Denture induced stomatitis, patient and denture related factors

Gareth David Calvert BDS MFDS FDS RCPS (Glas)

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College of Medicine, Veterinary and Life Science
University of Glasgow

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Abstract

Purpose: Denture hygiene habits are highly variable amongst denture wearers, and can be frequently non-existent. Approximately 20% of the UK population wear some form of denture prosthesis. Almost half of these individuals show signs of denture induced stomatitis. There is currently a lack of evidence of how certain denture hygiene habits influence denture plaque composition, and whether this directly contributes towards oral inflammation and associated morbidity. Moreover, *Candida albicans* is primarily attributed as the causative agent due to its biofilm forming ability, thought to be influenced by the denture material, and in turn their effectiveness of decontamination.

Aims: To assess denture hygiene habits and risk factors with respect to host and microbial factors on the clinical presence of denture induced stomatitis. Also, to conduct *in vitro* analyses of biofilm formation of clinical denture isolates on various denture substrates and the antimicrobial activity of common denture cleansers.

Materials and methods: Data regarding participant demographics and denture hygiene habits were collected using a standardised questionnaire. Denture plaque samples were analysed from participants wearing either a complete or partial denture, and *Candida* spp. enumerated. Data on the bacterial microbiome composition of each participant was analysed in respect to denture hygiene habits. *C. albicans* isolated from dentures of healthy and diseased individuals was quantified using real-time polymerase chain reaction and biofilm biomass assessed using crystal violet. Biofilm development on the denture substratum polymethylmethacrylate, Molloplast B and Ufi-gel was determined. Early and mature biofilms were treated with popular over the counter denture hygiene products and assessed using metabolic and biomass stains.

Results: Clinical data suggests the presence of denture induced stomatitis was positively associated with a history of smoking, denture design, poor denture hygiene, and retention of dentures whilst sleeping. Although *C. albicans* was detected in greater quantities in diseased individuals, it was not significantly associated with denture induced stomatitis. Microbiome analysis indicated that poor denture hygiene did not reveal any significant changes in microbiome composition in comparison to satisfactory oral hygiene. Neither did frequency of denture cleaning or sleeping whilst wearing a denture *in situ* reveal significant changes in the denture plaque composition. Denture substrata were shown to influence biofilm biomass, with polymethylmethacrylate providing the most suitable environment for *C. albicans* to reside. Of all the denture hygiene products tested, Milton had the most effective antimicrobial activity on early biofilms, reducing biofilm biomass and viability the greatest.

Conclusions: This study has shown that denture hygiene practices appear to have minimal direct influence on the composition of the denture microbiome and clinical presence of denture induced stomatitis, reinforcing the idea that denture induced stomatitis is a multifactorial disease, influenced by host, microbial and environmental factors.

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Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other Institution.

Gareth Calvert August 2017

Abbreviations

AS - Artificial saliva

CD4+ - Cluster of differentiation

CFE - Colony forming equivalents

CFI – Colony forming inhibition

CFU – Colony forming units

CV – Crystal violet

ddH_2O – Deionised water

DNA - Deoxyribonucleic acid

DIS – Denture induced stomatitis

g – Gram

GPI - Glycosylphosphatidylinositol

h – Hour

HIV – Human immunodeficiency virus

HBF – High biofilm former

LBF – Low biofilm former

MIC – Minimum inhibitory concentration

Min – Minute

mL – Milli litre

OTU – Operational taxonomic units

PBS - Phosphate buffered saline

PMMA – Polymethylmethacrylate

qPCR - Quantitative polymerase chain reaction

RDP - Ribosomal Database Project

RNA - Ribonucleic acid

Rpm – revolutions per minute

Saps - Secreted aspartyl proteases

Sec - Second

SEM – Scanning electron micrograph

WHO - World Health Organisation

YPD - Yeast peptone dextrose

XTT - 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide

µl - micro litre

µM – micro mitre

Chapter 1: Introduction

1. Introduction

1.1 General Introduction

Population data demonstrate that the UK edentulous population has fallen from 28% in 1978 to 6% in 2009. A further 3% of those classified as dentate will have an opposing edentulous jaw (Fuller et al. 2011). This represents 28% (approximately 18 million) of the UK population who wear a denture. The number of individuals who are edentulous increases more or less exponentially with age (Fuller et al. 2011).

When replacing missing teeth there are many prosthesis designs to consider and have been described elsewhere (Hargreaves 1981). Since 1937 the most commonly used removable denture prosthesis material is polymethylmethacrylate (PMMA) due to its favourable properties (Johnson and Jones1994). There are of course alternative materials from which to construct removable dentures, their use determined by individual clinical situations.

A common condition associated with wearing dentures is denture-induced stomatitis (DIS). DIS presents commonly as an erythematous inflamed area of the mucosa directly adjacent to the denture fitting surface. One form of DIS is also known as erythematous candidosis. This has been linked to colonisation by candidal species amongst other causative factors (van Reenen 1973). Patient, denture, and environmental factors such as hygiene, dental appliances, smoking, and decreased saliva production can also increase the risk of *Candida* sp. infection (Akpan and Morgan, 2002). Overall, these infections lead to high rates of morbidity, with the potential for systemic spread and increasing mortality rates (Guest and Morris 1997).

1.2 Denture induced stomatitis

DIS is the most common form of oral candidosis. Other forms include pseudomembraneous candidosis, chronic hyperplastic candidosis and angular cheilitis (Webb et al. 1998). DIS affects those who wear partial or complete dentures, and those at extremes of age. It is a commonly held belief that such infections are predominantly caused by *Candida albicans,* however, high throughput sequence analysis reveals the potential involvement of a more complex microflora (Song et al. 2009). Microbiological studies indicate DIS is associated with a quantitative increase in candidal colonisation and biofilm formation (Jeganathan and Lin 1992, Ramage et al. 2004). *C. albicans* is present in the oral cavity of between 45-75% of the population, but only causes acute infection when the host immune balance is altered (Vanden Abbeele et al. 2008, Mayer et al. 2013).

1.2.1 Definition

The term DIS has evolved from numerous historical descriptions since the 1800s (Black 1885). In 1936 Cahn described “rubber sore mouth’, subsequent terminology included “chronic denture palititis”, “stomatitis venenata” and “sore mouth under plates” (Cahn 1936, Fisher 1956). The term denture induced stomatitis was then adopted and described as the chonic inflammation of the oral mucosa covered by a removable denture (Budtz-Jorgensen 1972, Wilson 1998).

1.2.2 Classification

DIS classification has been generally based on the clinical appearance of the inflamed mucosa. Newton (1962) described the clinical stages of DIS based on inflammation severity and this has been widely adopted:

0 Healthy

1 Pin-point hyperaemia

2 Diffuse hyperaemia

3 Granular

Modifications to Newton's classification included (Bergendal 1983):

0 Healthy

1A Petechiae in normal palatal tissue

1B Localised area of inflammation of the denture bearing area

2 Generalised area of inflammation of the denture bearing area

3 Hyperplastic palatal surface with inflammation of the denture bearing area

While others attempted to describe the inflammation according to size (Schwartz 1988):

0 Healthy

1 Inflammation of the palate extending up to 25% of the denture bearing area

2 Inflammation of the palate covering between 25% and 50% of the denture bearing area

3 Inflammation covering more than 50% of the palatal denture bearing area

For the purpose of this research we will refer to Newton's simplified classification of DIS (Figure 1.1)

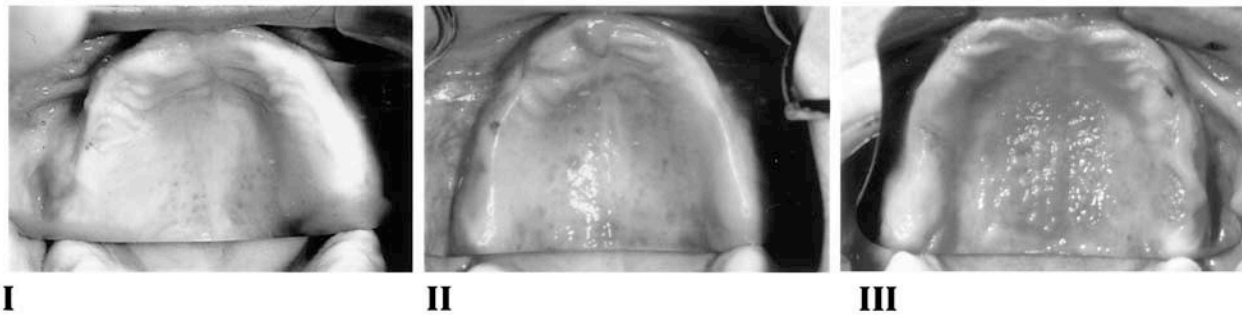

Figure 1.1 Newton's Type scale for the classification of inflammation present in DIS. I) Slight inflammation II) Diffuse inflammation III) Severe inflammation are shown in the figure (Barbeau et al. 2003)

1.2.3 Clinical relevance

In addition to the erythematous mucosa, patients may complain of discomfort, mucosal bleeding, swelling, a burning sensation, or a bad taste. However, most patients seem unaware of the problem (Budtz-Jorgensen 1974, Arendorf 1987, Webb 1998, Gendreau and Loewy 2011). Lack of salivary flow, xerostomia, has been reported in 14.7% of denture wearers and complaints include discomfort when eating, speaking and poor denture fit (Ikebe et al. 2005). Narhi and colleagues reported 41% of denture patients demonstrated xerostomia, which resulted in higher yeast counts than non-effected patients, though this ailment is probably causative and not a resultant of denture stomatitis due to lack of salivary flow to clear the *Candida* yeast (Narhi et al. 1993). Many *Candida* species can be regularly found on the palatal surface of dentures, which supports the theory that the upper denture functions as a reservoir for *Candida* (Kanli et al. 2005).

DIS can be associated with other oral lesions such as angular cheilitis and/or median rhomboid glossitis (Mullins 1962, Cawson 1965, Budtz-Jorgensen 1974, Russotto 1980, Arendorf 1987). A study involving terminally ill cancer patients, 90% who suffered from xerostomia, demonstrated that 65% had one or more of the mucosal irritations erythema (20%), median rhomboid glossitis (20%), atrophic glossitis (17%), angular cheiltis (11%) and pseudomembranous candidosis (9%) (Sweeney et al. 1998). Clearly, palliative care patients are an at-risk group for denture stomatitis due drug-related xerostomia, which leads to opportunistic yeast infections (Jobbins et al. 1992, Sweeney and Bagg 2000).

1.2.4 Prevalence

There are many figures quoted for the prevalence of DIS. This is likely because of the multifactorial aetiology of DIS and the impact of local and systemic co-morbidities in the populations examined. Studies suggest a range between 11-67% (Arendorf 1987) and 10-75% (Barbeau 2003) for healthy denture wearers.

In the United States, Shulman examined 3450 denture-wearing patients between the ages of 18-90 years and reported a prevalence of 27% (Shulman and Rivera-Hidalgo 2005). In Helsinki, 25% of 338 men and women living at home were found to have DIS (Nevalainen et al. 1997).

Investigators have tried to estimate the prevalence of DIS in older populations. Reichart examined 1367 Germans aged between 65-74 years old, concluding DIS was present in 18% (Reichart 2000). Chigstrom found 11% of 160 Swedish pensioners showed signs of DIS (Chigstrom 1970). A more recent study found 44% of pensioners displayed signs of DIS (Kulak-Ozkan, et al. 2002).

Some studies have targeted care homes across Europe to estimate the prevalence of DIS. Vigild found 28% of 64-85 year olds in care homes from Denmark had DIS (Vigild 1987). Peltola in Finland examined long term-hospitalised patients suggesting 25% exhibiting DIS (Peltola, et al. 2004). Finally, a Canadian study claimed one third of patients in a long term care for the elderly institution had DIS (Kuc 1999).

Research from University teaching and secondary care hospitals have reported the prevalence of DIS in their populations as 42% and 55% (Marchini L 2004, Dikbas et al. 2006). Based on these data it is difficult to put accurate figures on prevalence, but it is clear that DIS is a global problem.

1.2.5 Aetiology

Mucosal inflammation found adjacent to the fitting surface of the denture is multifactorial in nature. The associated factors can include: denture trauma, continuous denture wearing, poor denture hygiene, microbial plaque, denture base material, denture age, saliva, dietary factors, smoking, and systemic conditions

(Webb 1998, Ramage et al. 2004, Shulman et al. 2005, Emami 2008, Jainkittivong 2010, Salerno et al. 2011).

1.2.5.1 Patient related factors

From the available literature DIS would seem to be more prevalent in women (Nyquist 1952, Davenport 1970, Budtz-Jorgensen and Bertram 1972, Holbrook and Rodgers 1980, Nevalainen et al. 1997, Pires et al. 2002, Figueiral et al. 2007). No differences in gender were found in Peltola's study (Peltola, et al. 2004). A higher prevalence of DIS is seen in older people, likely due to long-term denture use, lack of dexterity in performing oral hygiene, polymedication, and decreased host immunity (Moskona and Kaplan 1998*)*. However, children and adults wearing acrylic partial dentures, obturator prostheses, and ortho-appliances can also be affected by DIS (Shimizu et al. 1987)

The majority of literature suggests the soft tissue of the hard palate is more commonly affected than the mandibular soft tissue (Nevalainen, et al. 1997, Jainkittivong et al. 2002). Shulman calculated an odds ratio of 6.2 and 5.2 when wearing complete maxillary and mandibular dentures respectively for the presence of DIS (Shulman et al. 2005). Furthermore, it has been shown oral mucosal lesions are more prevalent in complete denture wears than by partial dentures (Jainkittivong et al. 2002).

1.2.5.2 Medial conditions

The relationship between DIS and smoking status is not clear (Barbeau et al. 2003). Shulman suggests heavy smokers (>15 cigarettes/day) have an increased odds ratio (OR 1.31) of DIS (Shulman et al. 2005). While a number of other authors dispute this (Espinoza et al. 2003, Guggenheimer et al. 2000). Likewise, the association between diabetes and DIS is unclear. Individuals with a history of diabetes can include a wide range of disease severities which may affect the observed relationship with DIS. Schulman et al. found no association between DIS and diabetes history while Guggenheimer estimated an odds ratio of 1.52 with DIS when individuals glycosylated haemoglobin was more than 9% (Shulman et al. 2005, Guggenheimer et al. 2000)

Changes in the oral microbial flora due to immunological factors may predispose individuals to DIS (Iacopino and Wathen 1992). HIV supresses CD4+ lymphocytes and cells of the monocyte lineage resulting in a compromised cell mediated immunity (Perezous et al. 2005, Pereira-Cenci 2008). Individuals with HIV and wearing a denture have been shown to have increased prevalence of candidal carriage and oral candidiasis due to this increased susceptibility (Sweet et al. 1995).

Prescription medication, such as antibiotics, have the potential to inadvertently promote DIS development as they cause a change in the oral microbiome and allow other species such as *Candida* to flourish. However, because of the multifactorial aetiology of DIS this relationship is not always clear (Rossie and Guggenheimer 1997, Shulman et al. 2005). A further issue with drug prescribing is polypharmacy, especially in the elderly. This has the potential to effect both the quality and quantity of salivary flow, which can leave the oral cavity at risk to colonisation by opportunistic pathogens and thus DIS (Webb et al. 1998).

1.2.5.3 Saliva

Saliva has many functions, one of which is the protection of the oral mucosa, which is achieved in two ways. Firstly a physical cleaning effect, and secondly the presence of innate defensive molecules such as IgA, lysozyme, lactoferrin, calprotectin and histatin (Tanida et al. 2001, Dodds et al. 2005). The decrease or complete lack of saliva in xerostomia patients introduces an imbalance of the commensal microbiota and allows selective bacteria and yeast to proliferate (Narhi et al. 1993, Webb et al. 1998).

Candida must adhere to a suitable substrate and colonise, or risk being removed by saliva (Henriques et al. 2006). The protective role of saliva against *Candida* species on a plastic denture is controversial (Nikawa et al. 1992, Salerno et al. 2011). Saliva may have a role in *Candida* adhesion. For example components such as mucins, statherin, and proline rich proteins have been reported to absorb *C. albicans* facilitating its adherence to saliva coated denture materials (Salerno et al. 2011).

Several investigators have shown IgA to reduce the adherence of *Candida* to acrylic resin materials (Moura et al. 2006, Pereira-Cenci et al. 2007). Other studies have shown increased rates of candidal adhesion (Vasilas et al. 1992, Edgerton et al. 1993) or no effect at all (Nikawa et al. 1992, Jin et al. 2004, Tari et al. 2007). The variation in results may be due to the use of stimulated versus unstimulated saliva which will contain different proteins and hence may offer varied protection (Veerman et al. 1996). In addition, differing incubation periods, saliva temperature, and the morphological phase of C. *albicans* impact its adhesion over a 24 hour period (Elguezabalet al. 2004, Ramage et al. 2004).

The pH of the oral cavity has an effect on *Candida* adhesion. Lower pH values equal to 3 are optimal for the enzymatic activity of proteinases optimizing *Candida* adherence capacity (Webb 1998, Salerno et al. 2011). Such pH values can be maintained by *Candida* metabolizing carbohydrates present in saliva. The lower pH reduces the growth of some commensals facilitating the proliferation of *Candida* (Salerno et al. 2011).

