>0.05	2.03
Gammaproteobacteria	8.50	6.03	59.22	0.26	>0.05	10.02	7.56	3.57	30.65	0.39	>0.05	7.93
Other	0.02	0	0.39	0	>0.05	0.06	0.04	0	0.39	0	>0.05	0.09
Spirochaetes	0.11	0	2.60	0	>0.05	0.40	0.20	0	4.29	0	>0.05	0.76
Synergistia	0.10	0	2.21	0	>0.05	0.36	0.33	0	6.10	0	>0.05	1.21

Appendix XXIII: Pairwise comparisons of Bacterial Genera of Good and Poor Hygiene Mucosal Microbiome

Denture												
	Good						Poor					
Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinomyces	7.93	0	72.99	4.54	>0.05	12.40	8.57	0.23	40.39	6.10	>0.05	8.87
Atopbium	0.53	0.13	3.90	0	>0.05	0.96	0.57	0.19	3.38	0	>0.05	0.92
Bifidobacterium	0.50	0	15.84	0	<0.01	2.15	1.03	0.26	5.71	0	<0.01	1.56
Campnophaga	0.31	0.13	4.55	0	>0.05	0.76	0.47	0.13	3.51	0	>0.05	0.85
Campylobacter	0.23	0.13	1.82	0	>0.05	0.38	0.26	0.19	1.30	0	>0.05	0.31
Corynebacterium	1.01	0.13	11.30	0	>0.05	2.35	0.73	0.26	4.29	0	>0.05	1.10
Delftia	0.14	0	0.91	0	>0.05	0.19	0.11	0	1.69	0	>0.05	0.31
Dialister	0.06	0	1.56	0	>0.05	0.22	0.11	0	1.04	0	>0.05	0.27
Fusobacterium	0.26	0	2.47	0	>0.05	0.43	0.45	0.26	2.99	0	>0.05	0.65
Halomonas	0.79	0.26	8.05	0	>0.05	1.48	1.47	0.06	13.90	0	>0.05	3.35
Heamophilus	5.50	3.77	26.10	0	>0.05	6.61	5.52	1.62	28.57	0	>0.05	7.66
Jathingobacterium	0.68	0	12.60	0	>0.05	1.84	1.83	0.19	17.40	0	>0.05	4.06
Kingella	0.06	0	0.78	0	<0.01	0.13	0.23	0.13	1.43	0	<0.01	0.34
Lactobacillus	3.45	0	88.57	0	>0.05	14.73	3.32	0.39	57.14	0	>0.05	10.36
Leptotrichicia	0.95	0.39	6.49	0	>0.05	1.32	1.25	0.45	7.27	0	>0.05	1.85
Moryella	0.65	0.13	8.96	0	>0.05	1.41	0.89	0.26	11.43	0	>0.05	2.05
Nessieria	4.06	0.39	47.27	0	>0.05	8.71	2.27	0.26	20.65	0	>0.05	4.24
Porphyromonas	0.64	0	13.38	0	>0.05	1.92	0.39	0	2.99	0	>0.05	0.74
Prevotella	4.89	2.60	26.62	0	>0.05	5.95	5.89	4.81	20.75	0	>0.05	5.36
Rothia	10.21	5.84	50.52	0	>0.05	11.87	9.33	8.12	29.61	0.26	>0.05	7.57
Scardovia	0.04	0	0.52	0	>0.05	0.10	0.75	0.13	6.10	0	>0.05	1.45
Selenomonas	1.04	0.13	9.22	0	>0.05	1.72	2.07	0.52	16.36	0	>0.05	3.42
Staphylococcus	0.02	0	0.26	0	>0.05	0.06	0.02	0	0.39	0	>0.05	0.08

Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Streptococcus	40.57	35.97	87.92	2.73	>0.05	26.26	36.00	33.83	80.78	7.27	>0.05	18.78
Tanerella	0.07	0	1.82	0	>0.05	0.25	0.05	0	0.65	0	>0.05	1.14
Veillonella	4.57	3.77	13.90	0	>0.05	3.51	4.78	3.25	25.58	0	>0.05	5.35