1.2.5.4 Denture hygiene practice

Poor denture hygiene is accepted as a critical risk factor for the onset of DIS, such that good oral hygiene can alone be effective in its treatment (Jeganathan et al. 1992, Cross et al. 2004, Marchini 2004, Salerno et al. 2011). Several studies have shown a correlation between denture stomatitis and poor dental hygiene (Bergendal, 1983), including Butdz-Jørgensen et al who reported a denture stomatitis negative control group with 60% of patients with ‘excellent’ denture cleanliness compared to the 12% of patients positive for denture stomatitis (Budtz-Jorgensen et al 1970)

Pires and colleagues showed more than 80% of subjects with DIS had poor denture hygiene. Two thirds of the cases resolved with provision of new dentures and denture hygiene instruction (Pires et al. 2002). A similar study in nursing home patients produced similar improvement in DIS with denture hygiene (Webb et al. 2005). Only one study has shown no correlation with denture hygiene and DIS (Figueiral et al. 2007). Not removing the dentures at night while sleeping has also been associated with poor denture hygiene and an increased risk of developing DIS (Jeganathan and Lin 1992, Cross et al. 2004, Marchini et al. 2004, Figueiral et

al. 2007, Gendreau and Loewy 2011, Salerno et al. 2011). An anaerobic, low pH environment is likely to establish between the denture base and mucosa over extended periods of time promoting pathogenic yeast such as *Candida*.

Self-reported frequency of denture cleaning has been suggested to be irrelevant in the development and incidence of DIS (Nyquist 1953, Nikawa et al. 2000, Marchini et al. 2004). Schou et al. also demonstrated that the presence of denture plaque was not related to brushing habits. This is probably due to variation between cleaning techniques used as well as the duration and frequency (Schou et al. 1987). Also, patients' knowledge of denture hygiene practices has been shown to be inadequate compared to the recommended cleansing regime (Tarbet 1982, Milward et al. 2013, Mylonas et al. 2014). One study showed 60% of patients were shown to have less than ideal methods of cleaning (Dikbas et al. 2006, Johnson 2013, Milward et al. 2013). Two meta-analysis' have highlighted the lack of sound evidence base for the various recommended oral hygiene practices and the significant focus on systemic drug therapy rather than local measures (Emami et al. 2014 Hilgert et al. 2016). de Souza et al. are currently undertaking a randomised control trial in an attempt to address these issues, though the results of which are yet to be published (de Souza et al. 2017).

1.2.5.5 Denture base material

The type of material a denture is fabricated from and the greater its age are suggested to promote DIS (Budtz-Jorgensen 1970, Figueiral et al. 2007). Ramage and colleagues (2004) demonstrated the ability of *C. albicans* to adhere along imperfections and cracks on PMMA denture surfaces (Ramage et al. 2004). Materials with the roughest surface seem to exhibit the highest yeast counts, as penetration into an unpolished surface was greater than a polished surface (Radford et al. 1998, Webb 1998, Radford et al. 1999, Nevzatoglu et al. 2007, Pereira-Cenci et al. 2007). It has been suggested surface roughness values below 0.2 nanometres will not encourage microorganism adhesion (Quirynen et al. 1990). A review of the factors involved in microbial attachment and colonization to PMMA dentures confirmed that surface cracks and roughness facilitate attachment of microorganisms and biofilm development (von Fraunhofer and Loewy 2009).

Denture liners can be used in cases with thin, sharp, or irregularly formed alveolar ridges to prevent patient discomfort (Kawano et al. 1993). These come in a variety of materials including silicone, plasticized higher methacrylate polymers, hydrophilic polymethacylates or fluoropolymers. Denture liners can be added at the time of laboratory fabrication or subsequently at the chair side. Investigators have shown that chair-side addition soft denture liners have a higher surface roughness and the porous texture entraps yeast (Lefebvre et al. 2001, Pereira-Cenci et al. 2007, Tari et al. 2007).

Factors affecting the adhesion of *C. albicans* to soft denture lining materials include denture pellicle, hyphal invasion and specific interactions with the saliva coated denture liner (Nikawa et al. 2000). However, Moloplast B™, a laboratory added silicone liner has not shown increased growth of *Candida* species or DIS inflammation (Wright et al. 1985, Nikawa et al. 2000). Yoshijima and co-authors suggested that *C. albicans* adherence and colonization was decreased by the material hydrophobicity (Yoshijima et al. 2010). This finding has been challenged by Gedik and Ozkan, the authors tested Candidal adhesion on 4 different silicone reline materials including Moloplast B™ but found no significant difference. A proposed explanation was there was no significant difference in the surface roughness of the 4 silicone materials (Gedik and Ozkan 2009). A summary of the available literature suggested further in vitro and in vivo analysis of candidal adhesion to reline materials was required to conclude their impact on the clinical presentation of DIS (Radford et al. 2009). However it was noted the high rate of commercial turnover of dental materials can limit the clinical relevance of in vivo investigations.

1.2.5.6 Trauma

Nyquist hypothesised that denture trauma is the main aetiological factor of DIS, however, this was proven not to be the case (Nyquist 1953). The association between simple localised inflammation similar to Newton's class 1 and poor fitting dentures causing trauma was then described, but trauma alone would not lead to Newton class 2 and 3 (Cawson 1965, Budtz-Jorgensen 1970). Viglid suggested that patients who are wearing poorly fitting dentures are at a higher risk of developing DIS (Viglid 1987).

To examine this relationship further, Emami and co-workers employed a novel study design in patients with and without implant supported mandibular overdentures to examine the role of mucosal trauma. Dentures supported by two fixed implants will have increased support and retention, thus potentially reducing any trauma to the underlying mucosa. 173 completely edentulous patients were recruited, of which 97 received an implant supported mandibular overdenture, while 76 received a conventional mandibular denture. All patients had a new complete maxillary denture fabricated at the same time. After 1 year, patients with conventional mandibular dentures were significantly more likely to experience mild DIS of Newton's class 1 or 2 (OR: 4.5 95% CI: 2.2-9.1) than patients with implant supported mandibular dentures (Emami et al. 2008). This may indicate that the increased functional loading in this group contributed to the inflammation.

Immunohistochemical analysis has recently added support to a possible role of trauma. Trauma having been shown to cause variation in the expression of basement membrane antigens, potentially leaving the soft tissue susceptible to colonisation by microbes (Emami et al. 2008).

1.2.5.7 Denture plaque

In 1886 Black detected micro-organisms on the fitting surface of dentures in patients with DIS. This may be the first report indicating the microbiological relationship between denture plaque and DIS (Black 1886). Berkout (1923) classified the appearance of these organisms as that of *Candida* species (Berkhout 1923). In 1936 samples taken from the palatal mucosa of subjects with DIS suggested *C. albicans* was the causative factor (Cahn 1936). Later, Leathen et al investigated the microbial flora of dentures, again finding *C. albicans* (Leathen et al. 1960, Olsen 1975). Subsequently, the term denture plaque was introduced. Denture plaque is defined as a dense microbial layer comprised of micro-organisms and their metabolites, containing more than 10^{11} organisms per gram in wet weight (Budtz-Jorgensen et al. 1981). This complex microbial composition includes Gram positive bacteria such as *Streptococcus sanguis, S. gordonii, S. oralis, S. anginosus, Staphylococci* and rods (Salerno et al. 2011). Originally it was suggested that bacteria were the key participants in denture stomatitis (van Reenen 1973), but a quantitative relationship was later demonstrated between *Candida* spp. and severity of erythema (Tarbet, 1982).

Subsequent investigations have examined these findings in randomised studies to demonstrate that denture plaque is composed mainly of bacteria, though patients positive for denture stomatitis showed *C. albicans* cell components and decomposed serum (Budtz-Jorgensen et al. 1980). As *Candida* has a higher prevalence in DIS cases it has been suggested to be the predominant pathogen. Furthermore following antifungal treatment DIS improves. However recent literature emphasises the cooperative role played by bacteria, which must not be neglected (Baena-Monroy et al. 2005, Salerno et al. 2011).

1.2.6 Sampling of the oral cavity

Past studies have used a variety of techniques to obtain cultivable samples. Impression cultures, saliva cultures, oral rinse cultures, and palatal swabbing are the most common (Davenport 1970). Imprint cultures are taken by placing a sterile foam pad directly on the oral mucosa and left undisturbed for ~30 s. The pad is then removed and agar media is inoculated by inversion of the pad (Barbeau et al. 2003). The technique is advantageous for localisation of yeast culture in the oral cavity but does not harvest as many cells as oral rinses (Olsen 1975). The oral swab technique often results in mixed species cultures and may be of advantage when culturing specific lesions in the mouth (Zaremba et al. 2006). Collection from saliva has been promoted as a technique with >95% confidence in the detection of *C. albicans* (Epstein et al. 1980). Olsen and colleagues has recommended caution when depending on saliva collection as a method of quantifying *Candida* as the concentration of yeast in saliva can vary (Olsen and Stenderup 1990). Concentrated oral rinse sample collection has been recommended over other methodologies, though the yeasts isolated cannot be proven to directly relate to denture stomatitis (Samaranayake et al. 1986). Ease for the patient and adequate collection of yeasts makes the oral rinse a favoured methodology. For study of yeast biofilms in denture stomatitis, a novel collection technique by sonication of the dentures was used by Ramage et al. and used in the current study (Ramage et al. 2004).

1.3 *Candida's* role in denture stomatitis

Candida species are a common human commensal and generally do not cause harm to the host (Akpan and Morgan 2002, Ramage 2004). The yeast is commonly isolated from 30-40% of healthy individuals and usually found in the gastrointestinal and genitourinary tracts (Akpan and Morgan 2002). The incidence tends to increase when host defences are compromised or there is a shift in the normal microflora allowing the organism to flourish (Odds and Webster 1988, Akpan and Morgan 2002, Pappas et al. 2004).

The presence of a denture alters the conditions of the mouth by reducing access of saliva to the area between the denture acrylic and soft upper palate tissue, thereby minimising cleansing by physical forces in the oral cavity (Lamfon *et al.*, 2004). This means the denture acts as protective barrier for the growth and adhesion of *Candida spp*. (Fenlon et al. 1998). This aspect of *Candida spp.* colonisation is highlighted by the quantitative assessment of yeast cells with the saliva of dental patients. Higher numbers were found in denture-wearers than in dentate subjects (Parvinen et al. 1984). The significant association between DIS and yeast colonization was first described in 1970 and has been confirmed subsequently (Budtz-Jorgensen and Bertram 1970, Nanetti et al. 1993, Kulak-Ozkan et al. 2002, Dar-Odeh and Shehabi 2003, Baena-Monroy et al. 2005, Coco et al. 2008, Dagistan et al. 2009). Other common oral *Candida* spp. include *C. glabrata, C. parapsilosis* and *C. tropicalis*, but these species are found less commonly than *C. albicans* and historically have not been shown in a pathological role in DIS (Song et al. 2009, Gendreau and Loewy 2011). Interestingly, *C. glabrata* is emerging as the second most prevalent species isolated from dentures and palatal mucosa of patients with DIS. Furthermore there is also increasing evidence that more than one *Candida* species may simultaneously colonize the mucosa covered by dentures in both healthy and DIS patients (Snydman 2003). The worldwide increase in the use of immunosuppressive drugs is suggested to be one of the causes in increased *C. glabrata* infections (Pereira-Cenci et al. 2008). *C. glabrata* is innately resistant to the antifungal azole drugs, rapidly acquiring its resistance primarily by the over-expression of two ABC transporters, Cdr1p and Cdr2p or by the up-regulation of *ERG11* (Sanguinetti et al. 2005). This resistance has been put forward as a probable contributor to the clinical prevalence in countries where the use of fluconazole, a commonly-prescribed azole drug, is

high. Recent analysis that compared the overall prevalence of *C. glabrata* infections to the geographical distribution of fluconazole-resistant isolates, suggests that drug pressure may not be the only force responsible for the current prevalence of *C. glabrata* in bloodstream infections. The increased innate resistance of *C. glabrata* to azole antifungals is likely to be a contributing factor to the organism's clinical prevalence (Odds et al. 2007).

The role of *Candida* and predominantly *C. albicans* in the development of DIS is associated with pathogenic overgrowth on the denture surface and oral mucosa. Increased candidal load has been found to be related to the presentation of DIS (Webb 1998). How *C. albicans* achieves this is still controversial. No variant strains of this yeast have been identified as having unique virulence factors or pathogenic associations for DIS (Costa et al. 2008, Pinto et al. 2008).

Candida's virulence factors include morphological transitions between yeast and hyphal forms, expression of adhesins and invasins, thigmotropism, biofilm formation, phenotypic switching and hydrolytic enzyme secretion (Bilhan et al. 2009). DIS has been reported to be associated with the hyphal form of *C. albicans* when compared to denture wearers with no palatal inflammation. This has led to the hypothesis that the hyphal form has a greater capacity to invade host cells and denture surfaces (Budtz-Jorgensen 1975, Lo et al. 1997, Webb 1998, Bilhan et al. 2009).

1.3.2 *Candida* pathogenesis

C. albicans is a pleiomorphic fungus that can exist either as a commensal organism or opportunistic pathogen with the ability to cause a variety of infections, ranging from superficial to life threatening (Hobson 2003). Over the past several decades there has been intensive research effort to elucidate the molecular mechanisms involved in *C. albicans* pathogenesis. It would appear that the shift from unprovoked commensal to pathogen is triggered by a complex assortment of genetic virulence pathways (Sweet 1997). Genetic expression responsible for adhesion and colonisation (Calderone and Fonzi 2001), the development of hyphal cells (Saville et al. 2003), phenotypic switching (Soll 2002), extracellular proteolytic and lipolytic activity (Naglik et al. 2004) all play roles in pathogenicity.

1.3.3 Cell wall

Approximately 50% of the yeast cell wall weight of *C. albicans* is made up cross-linked glucans (Kapteyn et al. 2000), while the mannoproteins, whose adhesive properties are described below, constitute another 30-40% (Chaffin et al. 1998). Many cell wall proteins have membrane anchors characterized by glycosylphosphatidylinositol (GPI) on the C-terminus (Kapteyn et al. 2000). The cell wall proteins can also be glycosylated along the N- and C-termini. The N-glycans will often include branching chains of mannose that contain phosphodiester and glycosidic bonds. *C. albicans* can be classified into three serotypes, all of which respond differently to antifungal agents due to differences in mannan sugar used in cell wall composition (Masuoka 2004).

1.3.4 Adhesion

Adhesion using hyphae is termed active penetration and a fungal driven process. Though not fully understood, physical adhesion forces are believed to be crucial (Dalle et al. 2010, Wachtler et al. 2011). *C. albicans* also has a specialized series of proteins that mediate adherence to other *C. albicans* cells, other microorganisms, abiotic surfaces and host cells (Verstrepen and Klis 2006, Garcia et al. 2011). Such proteins found on the cell wall are called adhesins and bind to amino acid or sugar residues on other cell surfaces. For example the Als, EAP, and EPA glycoprotein families are involved in endothelial cell adhesion and fungal aggregation. Al proteins are expressed throughout *C. albicans* growth development, and as such it can be difficult to assign a function for each individual protein. Increased gene expression for ALS1-5 and 9 was shown in *C. albicans* from the oral cavity (Green et al. 2006), but variability of expression was noted. The Als family shows a wide generality exemplified by the overlap for peptide-binding specificity for Als1p and Als5p (Klotz et al. 2007). ALs1p, the most widely expressed Als family member, has been associated in the adhesion to human epithelial cells in the early stage of oropharyngeal candidosis (Kamai et al. 2002). A study by Rauceo and coworkers demonstrated that alterations in the threonine-domains of Als5p changed *C. albicans* ability to aggregate cells (Rauceo et al. 2006).

However the expression of these adhesins can be modified epigenetically and by environmental factors (Frieman and Cormack 2004). Using these specialized proteins to invade the host is called induced endocytosis and triggers engulfment of the fungal cell into the host cell (Mayer et al. 2013). The adhesion of *C. albicans* to denture base materials involves a similar complex series of virulence factor interactions and local factors (Serrano-Granger et al. 2005, Moura et al. 2006, Pereira-Cenci et al. 2007). An area of particular interest is surface free energy, defined as the interaction between forces of cohesion and adhesion and predicts if wetting occurs or not, implying the higher the surface free energy, the higher the adhesion of microorganisms (Klotz et al. 1985, Hazen 1989). Other factors should also be considered such as cell surface characteristics, diet, salivary composition, and antibody titres. The complex relationship between the different confounding factors may explain why many studies have failed to show a direct correlation with DIS and *Candida* adherence (Pereira-Cenci et al. 2007). Other factors will be discussed in the following sections.

1.3.5 Degradative enzymes

Candida sp. have evolved complex means of invading and entering host tissue following adhesion and colonization. Proteolytic enzyme production is expressed by several microbial pathogens and plays a key role in the pathogenicity of *Candida* sp. (Odds 1988). Degradative enzymes are primarily used to break down tissue for nutrition by the yeast, though the goal of a commensal organism is to co-exist with their host without causing unnecessary stress. Secreted aspartyl proteases (SAPs) are the most prolific degradative enzymes in *C. albicans* arsenal and the SAP gene family has been well characterised (Hube 1996).

Although not as destructive as the SAP family of enzymes, phospholipases also contribute to the virulence of *C. albicans*. These are ubiquitous enzymes that are involved in diverse processes such as membrane homeostasis, acquiring nutrients and the generation of bioactive molecules (Kohler et al. 2006). Phospholipases function by hydrolyzing the glycerophospholipids, commonly found in cell membranes and have been shown to aid invasion of mucosal epithelia of the host cell (Leidich et al. 1998).

1.3.6 Morphological variance

One of the most advantageous traits of *Candida*, and in particular *C. albicans*, is the ability to vary growth morphologies to fit an environment. Complex gene cascades have been deduced for *S. cerevisiase* to trace pseudohyphal growth (Pan and Heitman 1999) and similar research for *C. albicans* elucidated similar complex expression pathways (Sanchez-Martinez and Perez-Marti 2001). These variances in phenotypic growth allow the yeast to survive under duress and proliferate in a wide variety of conditions (Brown and Gow 1999).

1.3.7 *Candida* biofilms

Oral biofilms are highly structured communities of microorganisms that are either surface associated or attached to one another and enclosed within a self-produced protective extracellular matrix (Ramage et al. 2009). Immediately after cleaning, the denture surface will be coated in saliva pellicle and the first bacteria or early colonisers will begin to adhere. These are followed by a variety of other microorganisms if/when the conditions of the biofilm become amenable. The presence of a specific microorganism does not induce pathology, this depends on complex interactions with the host's inflammatory response.

A study comparing oral surfaces for biofilm growth noted that denture acrylic had a higher ratio of hyphal to yeast cells than did tooth enamel for the early phase of development, though there were fewer hyphal cells than yeast cells once matured (Lamfon et al. 2004). The growth surface was again reported as an important factor for biofilm development when several materials used for indwelling devices were shown to support varying levels of *Candida* yeast adherence and biofilm growth (Hawser and Douglas 1994), while it has also been reported that adherence of yeast cells can be promoted when a growth surface is coated with a glycoproteinaceous film (Cannon and Chaffin 1999).

C. albicans biofilm formation could be separated into three distinct developmental phases (Figure 1.2). These are:

Early phase, In the first two hours of formation, *C. albicans* were present as yeast forms (blastospores) which adhere to the growth surface, e.g.

polymethylmethacrylate or ‘denture material’. Chandra et al (2001) noticed that microcolonies appeared in the third and fourth hour and *C. albicans* communities materialized as thick lines of fungal growth after the eleventh hour (Chandra et al. 2001).

Intermediate phase, budding yeast cells transform to hyphal cells. Matrix mineral is produced due to gene expression and subsequent production of enzymes involved with carbohydrate synthesis. A monolayer structure is achieved between 12-30 h after colonisation.

Maturation phase, further growth and production of extra-polymeric matrix achieves a thee-dimensional structure containing pseudohyphae and hyphal forms. Microcolonies of yeast form the basal layer with networks of hyphal cells lining the top of the biofilm (Figure 1.3). Together a resilient biofilm structure forms after 31-72 h (Chandra et al. 2001, Ramage et al. 2001, Lamfon et al. 2004, Kruppa 2009).

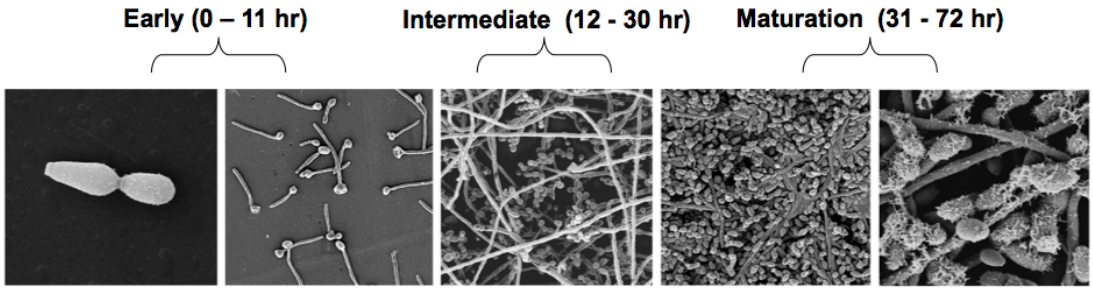

Figure 1.2: Stages of biofilm progression. Early stage occurs between 0 and 11 hours and results in initial hyphae formation. Production of matrix material in intermediate stage (12-30 hours) ensures that the biofilm gains structure and resilience. The final stage of maturation at 31-72 hours results in a resilient three dimensional structure (Ramage et al, 2006).

C. albicans grown as a biofilm has been shown to be 100-fold more resistant to fluconazole and 30-fold more resistant to amphotericin B than planktonically grown strains (Ramage et al. 2001, Chandra et al. 2001). *Candida* species are also capable of colonising other artificial surfaces and indwelling devices and can account for a considerable proportion of systemic infections (Potera 1999).

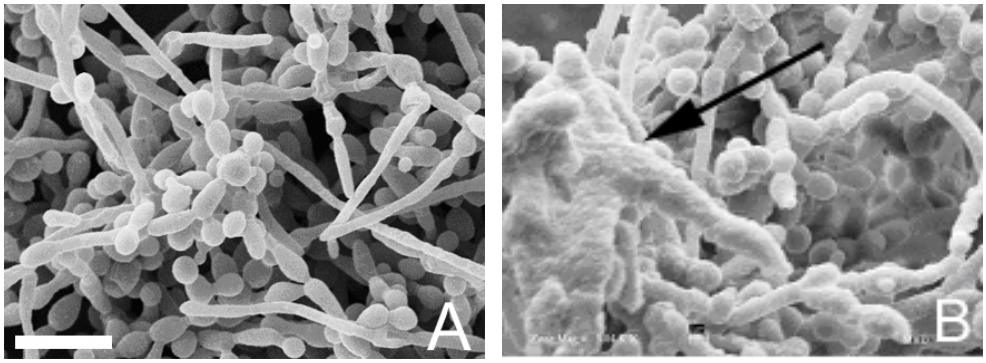

Figure 1.3 – *C. albicans* biofilm with EPS from static and continuous flow models (A) *C. albicans* biofilm grown under static conditions. (B) *C. albicans* biofilm grown in a continuous media flow model. Arrow indicates increase of EPS resulting in a cloudy film over the biofilm. 250 x magnification (Ramage et al. 2006)

1.3.8 Microbiome and interkingdom considerations

Historical data identified oral microbes by culture techniques which by todays standards have a number of short comings, the most notable a low sensitivity. Therefore, this technique is unlikely to provide a comprehensive representation of the polymicrobial population (O'Donnell et. al 2015). Denture related infections have historically been studied in the context of yeast infections, as indicated from the aforementioned focus on *Candida* spp. So to better understand the microbiome of denture wears, contemporary investigations have used PCR techniques and next generation sequencing analysis (Duyck et al 2016). Eren et al. highlighted the importance of resolving communities at more detailed taxonomic levels to better understand the ecological and functional diversity of the microbiota relevant to health and disease (Eren et al. 2014). This level of analysis of the denture microbiome is now receiving more notoriety. A recent predominantly class and phylum level study introduced modern sequencing approaches to the field (O'Donnell et al. 2015). A recent comprehensive genus and species level analysis found no significant difference between the bacteria residing in biofilms on dentures and remaining teeth of healthy patients and those with DIS, and the microbiota in disease and health (Shi et al. 2016). *Actinomyces*, *Capnocytophaga*, *Streptococcus*, *Veillonella*, and *Neisseria* were the most prevalent and abundant genera, independently of health or disease status (Shi et al. 2016). Of the *Streptococcus* species, *S. mitis, S. oralis* and *S. mutans* have been identified as the most abundant in denture plaque (Teles *et al*. 2012).

Microorganisms in the oral cavity interact with each other in many ways for their mutual benefit. For example, communicating directly though signalling molecules or using each other's metabolic end products to create a favourable environment to proliferate (Wright et al 2013). In addition to various forms of metabolic dependence, microorganisms may co-aggregate with two or more genetically distinct strains. This has been observed between *C*. *albicans* and several other microorganisms such as oral streptococci (Jenkenson et al 1990). In the oral cavity streptococci provide yeast with compounds that activate their virulence factors. For example, hyphal formation can be modulated by Gram-negative bacteria though quorum sensing, where secreted molecules triggers a genetic response in a polymicrobial community (Morales and Hogan 2010). Likewise, Hornby and coworkers were able to identify a molecule called farnesol which prevented *C. albicans* yeast to hyphal transition in a density dependent manner (Hornby *et al.*, 2001). *Candida* colonization has also been shown to be positively correlated with *lactobacilli* and negatively correlated with *Fusobacteria* (Shi et al. 2016). Though previous class level analysis indicated that *Candida* was not exclusive to DIS, and found in healthy participants, all be it in fewer numbers (Duyck et al. 2016, Shi et al. 2016). The data would therefore suggest one specific organism is unlikely to be responsible for disease or health, and this relationship can be even more complex in the situation of partial dentures with remaining teeth. Clinically, the implications of interkingdom biofilms poses a problem as it has been shown that *C. albicans* and *S. aureus* collectively enhance one another's resistance profiles to antibacterial (vancomycin) and antifungal (miconazole) (Kong al. 2016).

1.4 Chemotherapeutic approaches

The term denture hygiene covers a multitude of cleaning techniques for denture wearers. These range from simply cleaning with a tooth brush to utilising expensive ultrasonic equipment, or prescribed medicaments. Each of which have their own disadvantages and clinical contraindications. Therefore there is little universal consensus regarding the effectiveness of the these individual methods or in conjunction with one another, nor if they are cost effective for patients to invest in.

1.4.1 Mechanical disinfection

Mechanical cleaning of dentures with brushes or ultrasonic devices has limited success on complete removal of the biofilm and can damage the denture surface (Jagger and Harrison 1995, Nikawa et al. 1998). Brushing can increase surface roughness on the dentures (Oliveira et al. 2007), which aids attachment and colonisation of *Candida* sp. Biofilms, which form in any cracks or imperfections of the denture bases, are difficult to remove by brushing (Ramage et al. 2002). Microwave irradiation is an excellent alternative which damages *Candida* cell membranes (Campanha et al. 2007, Polyzois et al. 1995). However, there can be problems with shrinkage of denture-lining (Dixon et al. 1999, Seo et al. 2007).

1.4.2 Denture cleansers

Commercially available denture cleansers may be used to supplement good oral and denture hygiene to prevent colonisation and DIS. 76% of dentists recommend denture cleansers as a method of denture decontamination (Milward et al. 2013). Denture cleansers can be classified as alkaline hydrochlorites, alkaline peroxides, neutral peroxides with or without enzymes (Gajwani-Jain et al. 2015). They are usually in the form of a tablet that is dissolved in a specified volume of water and the denture is submerged for a varying length of time. Denture immersion alone has been shown to be ineffective against a biofilm and hence disrupting the biofilm by brushing the denture surface with a toothbrush is recommended. Unfortunately, this in turn can damage the denture surface potentially leading to increased *Candida* adherence (Ramage et al. 2004, Jose et al. 2010). 0.2% chlorhexidine gluconate has been reported to exhibit high inhibitory effects on *Candida* spp. as well as other oral microbes (Akpan and Morgan 2002). In contrast, a 2% solution is effective for overnight decontamination of dentures (Ellepola and Samaranayake 2000). Chlorhexidine is not only toxic to *Candida* spp., but also negatively influences candidal adhesion to surfaces, this property is particularly useful for the treatment of dentures (Ellepola and Samaranayake 2000). This is due to the chlorhexidine property of binding to proteins from the yeast cytoplasm which in turn results in disintegration of the cytoplasm and cell death. The practice of soaking dentures in sodium hypochlorite (a key compound in bleach) is recommended by some dentists. Similar to chlorhexidine, *Candida* spp. anti-

adhesive effects have also been demonstrated by soaking infected dentures in sodium hypochlorite (Webb et al. 1995).

1.4.3 Systemic drug therapy

Current therapy for advanced denture stomatitis consists of topically administered polyene antifungal agents or by systemic application of azole drugs (Ellepola and Samaranayake 2000). A major difficulty in treating *Candida* infections is the intrinsic antifungal resistance often associated with biofilm formation (Ramage et al. 2001). There are reports of resistance to particular types of antifungals including chlorhexidine, amphotericin B, and fluconazole (Chandra et al. 2001). Furthermore the efficacy of these interventions can be hindered by individual factors such as saliva dilution and failure to finish the prescription, which can in itself contribute to the development of antifungal resistance (Ellepola and Samaranayake 2000).

Nystatin, a polyene drug, is historically the most common topical treatment for denture stomatitis due to its efficacy and ease of application (Blomgren et al. 1998). Fluconazole, a drug distributed through gastrointestinal absorption, is favored for prophylaxis and low-level fungal infections based on its safety record but incidences of resistance by *C. albicans* and innate immunity from non-*albicans* species limits fluconazole's efficacy (Kullberg et al. 2005). Amphotericin B, a polyene, is also systemically distributed but is associated with problems with high toxicity (Walsh et al. 1998). For severe and deep tissue infections, a common clinical approach is to treat initially with a short course of amphotericin B followed by fluconazole once the *Candida* strain is known to be susceptible (Kullberg et al. 2005). Other members of the azole family, such as itraconazole and voriconazole, are also available though each particular compound has varied success against *Candida* infections. Echinocandins are a recently introduced class of antifungals that target a different component of the yeast cell (Karlowsky et al. 2006). Antifungal drugs target both components and enzymatic processes pertaining to the cell membrane and cell wall. However there are newer antifungals such as the echinocandins and liposomal formulations of amphotericin B which display increased activity against *Candida* biofilms (Ramage et al. 2004).

In many cases, antifungal penetration into the yeast biofilm can be difficult and leads to increased MICs. The biofilm matrix, consists of proteins, carbohydrates and other unknown components (Al-Fattani and Douglas 2006). It covers the biofilm and so it is thought it acts like a protective barrier by preventing penetration of antifungals, possibly by binding to them. The level of drug resistance does not appear to change with the varying levels of matrix produced (Douglas 2003). Another factor in the resistance of biofilms is the levels of ergosterol as it is the main target of most antifungal drugs. It appears that there is less ergosterol present in the later stages of biofilm formation and so the drugs have a smaller target, which does not affect the overall growth of the biofilm (Mukherjee et al. 2003). It has been hypothesized that changes in the ergosterol levels in the membrane could alter the permeability to drugs, therefore leading to a more resistant strain (Mukherjee et al. 2003).

1.5 Rationale & Hypothesis

Denture induced stomatitis has a complex multi-factorial aetiology. Because of ambiguity regarding the patient-denture-biofilm interactions, the risk factors for developing the inflammatory lesion are not a linear problem. It is hypothesised that both patient and denture related factors cumulatively influence the clinical presentation and microbial composition in patients with DIS.

This study will therefore prospectively investigate the patient and denture related factors of the clinical presentation of DIS in a Scottish subpopulation. Furthermore, in conjunction with a larger ongoing research project, this thesis will investigate how patient behavioural habits might effect the denture microbiome composition, and the roles played by different denture substrates in microbial adhesion.

Chapter 2: Patient and denture related factors of DIS

2.1 Introduction

Approximately 18 million people in the UK wear a denture (Fuller et al, 2011). A common condition associated with wearing dentures is DIS. It has been estimated that the presence of DIS can vary between 10-75% (Barbeau J 2003).

Mixed evidence exists whether patient related factors such as gender, age, and smoking status are associated with the presence of DIS in individuals wearing dentures (Figueiral et al. 2007, Guggenheimer et al. 2000, Peltola, et al. 2004, Shulman 2005). However, from a clinical stand-point most of these factors are out with the scope of a clinician's influence, but are informative as to identify at risk groups of patients for preventative advice/surveillance.

Denture related design factors also implicated in the development of DIS are denture age, construction material, and the trauma it may cause to the underlying soft tissue (Nyquist 1953, Pereira-Cenci et al. 2007, Tari et al. 2007). It is not unreasonable to hypothesise that due to function and degradation an older denture would acculuate more plaque, become less well fitting and traumatise the underlying tissues more than a newer denture. Furthermore, there is variation in surface roughness and rate of degredation of different denture fabricatoin materials that may influence plaque acculuation (Budtz-Jorgensen 1970, Lefebvre et al. 2001, Yoshijima et al. 2010). Clinically, this has prompted the dental field to consider replacing old ill fitting denutre as a way to resolve DIS.

Denture habits associated with DIS include continuous denture wearing, infrequent denture cleaning, and plaque accumulation. (Webb 1998, Ramage 2004, Shulman and Rivera-Hidalgo 2005, Emami et al. 2008, Jainkittivong 2010, Salerno et al. 2011). Despite the recommendations to remove denture at night while sleeping a significant number of edentulous patients still leave them *in situ*. (Jeganathan and Lin 1992, Cross, et al. 2004, Marchini et al. 2004, Figueiral et al. 2007, Gendreau and Loewy 2011, Salerno et al. 2011). Though validated guidelines for nocturnal storage conditions are missing (Duyck et al. 2016).

Commercially available denture cleansers may be used to supplement good oral and denture hygiene to prevent colonisation and DIS. Though, 60% of denture wearers were shown to have less than ideal methods of cleaning (Dikbas et al.

2006, Johnson 2013, Milward et al. 2013). A recent meta-analysis of randomised controlled trials attempted to evaluate various DIS antifungal treatment strategies (Emani et al. 2014). They did not detect statistically significant differences in both clinical and microbiological outcomes between antifungal treatments and different disinfection methods (Emani et al. 2014). Thus suggesting less invasive local methods of cleaning could be as effective as antifungal therapies to resolve DIS. However mechanical cleaning of a denture could simply be the use of a toothbrush or more involved cleaning with an ultrasonic. An ultrasonic could be of particular use for those patients that struggle with their manual dexterity to physically clean a denture. Duyck et al. (2016) suggested additional benefits of ultrasonically cleaning dentures when compared to soaking over night and/or tooth brushing alone.

While we have a tacit understanding of key factors driving mucosal health of a denture wearer, some of these recommendations are not supported by robust evidence. It is therefore important to continue building the evidence through site specific studies, which may either support existing theories or indeed introduce new ways of thinking about denture care.

2.2 Aims

It is hypothesised that denture hygiene habits influence oral health and disease, so this chapter will examine patient related factors in a Scottish cohort associated with the clinical presentation of DIS to determine whether any specific factors are of clinical importance.