Appendix XXV: Pairwise comparisons of Bacterial Classes for Cleaning Frequency at the Mucosal Microbiome

Mucosal												
	Once						Twice					
Class	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinobacteria	19.80	15.19	51.30	0	>0.05	12.65	21.20	16.95	83.38	0.39	>0.05	17.50
Alphaproteobacteria	0.52	0.26	4.03	0	>0.05	0.95	0.68	0.26	5.84	0	>0.05	1.10
Bacilli	40.17	38.44	99.22	4.56	>0.05	21.15	48.08	55.19	94.94	3.12	>0.05	26.52
Bacteroidia	8.86	7.27	37.92	0	>0.05	8.86	5.26	3.90	27.27	0	>0.05	5.89
Betaproteobacteria	7.38	2.60	47.90	0	>0.05	11.69	5.62	3.12	65.97	0	>0.05	9.66
Clostridia	10.62	8.44	26.23	0	>0.05	7.95	8.17	6.30	35.84	0.13	>0.05	1.40
Coriobacteriia	0.75	0.26	3.90	0	>0.05	1.16	0.77	0.13	6.23	0	>0.05	7.70
Epsilonproteobacteria	0.28	0.13	1.30	0	>0.05	0.35	0.23	0.13	1.82	0	>0.05	0.35
Flavobacteriia	0.39	0	3.34	0	>0.05	0.76	0.43	0.13	5.46	0	>0.05	0.92
Fusobacteriia	1.49	0.91	6.50	0	>0.01	1.61	1.36	0.65	7.27	0	>0.01	1.82
Gammaproteobacteria	9.21	4.68	59.22	0.26	>0.05	12.24	7.68	4.87	30.65	0.26	>0.05	7.65
Other	0.03	0	0.13	0	>0.05	0.06	0.02	0	0.39	0	>0.05	0.08
Spirochaetes	0.13	0	2.60	0	>0.05	0.51	0.14	0	4.30	0	>0.05	0.59
Synergistia	0.12	0	1.17	0	>0.05	0.35	0.22	0	6.10	0	>0.05	0.93

Appendix XXVI: Pairwise comparisons of Bacterial Genera for Cleaning Frequency at the Mucosal Microbiome