2.3 Methods

2.3.1 Recruitment

A cross-sectional study designed was developed to investigate patient and biological factors associated with denture-related stomatitis. Ethical approval was sought and approved by the West of Scotland Research Ethics Service (12/WS/0121), which was performed by Dr Douglas Robertson and Prof Gordon Ramage. Patients who wore a removable denture were identified by receiving clinicians at routine appointments in Oral Surgery, Oral Medicine, and Restorative dentistry departments at Glasgow Dental Hospital & School. Convenience sampling was used based on the patient availability on recruitment days. Patients were recruited by a designated research nurse or a PhD student (Lindsay O'Donnell), who explained the premise of the study and what was required from the patient. An information sheet was provided to each patient and written consent was gained for inclusion in the study. The inclusion criteria were that patients (>18 years) were wearing either a partial or complete denture, and the exclusion criteria included the following: pregnancy, breast feeding, current steroid or antibiotic use (<6 weeks), current immunosuppressant therapy (<6 weeks), previous radiotherapy for the treatment of head and neck malignancy, patients who are unable to give consent due to incapacity or those who do not speak English and no interpreter was present, and patients who are involved in current research or have recently been involved in any research prior to recruitment. Patient confidentiality was protected by assigning a unique patient code to each of the participant's data set. Access to the clinical data recorded was limited to only those directly involved in the study.

2.3.2 Descriptive data

The receiving qualified dentist recorded demographic data including age, gender, oral and denture hygiene regimens, and a medical history including recent antimicrobial or antifungal medication.

2.3.3 Clinical examination

Clinical assessments were carried out by six experienced dentists working in the prosthodontic department of the University of Glasgow Dental Hospital and School. All prosthodontists received personal training from Douglas Robertson (senior clinical lecturer in restorative dentistry and principle investigator) in order to standardise the assessment of the dentures retention, stability, occlusion and cleanliness. The experienced prosthodontists were asked to make a clinical judgement about whether the dentures were a good fit and if not whether the retention, stability or occlusion was unacceptable. Oral hygiene was graded after training and discussion as *good*: with little or no signs of denture or dental plaque visibly present, and *poor*: generalised or gross denture or denture plaque evident. Newton's classification method for DS was used to score the appearance of the patient's palatal mucosa (Newton, 1962), with all six examiners trained in the use of this DS classification based on photograph examples of each condition available at the time of examination. The following scores were applied; 0= healthy mucosa, 1= pinpoint hyperaemic lesions (localized erythema), 2= diffuse erythema (generalized simple inflammation), and 3= hyperplastic granular surface (inflammatory papillary hyperplasia). All examiners were trained but no formal calibration calculations were carried out. The patient demographic and clinical examination data was recorded on a standardised data collection sheet. There was no age related exclusion criteria for this study. Patients were excluded from the study if they were pregnant, had previous radiotherapy for the treatment of head and neck malignancy, had periodontitis, had been receiving antimicrobial/antifungal treatment, using prescription mouthwashes or had received immunosuppressant therapy within six months previous to sampling. The standardised data collection sheet can be seen in appendix 1. Dr Lindsay O'Donnell collected and collated the majority of this information between Feb 2013 and Oct 2014.

2.3.7 Statistical Analysis

For the purposes of statistical analysis, inflammation grades 2 and 3 were grouped together (n=24). Therefore the sample is similar in size to the grade 1 inflammation group (n=25). Clinically there is little difference between grade 2 and 3 inflammation as both are well established chronic lesions.

Patient demographic and inflammation grade data was transferred to SPSS ver. 22 (SPSS® Inc Chicago, Illinois, USA) to perform statistical analysis. Pie charts were produced from Microsoft Excel 2016 (Microsoft Office 365). Odds ratios were calculated. A p value of less than 0.01 was considered significant due to multiple testing to reduce the potential of a chance finding.

Participant and denture ages were not normally distributed, therefore a Kruskal Wallis test was used. T-tests were used to test for differences between health and DIS with denture related habits.

2.4 Results

2.4.1 Participant demographics

Over a 12-month basis 131 participants were recruited to the study. The prevalence of DIS was 37.4%. 82 participants had no signs of DIS and were termed healthy, with 49 experiencing signs of DIS (Table 2.1). The sample (n=131) included 46 males (35.1%) and 85 females (64.8%). The mean participant age was 70.2 ± 11.5 years (median 72 years). The mean ages of the healthy and DIS categories can be seen in table 2.1. The mean denture age for the entire sample was 4.5 ± 5.1 years old, with a range of 0.25 to 40 years. The mean complete denture age was 4.5 ± 3.9 years and 4.7 ± 7.4 for partial dentures. Twenty-four (18.3%) of the participants were smokers.

Table 2.1: Demographic data of the study participants separated into healthy and denture induced stomatitis groups (n=131)

		Denture induced stomatitis		
	Healthy	Grade 1*	Grade 2** & 3***	Total
N (%)	82 (62.6%)	25 (19.2%)	24 (18.3%)	49 (37.4%)
Male	26 (56.5%)	11 (23.9%)	9 (19.6%)	20 (43.5%)
Female	56 (65.9%)	14 (16.5%)	15(17.6%)	29 (34.1%)
Mean Age	71.6 ± 10.4	69.0 ± 14.6	67.5 ± 11.1	68.2 ± 11.9
Current smoker	11 (8.3%)	5 (3.8%)	8 (6.1%)	13 (9.9%)
Mean denture age	4.5 ± 5.5	4.4 ± 4.2	4.6 ± 4.9	4.5 ± 4.1
Complete dentures	61 (46.5%)	16 (12.2%)	12 (9.2%)	28 (21.3%)
Partial dentures	21 (16.0%)	9 (6.9%)	12 (9.2%)	21 (16.0%)

* = Newton's Pin-point hyperaemia. ** = Newton's Diffuse hyperaemia. *** = Newton's Granular hyperaemia

2.4.2 Relationship between participant factors and denture induced stomatitis

2.4.2.1 Participant age and denture induced stomatitis

The median age for the sample (n=131) was 72 (IQR 64 – 77) years old, ranging between 33 and 95 years old, with a mode of 76 years of age. The median participant ages for healthy, grade 1 and grade 2 & 3 inflammation groups were 72, 71, and 68, respectively. Figure 2.1 shows the trend between participant age and healthy/inflammation groups. Given that participant ages were not normally distributed, a Kruskal-Wallis test was performed that confirmed there is no statistical difference between these groups (p=0.19). This suggests age has no influence on the presence of DIS.

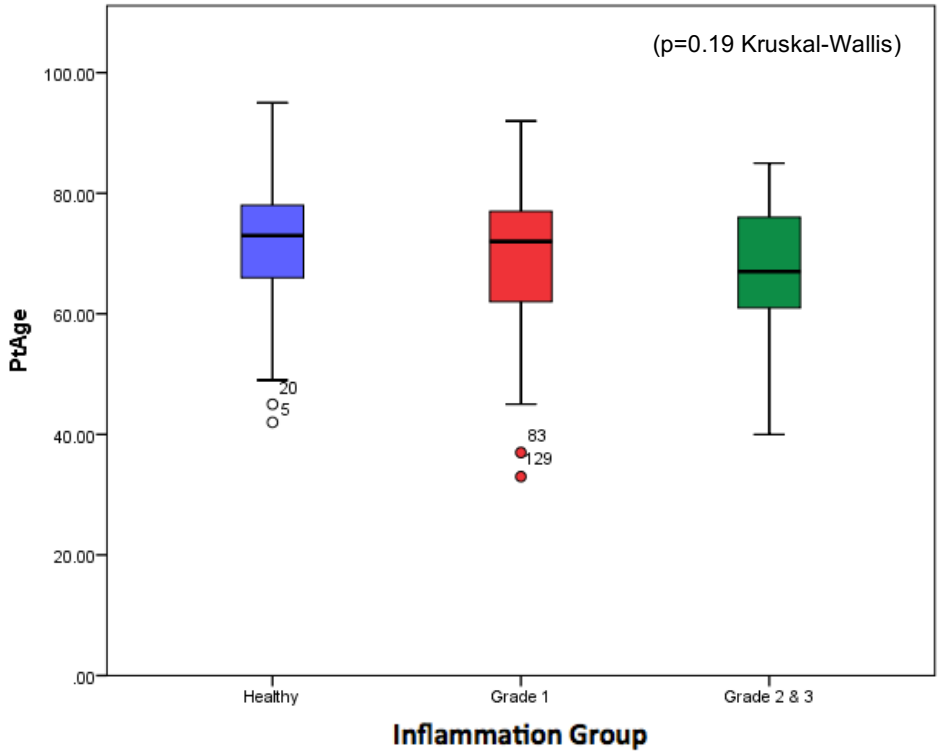

Figure 2.1: Median and range of participant age for each healthy and inflammation groups of the study participants. The samples (n=131) were categorised into different Newton's types (Healthy, Grade 1, 2 & 3) by age and data analysed non-parametrically using a Kruskal-Wallis test (p= 0.19).

2.4.2.2 Gender and denture induced stomatitis

There were consistently more females present across healthy and inflamed groups. The odds ratio of female to males between healthy and inflammation groups was 0.67, though this was not significant (p=0.29). This suggests no association between gender and DIS. It should be noted that the majority of each of the 3 groups were female.

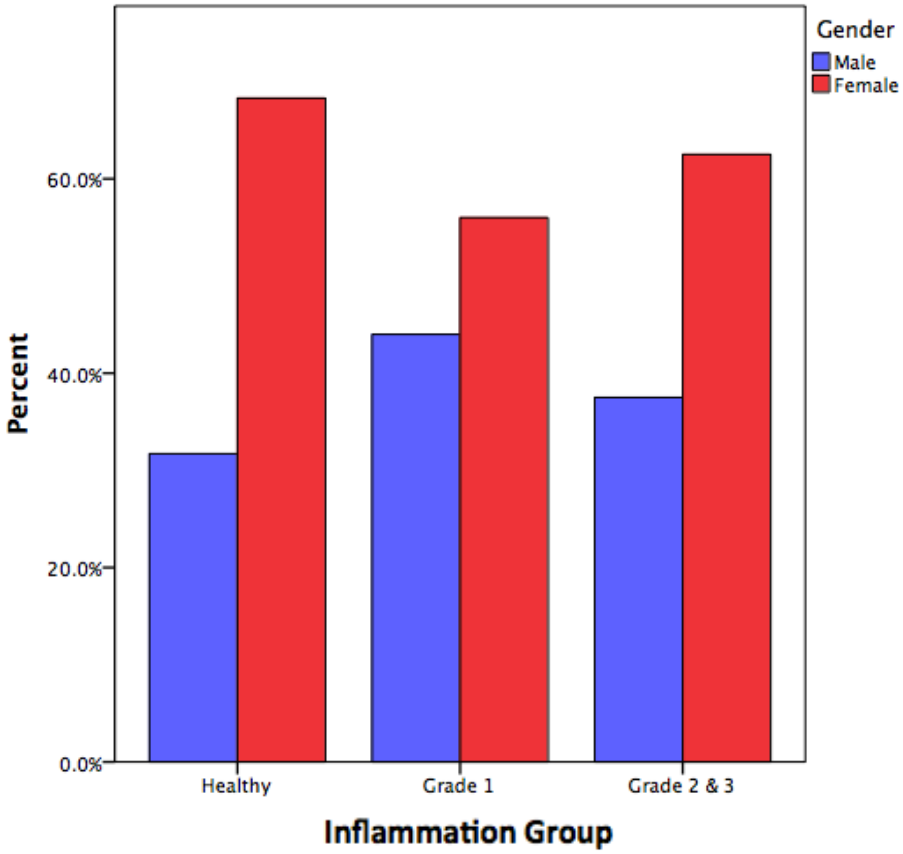

Figure 2.2: Gender percentage distribution for the healthy and inflammation groups of the study participants. The samples (n=131) were categorised into different Newton's types (Healthy, Grade 1, 2 & 3) by gender. More females were present in each group, data was analysed using an odds ratio calculation (OR 0.67 and p=0.29).

2.4.2.3 Smoking status and denture induced stomatitis

There were 11 (13.4%) current smokers in the healthy group. In the grade 1 and 2 & 3 inflammation groups there were 5 (20.0%) and 8 (33.3%) current smokers, respectively. A significant trend was observed, current smokers were associated with DIS (p=0.002). The odds ratio of smoking and experiencing DIS was 3.7.

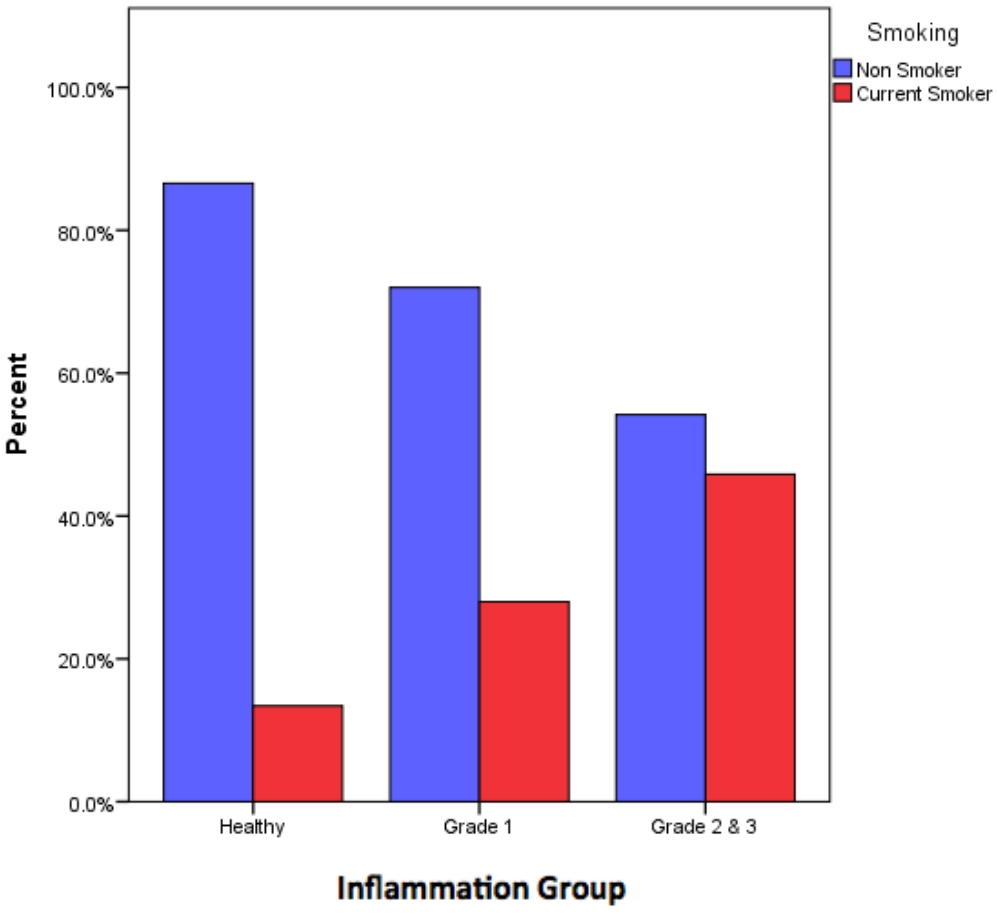

Figure 2.3: Percentage of current smokers distribution for the healthy and inflammation groups of the study participants. The samples (n=131) were categorised into different Newton's types (Healthy, Grade 1, 2 & 3) by smoking status (current or non-smoking). The proportion of current smokers increases with the level of inflammation. Data was analysed using an odds ratio calculation (OR 3.7 and p=0.002).

2.4.3 Relationship between denture related factors and denture induced stomatitis

2.4.3.1 Denture design and denture induced stomatitis

Eighty-nine (67.9%) participants wore complete dentures; 55 both complete upper and lower, 31 complete upper, and 3 complete lower. Eighty-eight (99%) were constructed with PMMA, one was constructed with cobalt chromium metal. Forty-two (32.1%) participants wore partial dentures; 7 partial upper and lower, 33 partial upper, and 2 partial lower. 26 (61.9%) were constructed with PMMA, 15 cobalt chromium metal and one made of a non-PMMA based acrylic.

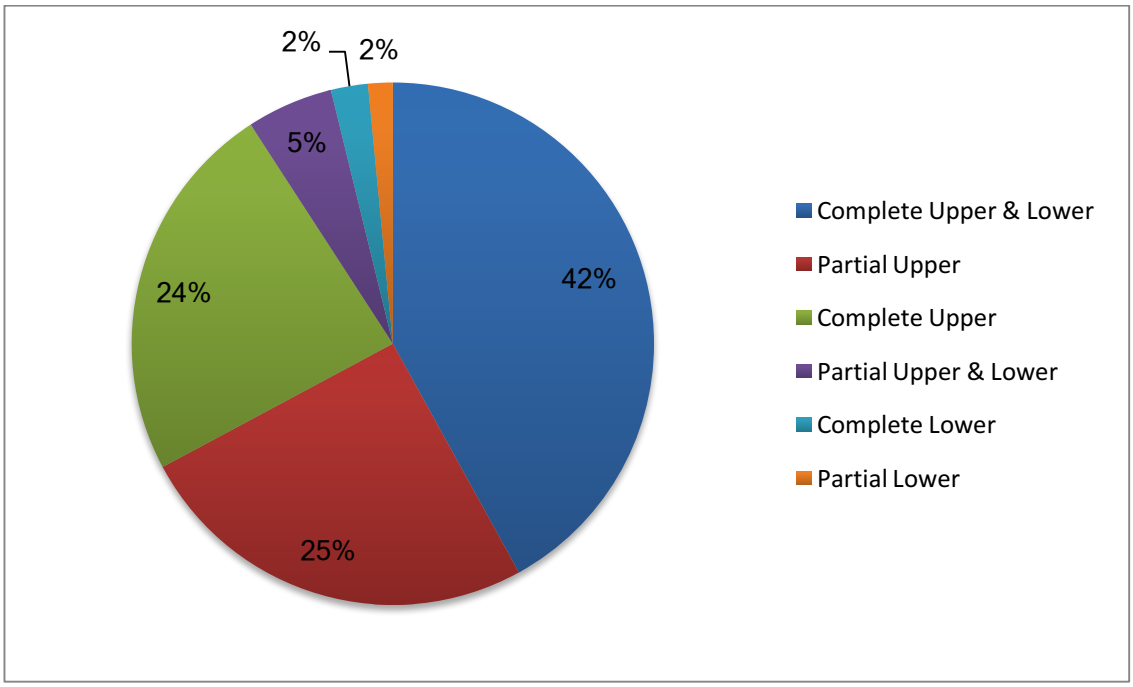

Figure 2.4: Distribution of denture type and design amongst the study participants.

In the study population more healthy participants were wearing complete dentures (74.4%) than partial dentures. This group of healthy participants wearing complete dentures is higher than participants with complete dentures with grade 1 (64.0%) or grade 2 & 3 (50.0%) inflammation. Partial denture wearers were twice as likely to have DIS when compared with complete denture wearers (OR 2.2, p=0.04).

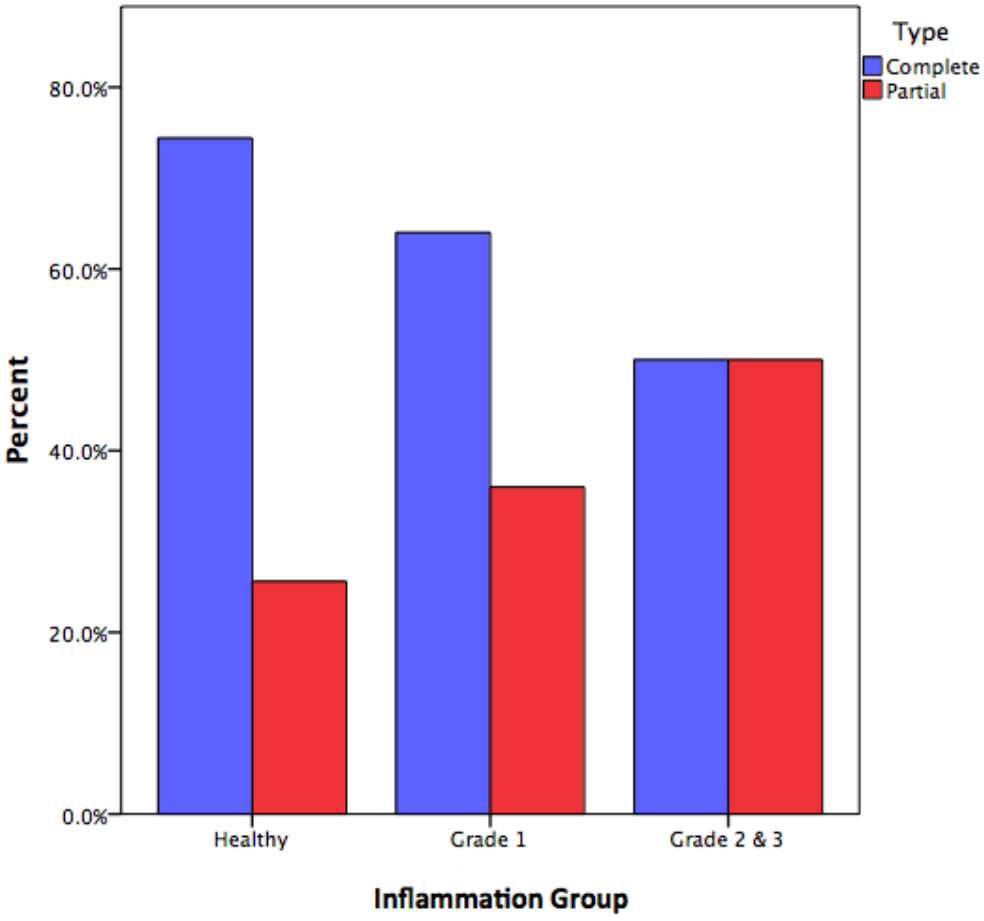

Figure 2.5: Percentage distribution of partial and complete dentures for each of the healthy and inflammation groups. The samples (n=131) were categorised into different Newton's types (Healthy, Grade 1, 2 & 3) by denture design (partial denture or complete denture). There is a trend that DIS increase with partial dentures. Data was analysed using an odds ratio calculation (OR 2.2 and p=0.04).

2.4.3.2 Denture fit and denture induced stomatitis

Thirty-eight (42.7%) of the 89 complete dentures were assessed to be of good fit. This included 25 complete upper and lower, 11 complete upper and 2 complete lower dentures. Twenty-nine (69.0%) of the 42 partial dentures were assessed to be of good fit. This included 6 partial upper and lower, and 23 partial upper dentures.

The summary of technical inadequacies as evaluated by the operating clinician can be seen below. Of the dentures that were technically not satisfactory, the most common finding was the denture was unretentive and/or unstable (84%).

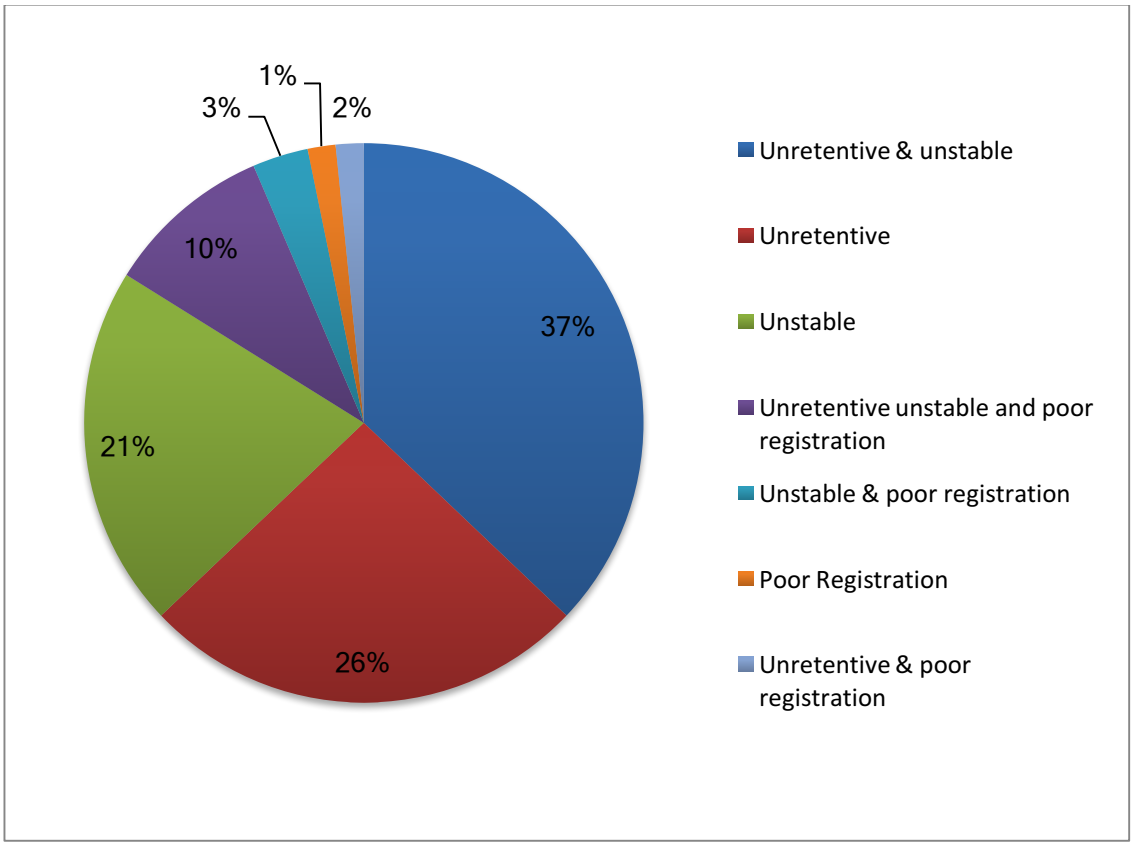

Figure 2.6: Distribution of design short-comings of the participant's dentures.

As can be observed from the Figure 2.7 there is no apparent relationship between the fitting accuracy of a denture and DIS. Each of the healthy, and inflammation

groups have similar proportions of good (yes) and poorly (no) fitting dentures. The odds ratio was calculated as 1 (p=0.98).

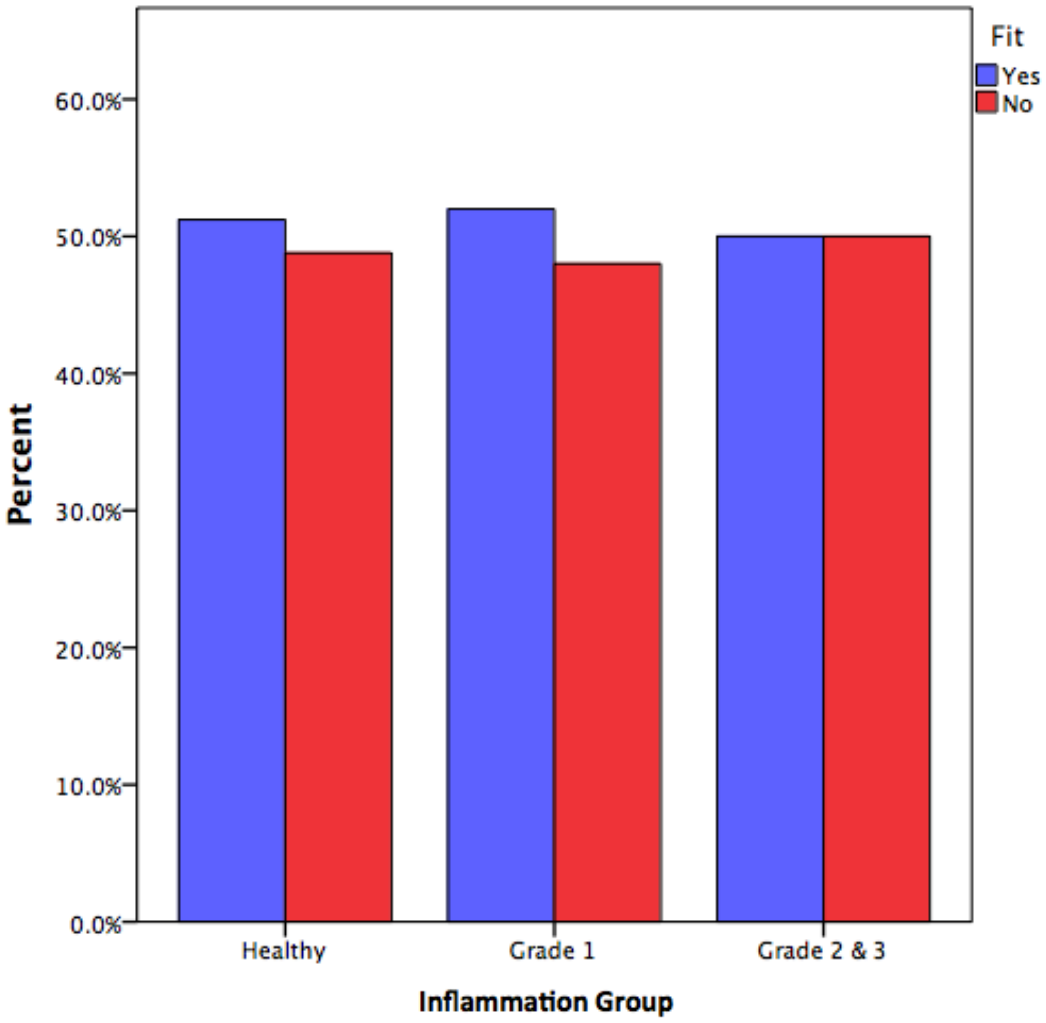

Figure 2.7: Percentage distribution of dentures fit for each of the healthy and inflammation groups. The samples (n=131) were categorised into different Newton's types (Healthy, Grade 1, 2 & 3) by well fitting (yes) or poorly fitting (no) dentures. There appears to be no trend. Data was analysed using an odds ratio calculation (OR 1 and p=0.98).

2.4.3.3 Denture age and denture induced stomatitis

The median denture age for healthy, grade 1 and grade 2 & 3 inflammation groups was 3.5, 3.0, and 2.75 respectively. A Kruskal-Wallis test shows there is no significant difference between the age of the dentures amongst the healthy and inflammation groups (p=0.97).

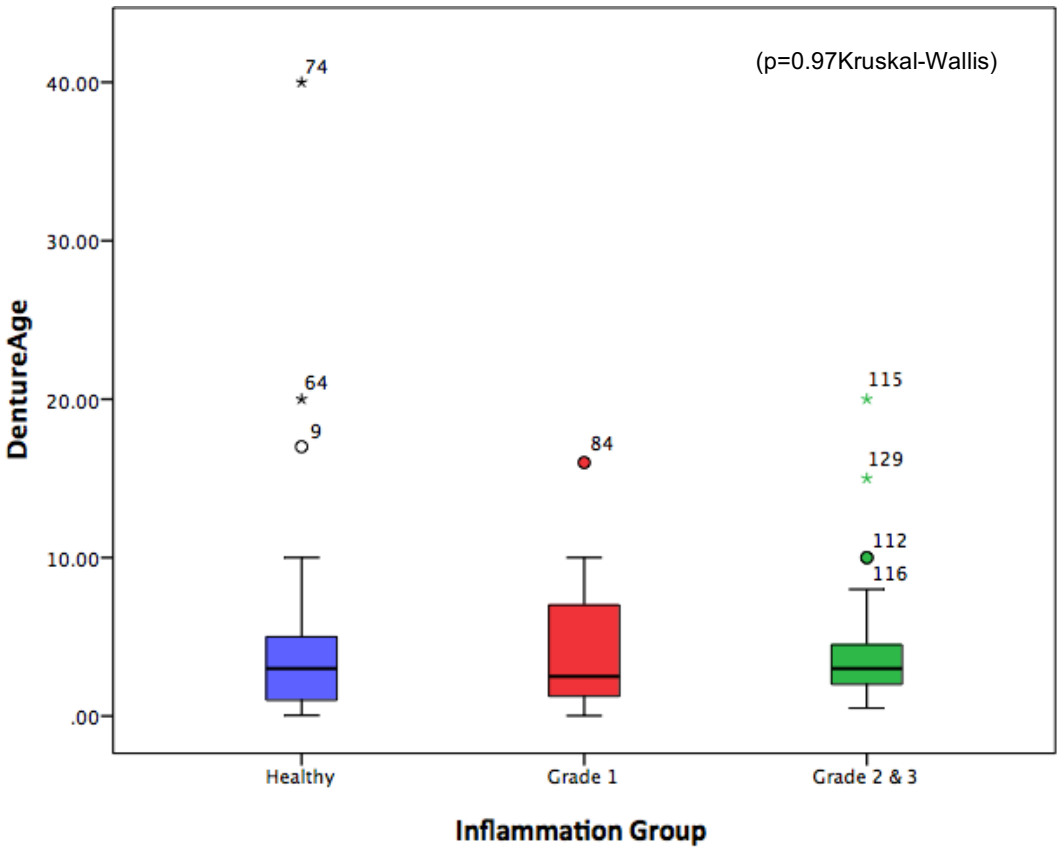

Figure 2.8: Median and range values of the participants denture age for each of the healthy and inflammation groups. The samples (n=131) were categorised into different Newton's types (Healthy, Grade 1, 2 & 3) by the age of the denture. Data was analysed non-parametrically using a Kruskal-Wallis test (p= 0.97), showing no significant difference between denture age and DIS.

2.4.4 Relationship between hygiene habits and denture induced stomatitis

2.4.4.1 Frequency of denture cleaning and denture induced stomatitis

Healthy participant's most frequent method of cleaning their denture was using a denture cleanser (36.6%), followed by toothpaste (31.7%) and then a combination of denture cleanser and toothpaste (14.6%). Whereas, participants experiencing DIS most frequent method of cleaning their denture was toothpaste (49.0%), then denture cleanser (20.4%) and a combination of denture cleanser and toothpaste (18.4%).

Participant's with grade 2 & 3 inflammation reported cleaning their denture less than once a day (4.2%), which was less often than participants in the healthy group (1.3%). Likewise, participant's in the healthy group more commonly cleaned their denture more than twice a day (20.7%) than those with grade 2 & 3 inflammation (16.7%). The odds ratio of experiencing DIS when cleaning once daily rather than twice daily was 1.5, which was not significant (p=0.33).