Mucosal												
	Once						Twice					
Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinomyces	8.53	5.58	50.76	0	>0.05	10.69	7.97	3.25	72.99	0	>0.05	9.87
Atopbium	0.62	0.26	3.90	0	>0.05	1.05	0.51	0.13	3.51	0	>0.05	0.89
Bifidobacterium	0.76	0.26	5.71	0	>0.05	1.35	0.66	0.13	15.94	0	>0.05	2.20
Bradyrhizobium	0.33	0	3.51	0	>0.05	0.74	0.39	0.13	4.54	0	>0.05	0.83
Campnophaga	0.28	0.13	1.30	0	>0.05	0.35	0.23	0.13	1.82	0	>0.05	0.35
Campylobacter	0.86	0.13	8.31	0	>0.05	1.90	0.93	0.13	11.30	0	>0.05	2.03
Corynebacterium	0.86	0.1	8.31	0	>0.05	1.90	0.93	0.13	11.30	0	>0.05	2.03
Delftia	0.16	0	1.67	0	>0.05	0.33	0.08	0	0.91	0	>0.05	0.17
Dialister	0.10	0	1.56	0	>0.05	0.30	0.07	0	1.04	0	>0.05	0.20
Fusobacterium	0.32	0.13	1.56	0	>0.05	0.41	0.33	0.06	2.99	0	>0.05	0.57
Halomonas	1.11	0.26	13.90	0	>0.05	2.82	1.02	0.39	13.90	0	>0.05	2.12
Heamophilus	4.44	2.08	28.57	0	>0.05	6.19	5.99	2.86	26.49	0	>0.05	7.29
Jathingobacterium	1.06	0	17.40	0	>0.05	3.33	1.13	0	15.06	0	>0.05	2.70
Kingella	0.11	0	1.43	0	>0.05	0.28	0.23	0	6.23	0	>0.05	0.82
Lactobacillus	3.54	0.13	65.97	0	>0.05	12.67	3.33	0	88.57	0	>0.05	13.57
Leptotrichicia	1.13	0.78	6.49	0	>0.05	1.41	1.03	0.39	7.27	0	>0.05	1.59
Moryella	1.05	0.52	11.43	0	>0.05	2.23	0.60	0.13	8.96	0	>0.05	1.34
Nessieria	5.16	0.26	47.27	0	>0.05	11.51	2.61	0.39	21.17	0	>0.05	4.42
Porphyromonas	1.06	0	13.38	0	>0.05	2.63	0.32	0	3.12	0	>0.05	0.68
Prevotella	6.78	6.10	23.12	0	>0.05	6.15	4.58	2.79	26.62	0	>0.05	5.44
Rothia	10.14	6.62	50.52	0	>0.05	11.33	9.78	6.62	42.47	0	>0.05	10.14
Scardovia	0.06	0	0.52	0	>0.05	0.13	0.41	0	6.10	0	>0.05	1.13
Selenomonas	1.03	0.32	9.22	0	>0.05	2.15	1.42	0.26	16.36	0	>0.05	2.87

Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Staphylococcus	0.01	0	0.26	0	>0.05	0.05	0.02	0	0.39	0	>0.05	0.08
Streptococcus	32.61	31.43	87.92	2.86	>0.05	18.69	41.71	41.95	87.66	0	>0.05	23.37
Tanerella	0.04	0	0.65	0	>0.05	0.13	0.07	0	1.82	0	>0.05	0.25
Veillonella	6.21	4.81	25.28	0	>0.05	5.56	3.94	3.38	17.27	0	>0.05	3.32

Appendix XXVII: Pairwise comparisons of Bacterial Classes for Sleeping with or without a Denture *in situ* at the Mucosal Microbiome

Mucosal												
	Yes						No					
Class	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinobacteria	17.35	15.97	41.43	0	>0.05	10.62	22.02	16.23	83.38	1.04	>0.05	18.30
Alphaproteobacteria	0.73	0.26	5.84	0	>0.05	1.27	0.54	0.26	3.25	0	>0.05	0.79
Bacilli	45.88	43.77	99.22	4.55	>0.05	25.29	44.93	45.58	83.70	9.48	>0.05	25.41
Bacteroidia	8.96	6.95	37.92	0	<0.05	1.33	4.51	3.90	15.19	0	<0.05	0.71
Betaproteobacteria	4.30	2.60	16.23	0	>0.05	4.65	9.04	3.25	65.97	0.13	>0.05	14.91
Clostridia	10.67	7.39	35.84	0	<0.05	8.76	7.21	6.36	20.91	0.13	<0.05	5.73
Coriobacteriia	1.11	0.39	6.23	0	<0.05	1.63	0.45	0.13	3.51	0	<0.05	0.80
Epsilonproteobacteria	0.35	0.19	1.82	0	<0.05	0.44	0.15	0.13	0.91	0	<0.05	0.20
Flavobacteriia	0.36	0.13	3.64	0	>0.05	0.63	0.49	0.13	5.46	0	>0.05	1.14
Fusobacteriia	1.81	1.17	7.27	0	<0.01	1.82	1.07	0.26	6.88	0	<0.01	1.71
Gammaproteobacteria	7.78	5.58	30.65	0.26	>0.05	7.14	9.17	2.21	9.22	0.39	>0.05	11.28
Other	0.03	0	0.39	0	>0.05	0.08	0.02	0	0.39	0	>0.05	0.07
Spirochaetes	0.20	0	4.29	0	>0.05	0.78	0.09	0	1.30	0	>0.05	0.26
Synergistia	0.24	0	6.10	0	>0.05	0.98	0.18	0	3.25	0	>0.05	0.65