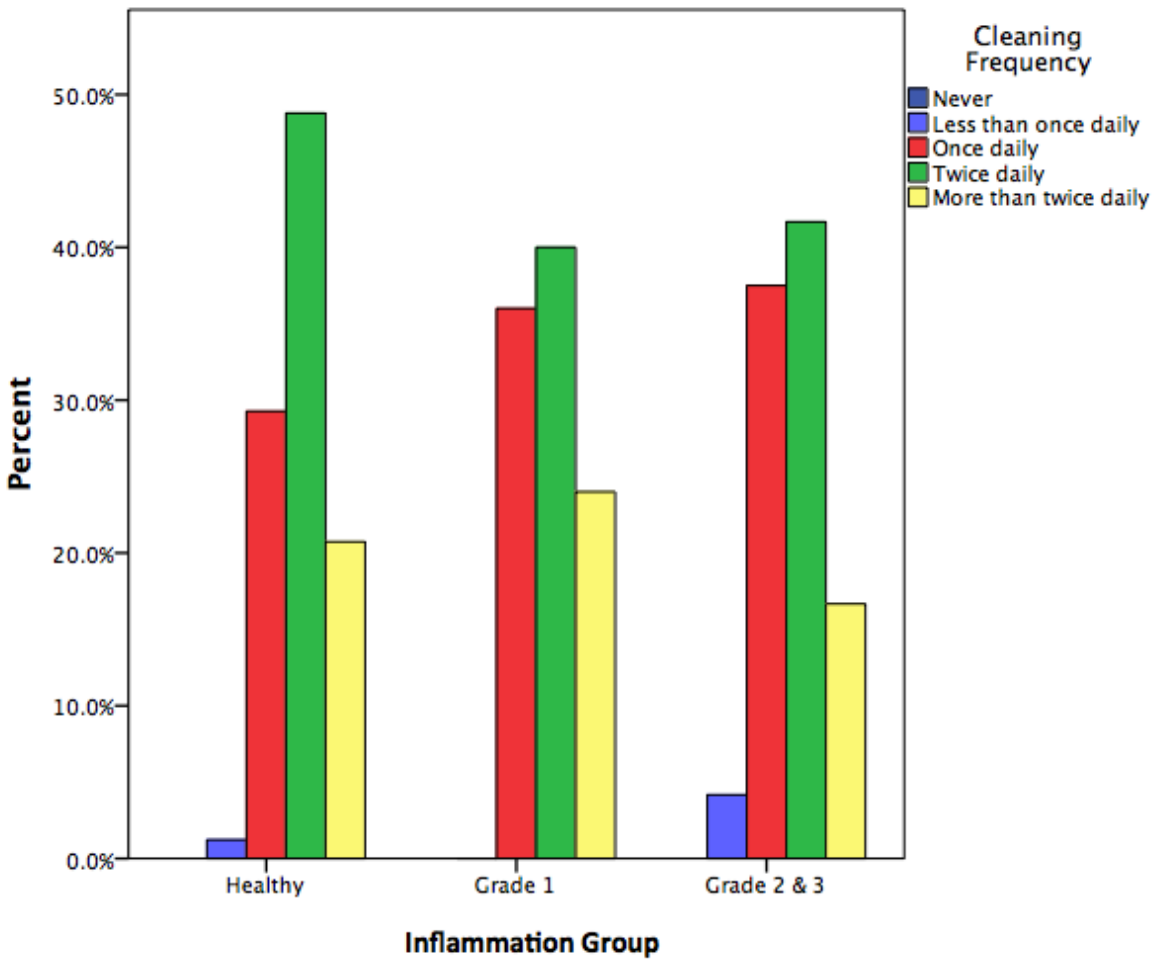

Figure 2.9: Percentage distribution of denture cleaning frequency for each of the healthy and inflammation groups. The samples (n=131) were categorised into different Newton's types (Healthy, Grade 1, 2 & 3) by the reported frequency of denture cleaning (never, less than once a day, once daily, twice daily, more than twice daily). Data was analysed using an odds ratio calculation (OR 1.5 and p=0.33) showing no significant association between cleaning frequency and DIS.

2.4.4.2 Denture hygiene status and denture induced stomatitis

Figure 2.10 shows healthy participants more often have good or excellent denture hygiene (80.3%) when compared to participants with grade 1 inflammation (55.0%). Likewise healthy participants infrequently have poor denture hygiene (20.7%) compared with 44% of participants with grade 1 inflammation. The odds ratio indicates participants are 3.5 times more likely to experience DIS if they had poor rather than good denture hygiene (p=0.03).

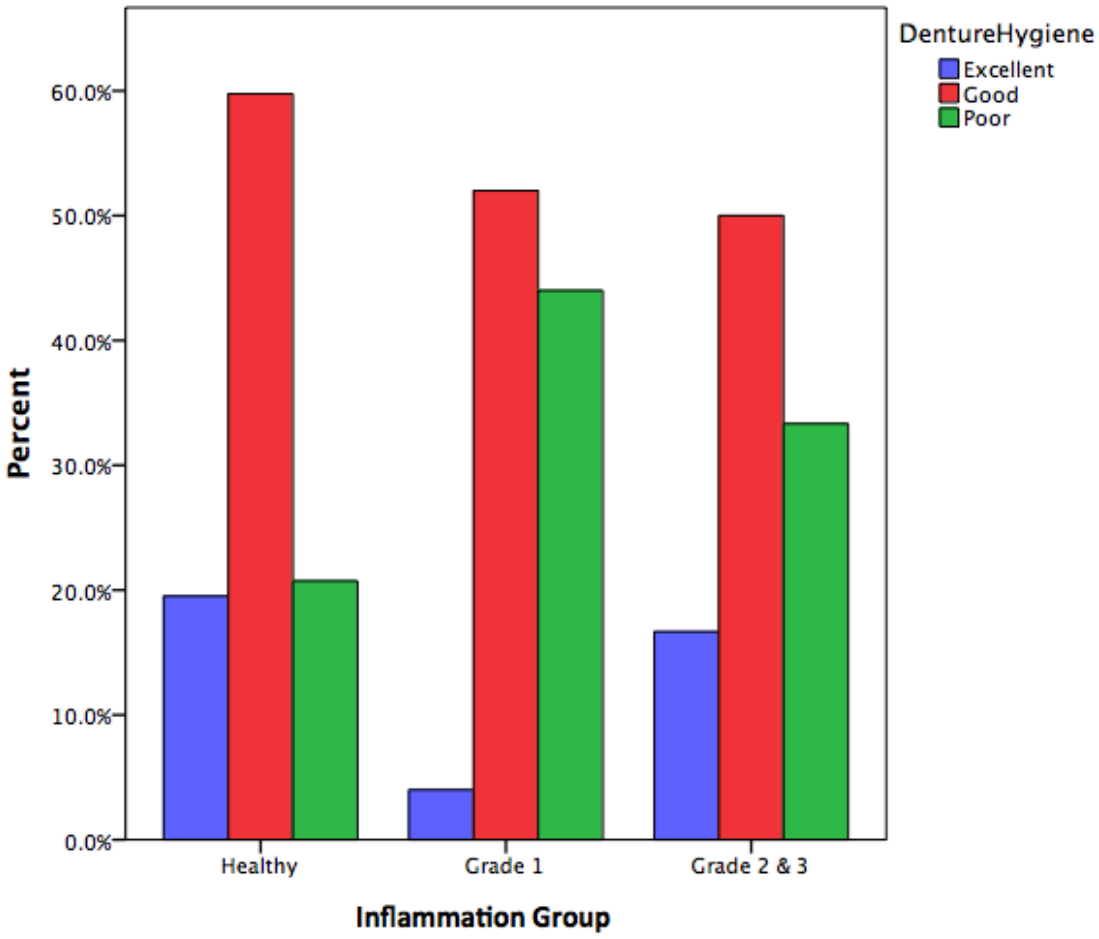

Figure 2.10 Percentage distribution of the study participant's denture hygiene, for each of the healthy and inflammation groups. The samples (n=131) were categorised into different Newton's types (Healthy, Grade 1, 2 & 3) by their level of denture hygiene (excellent, good, poor). Data was analysed using an odds ratio calculation (OR 3.5 and p=0.03). Poor denture hygiene was associated with DIS.

2.4.4.3 Wearing dentures while sleeping and denture induced stomatitis

Seventy-three (55.7%) participants admitted that they wore their denture while sleeping. 69.4% of participants with inflammation sometimes or always wore their denture while sleeping, compared to 34.5% of healthy participants.

A trend can be seen that healthy participants less frequently wear their denture while sleeping (35.4%) compared to inflammation group 1 (56.5%) and group 2&3 (62.0%). The odds ratio of experiencing DIS when wearing dentures at night is 2.8 times higher compared to not wearing dentures at night. Wearing a denture while sleeping was related to an increase in the risk of experiencing denture stomatitis (OR 2.8 p=0.01).

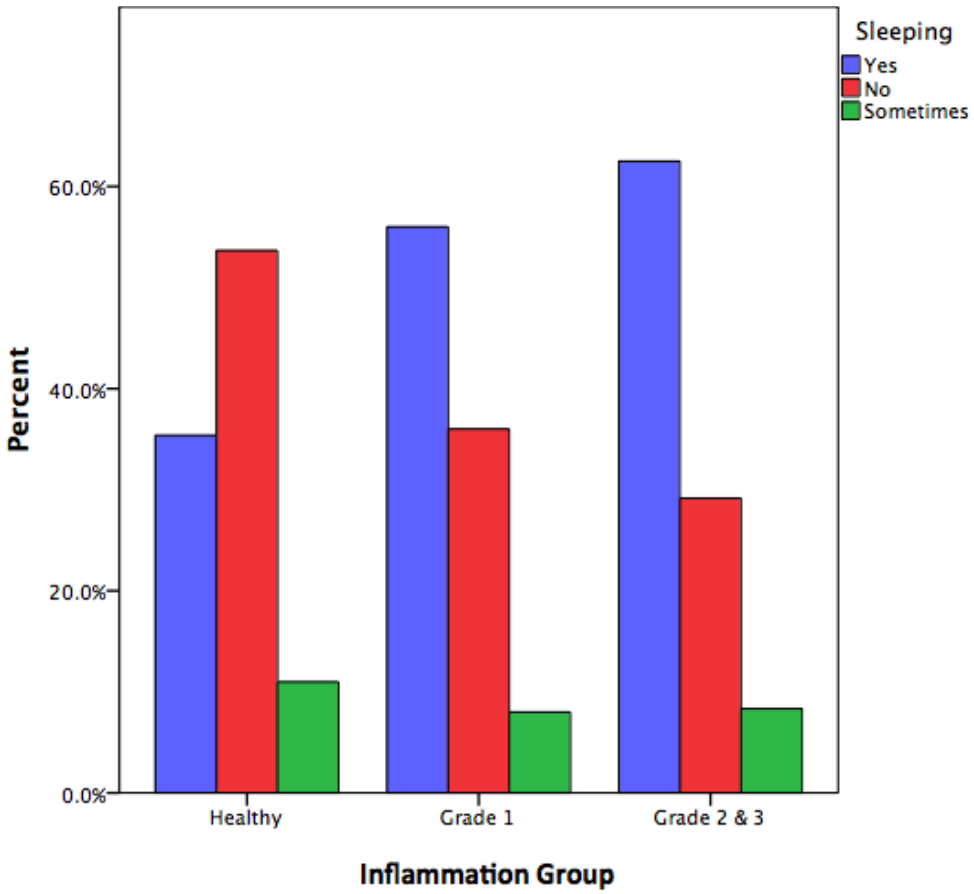

Figure 2.11 Percentage distribution of participants sleeping with their dentures at night for each of the healthy and inflammation groups. The samples (n=131) were categorised into different Newton's types (Healthy, Grade 1, 2 & 3) by which participant wore their dentures while sleeping (yes, no, sometimes). Data was analysed using an odds ratio calculation (OR 2.8 and p=0.01). Wearing dentures while sleeping was associated with DIS.

2.5 Discussion

Denture health and disease is governed by multiple factors, including some that can be directly influenced daily (e.g. patient hygiene habits) and some that are indirectly influenced daily (e.g. denture microbiome). This component of the study aimed to understand which patient variables had the capacity to influence denture health and disease. The data overall suggests that the presence of denture induced stomatitis was positively associated with a history of smoking, denture design, poor denture hygiene, and retention of dentures whilst sleeping.

There are a number of postulated reasons why age maybe associated with the presence of DIS. The aging population is more associated with complex medical conditions, comorbidities, and polypharmacy. This potential for an immunocompromised host allows microorganisms such as *Candida* species to flourish and potentially cause the development of DIS (Figueiral et al. 2007). A common oral complication of polypharmacy is xerostomia, potentially increasing the patients risk to oral infections. Furthermore, you might expect the older participant to have reduced manual dexterity, therefore finding it challenging to physically clean the denture. However, the current study found no association between participant age and gender with DIS, though the cross sectional nature of the study may account for lack of supporting evidence.

It is unclear if smoking affects the presence of DIS. Shulman (2005) identified heavy smokers (15 cigarettes/day) were 1.3 more likely to develop DIS (Shulman 2005). The current study also demonstrates participants had a higher risk of developing DIS if they smoked (OR 3.7). Though, Espinoza (2003) disputes this association, and as such there is no consensus on the subject. Furthermore, the mechanism by which smoking may affect the inflammatory lesion is uncertain (Espinoza, 2003).

The work within has shown that the accuracy of the denture fit was not associated with the presence of DIS. Though a poorly fitting denture has the potential to move and traumatise the underlying mucosa. The research to support the relationship between trauma and DIS is not clear (Budtz-Jorgensen and Bertram 1970, Cawson 1965, Emami et al. 2008). Budtz-Jorgensen and Bertram (1970) did report an association of simple localised inflammation with poorly fitting dentures

(Budtz-Jorgensen and Bertram 1970), though it is not unreasonable to consider the movement of a poorly fitting hard acrylic denture across the mucosa could promote a mild inflammatory reaction.

Poor denture hygiene is an accepted risk for developing DIS. The findings from this body of work suggest patients are 3.5 times more likely to experience DIS if they have poor denture hygiene. Good denture hygiene practice is not always commonplace amongst denture wearers. 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 (Leathen et al. 1960, Olsen 1975). There are many factors that can alter the composition of denture plaque, with denture hygiene thought to have a strong influence (Jeganathan and Lin et al. 1992, Cross et al. 2004, Marchini et al. 2004, Salerno et al. 2011). The accumulation of this denture plaque could therefore be related to denture hygiene habits, or the lack thereof.

There may be a number of participant related reasons for these observations described herein, such as the *'Hawthorne effect'.* Here, participants may know the professionally recommended regime for denture hygiene and because they are recruited to a study record this in their response. However, this response may vary from their actual denture hygiene habit. Or, participants believe the more frequently you clean the denture the better, but in actual fact their cleaning technique is not efficient. Responses included using a number of techniques such as a tooth-brush and toothpaste, soaking in various solutions, and simply using soap and water with various frequencies per week. As DIS and biofilms are better understood (Ramage et al. 2001), undoubtedly the frequency of denture cleaning may be less important than the technique used. Physical removal and disruption of the biofilm by brushing would be more likely to resolve the disease when compared with merely soaking the denture, irrerespective of how many times thoughout the day. This variation in denture care is not uncommon, the majortity of patients in the general population have been shown not to practise optimal denture hygiene (Dikbas et al. 2006, Johnson 2013, Milward et al. 2013).

It was also demonstrated that there was a strong relationship (OR 2.8) between wearing a denture while sleeping and developing DIS. Such a relationship has been demonstrated elsewhere (Jeganathan and Lin 1992, Cross, et al. 2004,

Marchini et al. 2004, Figueiral et al. 2007, Gendreau and Loewy 2011, Salerno et al. 2011). Given the unique site of dentures, in direct contact with the mucosa, the already accumulated plaque on the denture surface has a greater opportunity to colonise and subsequently infect the mucosa if not removed. This is likely because the denture is in contact with the mucosa for longer periods of time preventing the cleansing action of saliva, favouring a more pathogenic biofilm colonisation (Salerno et al. 2011). Also, constantly wearing the denture maintains relatively anaerobic and low pH conditions between the denture base and the mucosa, which may promote opportunistic overgrowth of pathogenic yeasts, such as *Candida (*Kulak-Ozkan, et al. 2002).

Interestingly, the study participants that wore partial dentures were more likely to experience DIS. Previously it has been described that DIS is more common with complete dentures as they cover a greater surface area of the mucosa (Jainkittivong et al. 2002). However, as discussed previously denture plaque is a complex biofilm, not just comprised of yeast. Therefore, participants with partial dentures that have some remaining teeth have the potential to accumulate dental plaque, another complex polymicrobial reservoir that may contibute to the contemporary understanding of the biofilm producing DIS.

The current cohort only included one denture that was not fabricated from PMMA or metal. Therefore, the clinical data was not sufficent to anlyse a relationship between different denture base materials and DIS. Alternative denture base materials include silicone, plasticized higher methacrylate polymers, hydrophilic polymethacylates and fluoropolymers, or collectively called denture-lining materials. Denture lining materials can also develop increased surface roughness as they age. Tari and co-workers suggested that these materials after prolonged use are also susceptible to attachment and colonization with *C. albicans* (Tari et al. 2007). Though in the current study prolonged use of PMMA and cobalt chrome dentures had no influence on the presence of DIS. Surface hydrophobicity has been shown to selectively increase the ability of hyphal forms of *C. albicans* to colonize denture surfaces, and *in vitro* studies have shown that decreasing surface hydrophobicity by using hydrophilic coating materials like silicone can decrease the ability of *C. albicans*, but not other yeasts, to attach and colonize the denture surface (Wright et al. 1985, Nikawa et al. 2000). Though it has been shown that saliva immersion decreases the surface roughness and *Candida* biofilm growth on

various soft denture liners had no significant differences in the quantity of cells recovered between materials (Radford 1998).

2.6 Summary

The prevalence of DIS in the study was 37.4%. Patient related factors such as age and gender did not influence the presence of DIS. Though participants were 3.5 times more likely to experience DIS if they smoked. Denture related factors such as denture hygiene and wearing them while sleeping were significantly associated with the presence of DIS. Thought, the clinical data of participants hygiene habits provides no information on how these may affect the oral microbial composition.

Chapter 3: Denture and mucosa microbiome

3.1 Introduction

Interest into the pathogenesis of oral disease has become more relevant because of the increasing associations found between oral health and systemic diseases, including rheumatoid arthritis, cardiovascular disease and respiratory disease (Inaba & Amano, 2010, Farquharson et al. 2012, O'Donnell et al. 2015). Furthermore, the population groups most at risk of developing these diseases are elderly populations, 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/disease (Iinuma et al. 2015). However, before such links can be investigated, a more in depth understanding of the complex microbiology is required.

The introduction of a denture prosthesis into the oral environment changes the microbial niche and ultimately adds to the complexity of intra-oral microbial relationships, which in turn influences oral diseases of microbial origin. More recently interactions between *Candida* and bacteria have been identified as an important factor in denture plaque (O'Donnell et al 2015), including defined associations between *C. albicans* and streptococci. For example, candidal hyphal formation can be modulated by Gram-negative bacteria through quorum sensing, where an excreted molecule triggers a genetic response in a polymicrobial community (Morales and Hogan 2010).

There are many factors that can alter the composition of denture plaque, with denture hygiene thought to have a strong influence. Good denture hygiene practice is not always commonplace amongst denture wearers (Pires et al. 2002), which could be attributed to the fact that the majority of denture wearing 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 & 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. Understanding how key factors such as hygiene habits and practices can impact the denture plaque composition is crucial to further our basic knowledge in this arena.

DIS is primarily thought of as a disease of yeast origin, with *Candida spp.* colonising the denture surface to form co-aggregates with bacteria and other yeasts to build complex microbial communities known as biofilms (Shirtliff et al. 2009, Morales & Hogan 2010). However, there is very little evidence of the microbial changes that occur when going from a healthy to the diseased DIS phenotype. 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, Sumi et al. 2003, Daniluk et al. 2006, Ealla et al. 2013). 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 oral cavity is a multifaceted arena, with over 700 bacterial species known to colonise this environment (Chen et al. 2010). Thus, understanding the intricate microbial relationships and interactions between natural hard and soft tissues is complex. We know that some oral diseases such as periodontitis has a 'pattern' or 'signature' of particular bacteria associated with the disease (Socransky et al. 1998), though our knowledge related to oral prostheses is somewhat lacking. The advent of high throughput sequencing has revolutionised our understanding of microbial ecosystems, and thus using this superior method we can gain an insight into the oral microbiome of denture wearers (O'Donnell et al. 2015).

3.2 Aim

The purpose of this chapter was to investigate the influence of denture hygiene habits on the oral microbiome of a denture wearer. It is hypothesised that denture hygiene influence both the denture and mucosal microbiome, and the presence of disease.

3.3 METHODS

3.3.1 Clinical sample collection

Patients were recruited and clinical data recorded as described in chapter 2. Denture hygiene categories ‘excellent’ and ‘good’ were grouped together and labelled satisfactory for ease of statistical analysis. The following clinical samples were collected; ethylene oxide sterilised swabs (Fisher Scientific, Loughborough, UK) were used to take samples from the denture surface in contact with the palatal mucosa, and the palatal mucosal surface covered by the denture. Samples were collected and processed as previously described (O'Donnell et al. 2015). In total samples from 131 patients were collected, which included 131 denture swabs and 131 mucosal swabs. However, during DNA extraction process not all samples had sufficient DNA, and therefore only DNA from 108 denture samples, and 87 mucosal samples could be used.

In parallel, dentures were removed from the patients’ mouth and placed in sterile bags (Fisher Scientific) containing 50 ml PBS (Sigma-Aldrich, Dorset, UK). Adherent denture plaque was then removed by sonication (Ultrawave, Cardiff, UK) for 5 min, as previously described (O'Donnell et al. 2015). The denture sonicate was then used to prepare ten-fold serial dilutions (10^{0}–10^{-3}) in order to perform colony forming unit (CFU) counts on Colorex*Candida* plates (E&O Laboratories, Bonnybridge, UK). One hundred µl of each serial dilution was spread across each plate, and then incubated at 30°C for 48 hrs. CFUs forming on plates were then counted and the mean number of *Candida* cells colonising each denture was calculated. Pure culture isolates were stored in Microbank® vials (Pro-Lab Diagnostics, Cheshire, UK) at −80°C until further use. Dr Lindsay O’Donnell undertook in the collection and processing of the samples between Feb 2013 and Oct 2014.

3.3.2 Illumina sequencing

All samples were prepared for DNA isolation as previously described (O'Donnell et al. 2015), using a combination of chemical and mechanical lysis. The concentration of DNA per sample was then calculated by qPCR using the Light cycler LC480-II (Roche Diagnostics, Switzerland). Bacterial 16S rDNA

concentrations (CFU/ml) were determined from standard curves of *E. coli* K12 cultures. For each individual sample amplicon libraries of the V4 hypervariable region of the 16S rRNA gene were generated. The amplification mix and PCR conditions used were previously described (O'Donnell et al, 2015). 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).

3.3.3 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 quality filtered with usearch (maxee 0.5). The representative sequence of each cluster was assigned a taxonomy using the RDP classifier (Cole 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. Data sets are available at NCBI project ID**:** PRJNA324548 (http://www.ncbi.nlm.nih.gov/bioproject/324548). The introductory analysis was performed in ACTA (Amsterdam, Netherlands) by Dr Bernd Brant.

3.3.4 Statistical analyses

The data set was randomly sub-sampled to 770 reads per sample (minimum number of reads per sample was 776), the use of a rarefaction curve based on our data deemed that 770 reads per sample was sufficient to avoid minimal loss in diversity which allowed for inclusion of the maximum number of samples for analysis. To carry out diversity statistics (Shannon diversity index and Dominance index) OTU datasets were reduced by $\log_2$ transformation the analysis was carried out using PAST software (Hammer O, 2001).

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 included a Shannon diversity index,

which measures the species diversity in a sample, the higher the value the more diverse the sample. As for the dominance index, this assesses how dominant species are within a biological sample and are assigned a value between 0 (all taxa equally represented) and 1 (1 taxa dominates the whole sample). Prism 5.0 (Microsoft) statistics was used for statistical tests and graph production. 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. Dr Lindsay O'Donnell assisted in the analysis of these data.

3.4 RESULTS

3.4.1 Presence of *Candida* and denture induced stomatitis

Two thirds of participants in the study (66.4%) had *Candida* species present. *Candida* species were detected in the majority (62% n=50) of the healthy participants. Similarly the majority participants with DIS were colonised with *Candida* species (75.5% n=37). Participants are 1.9 times more likely to experience DIS in the presence of *Candida* species, though this was not deemed statistically significant (p=0.09).

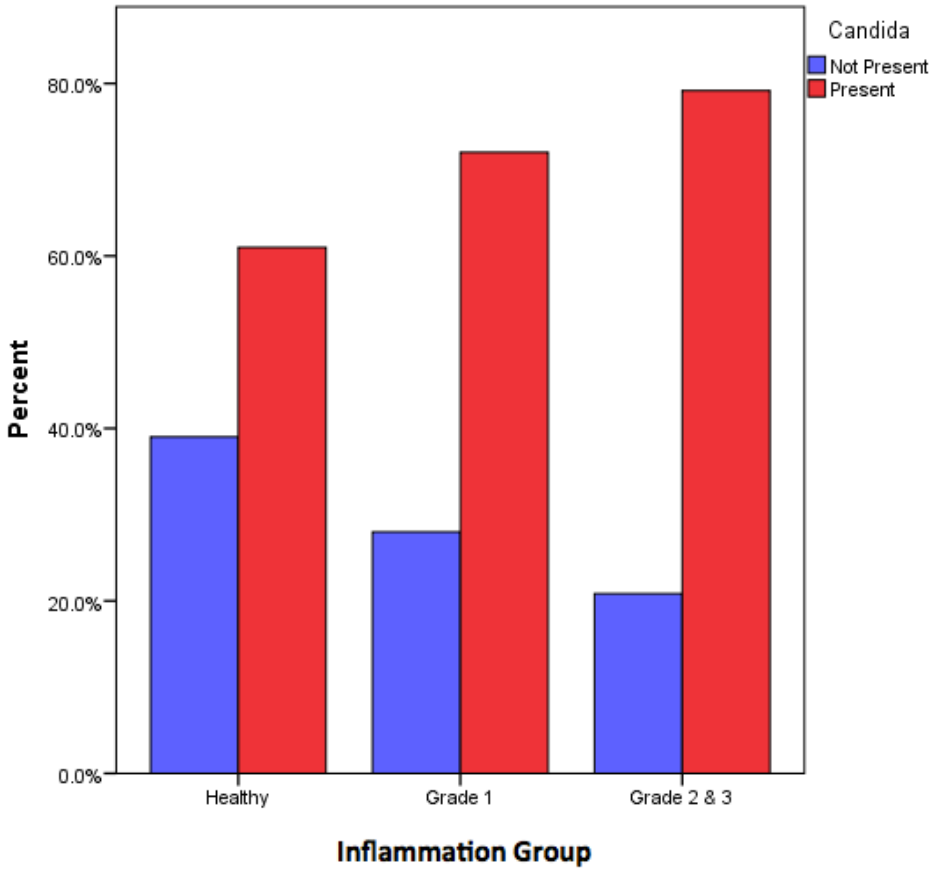

Figure 3.1: Percentage distribution of participants with and without *Candida* for each of the healthy and inflammation groups. The samples (n=131) were categorised into different Newton's types (Healthy, Grade 1, 2 & 3) by the presence or absence of *Candida* species in the oral cavity. Data was analysed using an odds ratio calculation (OR 1.9 and p=0.09). The presence of *Candida* species was not associated with DIS.

3.4.2 Influence of denture hygiene on the denture *Candida*

The influence of *Candida* load on dentures was compared across a number of patient and denture hygiene practices (Figure 3.2). In terms of hygiene status, no statistical significance was observed between satisfactory and poor hygiene groups with respect to candidal load (Figure 3.2A). Whether dentures were retained *in situ* when sleeping appeared to have no effect (Figure 3.2B), nor did cleaning once or twice per day have an impact on candidal colonisation (Figure 3.2C).

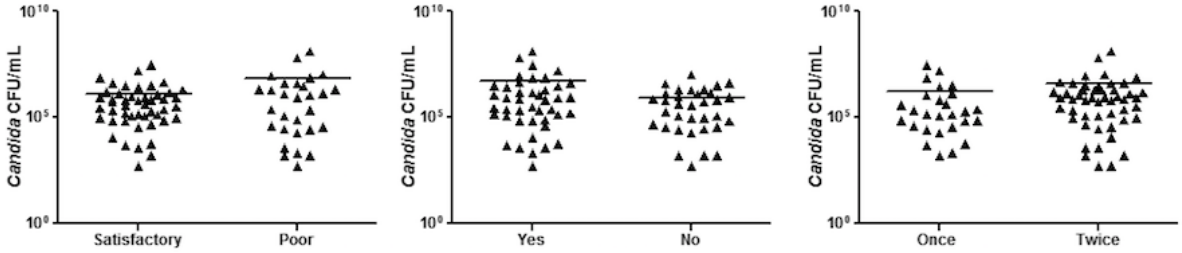

Figure 3.2: **Comparison of changes in *Candida* species as a result of denture hygiene practices.** Candidal colony forming units (CFU) were quantified from participants denture sonicates for (A) hygiene status, (B) sleeping with a denture *in situ* and C) denture cleaning frequency. Data represents individual CFU, and the line represents the mean. Statistical analysis was performed using a Mann Whitney test, as data did not conform to a normal distribution.

3.4.3 Influence of denture hygiene on the denture microbiome

The Shannon diversity index and dominance index were employed to compare the diversity and dominance of the denture microbiome for the different patient and denture hygiene practices (Figure 3.3). As for hygiene status, no statistical significance was observed between satisfactory and poor hygiene groups in terms of diversity (Figure 3.3A) or dominance (Figure 3.3B). Whether dentures were cleaned once or twice per day appeared to have no effect on the diversity or dominance of the denture microbiome (Figure 3.3D & E), and this also applied to sleeping with or without a denture *in situ* (Figure 3.3G & H). Comparison of the average bacterial class proportion between the groups revealed few changes other than elevated levels of *Alphaproteobacteria* on the denture in those with poor denture hygiene (Mean: 1.76 v 2.57, $p<0.05$ [Figure 3.3C]). As for cleaning frequency, few significant changes were observed between groups, including an increase in *Bacilli* in those that cleaned twice a day (Mean: 25.06 v 37.14, $p<0.01$, [Figure 3.3F]). The denture microbiome of those that slept with their dentures *in situ* had significantly higher levels of *Bacteroidia* (Mean: 6.25 v 2.16, $p<0.05$), *Clostridia* (Mean: 14.88 v 5.57, $p<0.001$), *Epsilonproteobacteria* (Mean: 0.13 v 0.03, $p<0.05$) and *Fusobacteria* (Mean: 0.18 v 0.05, $p<0.05$), and significantly lower levels of *Bacilli* (Mean: 0.18 v 0.05, $p<0.05$), *Betaproteobacteria* (Mean: 0.18 v 0.05, $p<0.05$) and *Gammaproteobacteria* (Mean: 0.18 v 0.05, $p<0.05$) (Figure 3.3I).

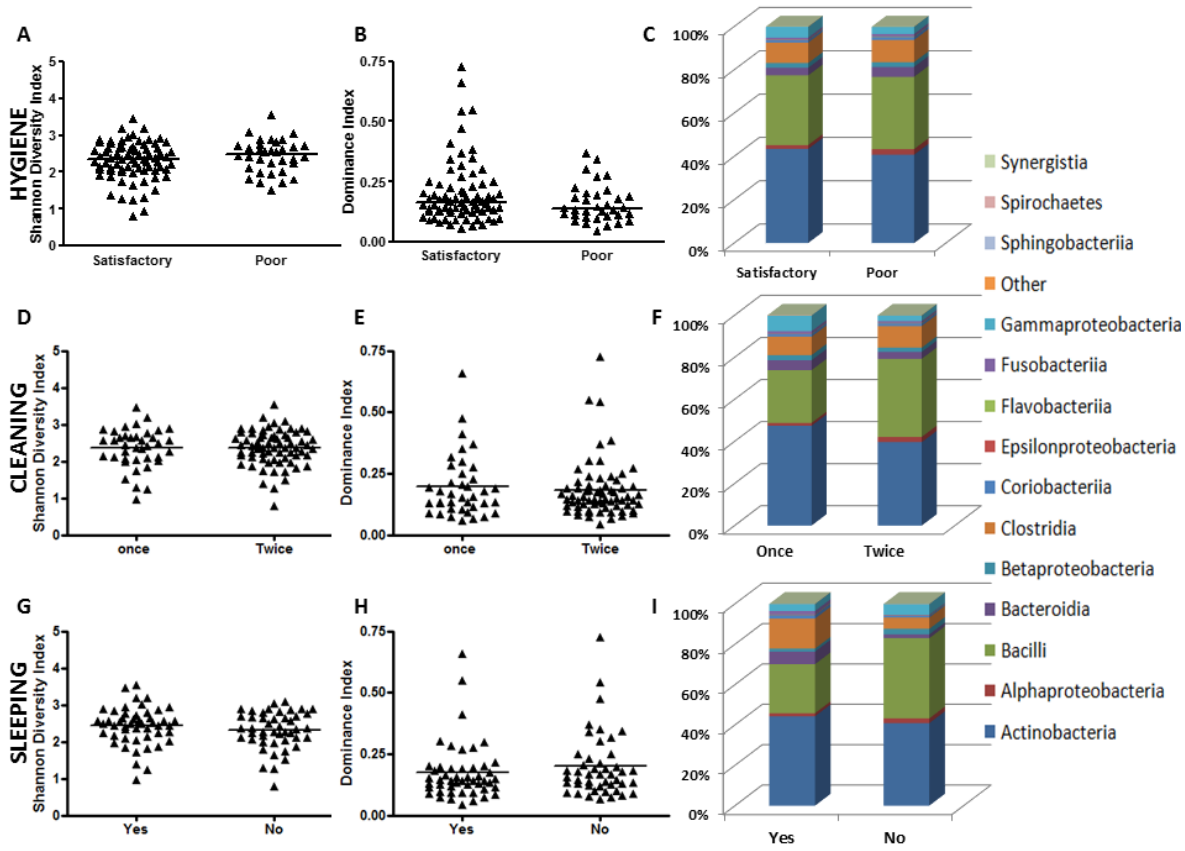

Figure 3.3: **Comparison of changes in the denture microbiome as a result of denture hygiene practices.** The microbiome was assessed for differences in taxonomic diversity and dominance of denture plaque for hygiene status (A) and (B), denture cleaning frequency (D) and (E) and sleeping with a denture in situ (G) and (H). Differences in denture microbiome composition for hygiene status (C) denture cleaning frequency (F) and sleeping with a denture in situ were compared (I). Data represents mean. Statistical analysis was performed using a Mann Whitney test as data did not conform to a normal distribution.

Diversity and dominance was further assessed across the hygiene status, cleaning frequency and sleeping with the denture *in situ* categories with respect to health and presence of DIS. No changes were noted between any of the groups, across any of the categories (Figure 3.4).