Appendix XXVIII: Pairwise comparisons of Bacterial Genera for Sleeping with or without a Denture *in situ* at the Mucosal Microbiome

Mucosal												
	Yes						No					
Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Actinomyces	8.21	5.58	50.91	0	>0.05	9.15	8.10	3.25	72.99	0	>0.05	13.80
Atopbium	0.71	0.26	3.90	0	>0.05	1.09	0.30	0	2.86	0	>0.05	0.59
Bifidobacterium	0.83	0.13	15.84	0	>0.05	2.37	0.49	0.13	5.71	0	>0.05	1.10
Campnophaga	0.32	0.13	3.51	0	>0.05	0.59	0.44	0	4.55	0	>0.05	1.04
Campylobacter	0.32	0.13	3.51	0	>0.05	0.59	0.46	0	4.55	0	>0.05	1.03
Corynebacterium	0.59	0.13	5.20	0	>0.05	1.14	1.39	0.26	11.30	0	>0.05	2.75
Delftia	0.11	0	0.91	0	>0.05	0.19	0.09	0	1.69	0	>0.05	0.29
Dialister	0.11	0	1.56	0	>0.05	0.30	0.06	0	1.04	0	>0.05	0.18
Fusobacterium	0.33	0.13	1.56	0	>0.05	0.42	0.32	0.13	2.99	0	>0.05	0.65
Halomonas	1.08	0.32	13.90	0	>0.05	2.37	0.97	0.52	13.90	0	>0.05	2.32
Heamophilus	5.24	2.27	26.49	0	>0.05	6.84	5.91	2.99	28.57	0	>0.05	7.23
Jathingobacterium	1.03	0	15.06	0	>0.05	2.79	1.23	0.13	17.40	0	>0.05	3.08
Kingella	0.12	0	1.43	0	>0.05	0.27	0.30	0	6.23	0	>0.05	1.05
Lactobacillus	3.44	0.13	88.57	0	>0.05	14.44	3.34	0	65.97	00	>0.05	11.40
Leptotrichicia	1.28	0.71	7.27	0	<0.05	1.60	0.75	0.13	6.49	0	<0.05	1.37
Moryella	0.98	0.39	11.43	0	>0.05	2.06	0.37	0	2.60	0	>0.05	0.66
Nessieria	2.26	0.32	13.64	0	>0.05	2.54	5.10	0.39	47.27	0	>0.05	10.73
Porphyromonas	0.69	0	13.38	0	>0.05	1.96	0.33	0	2.99	0	>0.05	0.72
Prevotella	6.19	4.22	26.62	0	>0.05	6.56	3.88	2.86	15.06	0	>0.05	3.90
Rothia	12.87	7.27	50.52	0.65	<0.05	12.57	6.69	5.65	28.70	0	<0.05	6.30
Scardovia	0.47	0	6.10	0	>0.05	1.30	0.17	0	1.82	0	>0.05	0.37
Selenomonas	1.81	0.45	16.36	0	>0.05	3.26	0.52	0.13	4.68	0	>0.05	0.89
Staphylococcus	0.02	0	0.39	0	>0.05	0.07	0.02	0	0.26	0	>0.05	0.07

Genus	Mean	Median	Max	Min	P value	SD	Mean	Median	Max	Min	P value	SD
Streptococcus	38.83	35.78	87.92	2.72	>0.05	23.25	38.97	32.08	82.34	3.12	>0.05	24.89
Tanerella	0.06	0	0.65	0	>0.05	0.13	0.08	0	1.82	0	>0.05	0.31
Veillonella	4.61	3.51	25.58	0	>0.05	4.36	4.69	3.77	17.27	0	>0.05	4.14

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