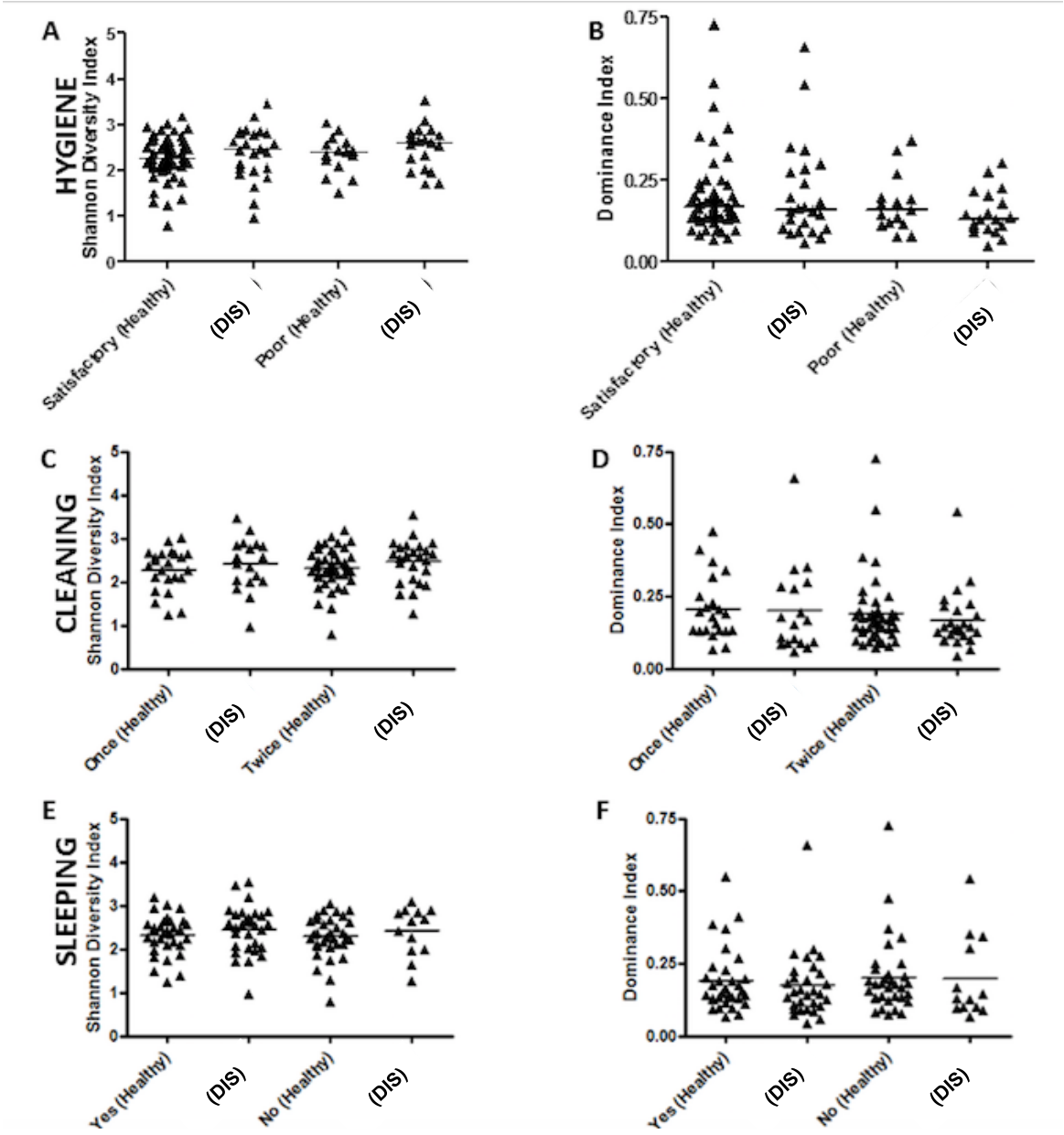

Figure 3.4: **Comparison of changes in diversity of the denture microbiome as a result of denture hygiene practices.** The microbiome was assessed for differences in taxonomic diversity and dominance of denture plaque, healthy and diseased patient groups were further split based on hygiene status (A) and (B), denture cleaning frequency (D) and (E) and sleeping with a denture in situ (G) and (H). Data represents mean. Statistical analysis was performed using a Kruskall Wallis test with Dunn's post-test to compare all groups to each other.

3.4.4 Disease status and denture microbiome composition

The changes in the average abundance of each bacterial class was compared between the healthy and DIS groups. No notable changes were detected across the groups within the denture hygiene category for any classes of bacteria (Figure 3.5A) or within the cleaning category (Figure 3.5B). However, sleeping with a denture *in situ* saw notable changes (Figure 3.5C). 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* (Mean: 12.54 v 4.88, $p<0.01$), as well as diseased individuals that slept whilst wearing their denture (Mean: 13.27 v 4.88, $p<0.001$). Levels of *Bacteroidia* was significantly elevated in the diseased group that slept with their denture *in situ* in comparison to the healthy group that did not (Mean: 6.53 v 2.03, $p<0.05$). *Fusobacteria* was more abundant in the healthy group that slept with their dentures *in situ* when compared to the healthy group that did not (Mean: 1.56 v 0.43, $p<0.05$), in addition the healthy ‘no’ group had significantly lower levels than the diseased ‘no’ group (Mean: 0.43 v 1.38, $p<0.05$).

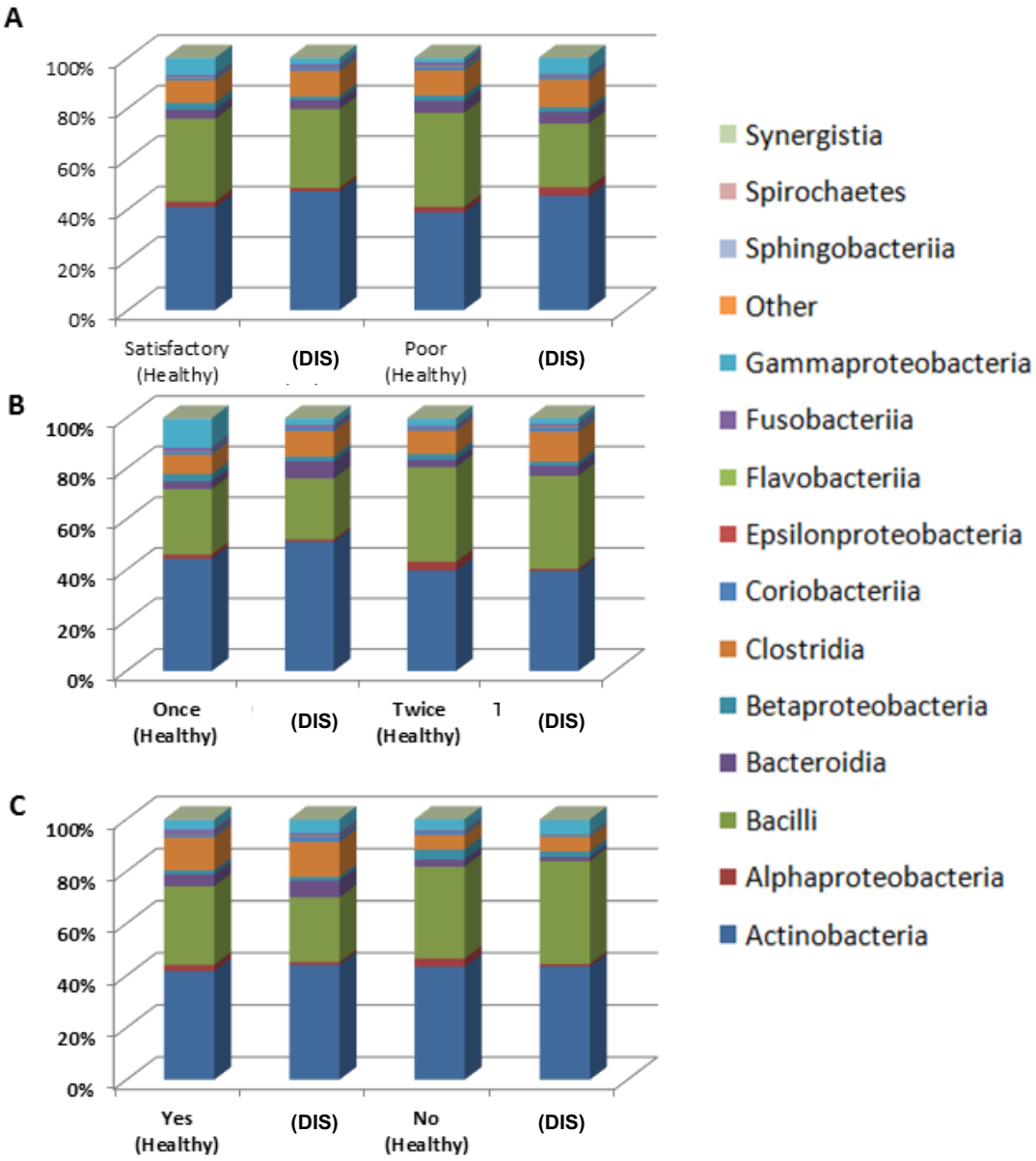

Figure 3.5: **The influence of disease status on the denture microbiome as a result of denture hygiene practices.** The microbiome was assessed for taxonomic differences in denture plaque, healthy and diseased patient groups were further split based on hygiene status (A), denture cleaning frequency (B) 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.

3.4.5 Influence of denture 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 (Figure 3.6). Comparing satisfactory and poor denture hygiene at the mucosa revealed that those with poor denture hygiene have a significantly more diverse mucosal microbiome than those classed as having satisfactory denture hygiene (Mean: 2.3 v 2.68, $p<0.05$ [Figure 3.6A]). Furthermore, the poor hygiene group had significantly less dominant taxa than the satisfactory hygiene group (Mean: 0.23 v 0.15, $p<0.05$ [Figure 3.6B]). Cleaning dentures either once or twice per day appeared to have no significant effect on the diversity or dominance of the mucosal microbiome (Figure 3.6D & 3.6E). This was also unaffected by sleeping with a denture in situ in terms of diversity or dominance (Figure 3.6G & 3.6H). Bacterial class abundance levels showed no significant variation between groups in terms of hygiene status (Figure 3.6C) or cleaning frequency (Figure 3.6F). However, sleeping whilst wearing a denture revealed significantly elevated levels of Bacteroidia (Mean: 0.18 v 0.05, $p<0.05$), Coriobacteria (Mean: 0.18 v 0.05, $p<0.05$), Epsilonproteobacteria (Mean: 0.18 v 0.05, $p<0.05$) and Fusobacteria (Mean: 1.73 v 0.46, $p<0.001$) (Figure 3.6I).

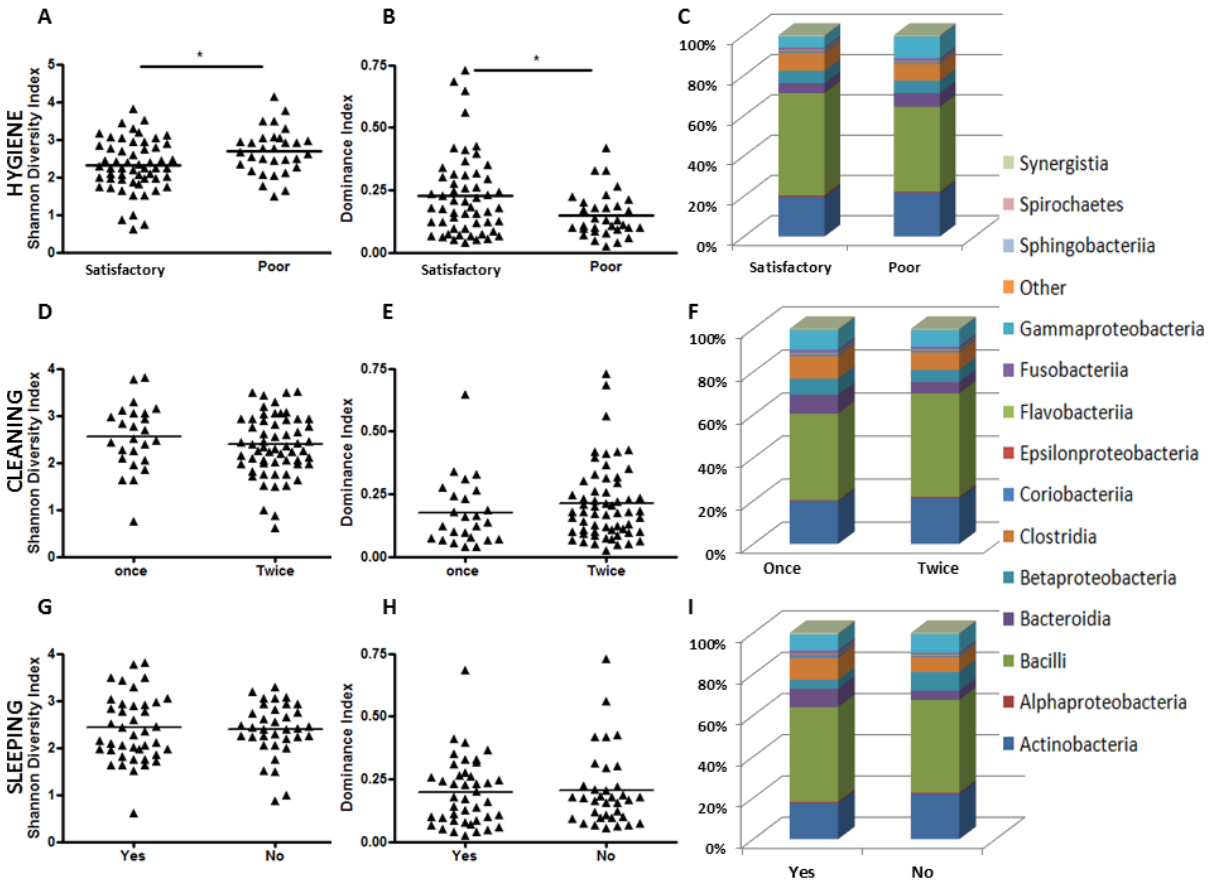

Figure 3.6: **Comparison of changes in the mucosal microbiome as a result of denture hygiene practices.** The microbiome was assessed for differences in taxonomic diversity and dominance of mucosal plaque for hygiene status (A) and (B), denture cleaning frequency (D) and (E) and sleeping with a denture 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 mean (* $p<0.05$). Statistical analysis was performed using a Mann Whitney test as data did not conform to a normal distribution.

As previous, the groups within the hygiene, cleaning and sleeping categories were further separated into healthy and DIS groups to investigate the potential impact on disease. The diversity of the mucosal microbiome was significantly higher in the poor hygiene group with DS when compared to the healthy group with satisfactory hygiene (Mean: 2.15 v 2.77, $p<0.05$ [Figure 3.7A]); however, no significant changes in dominance were detected (Figure 3.7B). Neither diversity nor dominance analysis revealed significant variation between groups in the cleaning frequency category (Figure 3.7C & 3.7D). This also applied to sleeping with the denture *in situ* (Figure 3.7E & 3.7F).

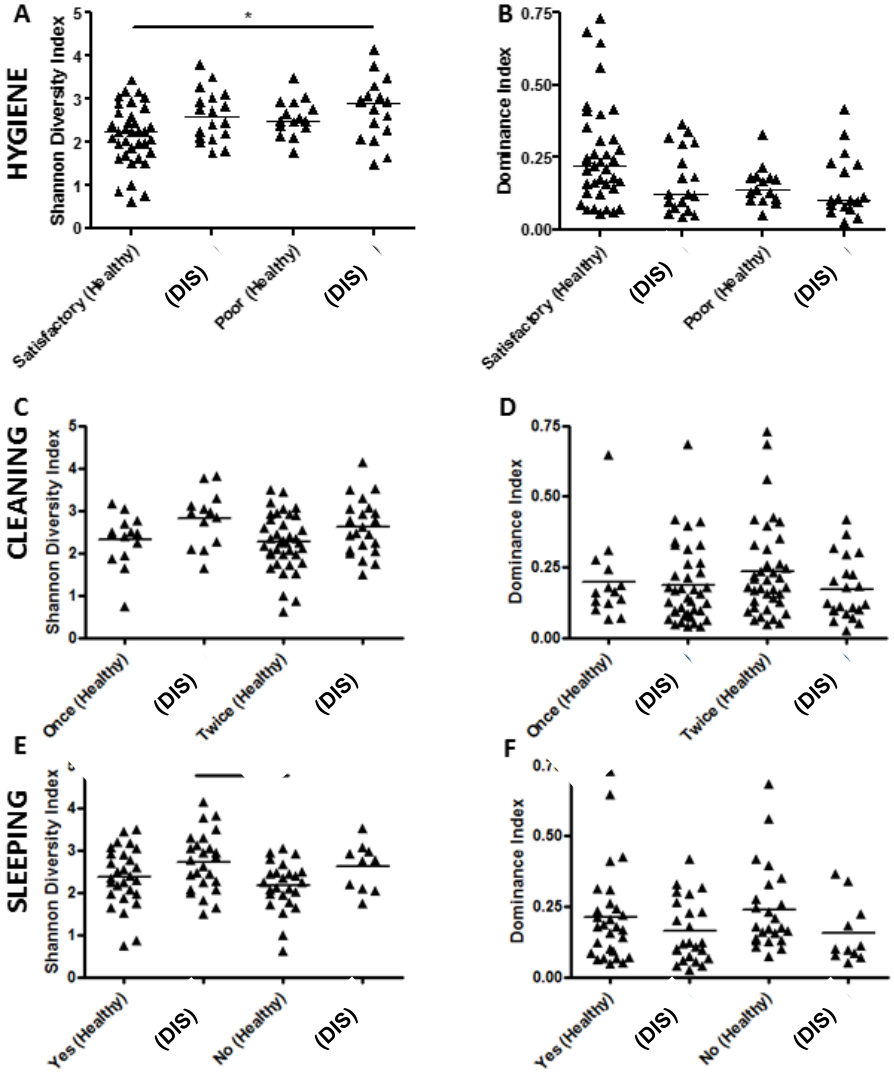

Figure 3.7: **Comparison of changes in diversity of the mucosal microbiome as a result of denture hygiene practices.** The microbiome was assessed for differences in taxonomic diversity and dominance of mucosal plaque, healthy and diseased patient groups were further split based on hygiene status (A) and (B), denture cleaning frequency (D) and (E) and sleeping with a denture in situ (G) and (H). Data represents 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.

3.4.6 Disease status and mucosal microbiome composition

The abundance of *Bacteroidia* was significantly elevated in diseased patients in comparison to healthy patients with satisfactory hygiene (Mean: 10.28 v 4.13, $p<0.05$ [Figure 3.8A]). Cleaning frequency also related to an increase in *Bacteroidia* between the healthy group that cleaned twice per day and the diseased group that cleaned only once (Mean: 4.52 v 12.22, $p<0.05$ [Figure 3.8B]). *Alphaproteobacteria* levels were also significantly higher in the healthy group that slept with their denture *in situ* when compared to the healthy group that did not (Mean: 0.99 v 0.52, $p<0.05$), as well as the diseased group that slept with their denture *in situ* (Mean: 0.99 v 0.34, $p<0.01$ [Figure 3.8C]).

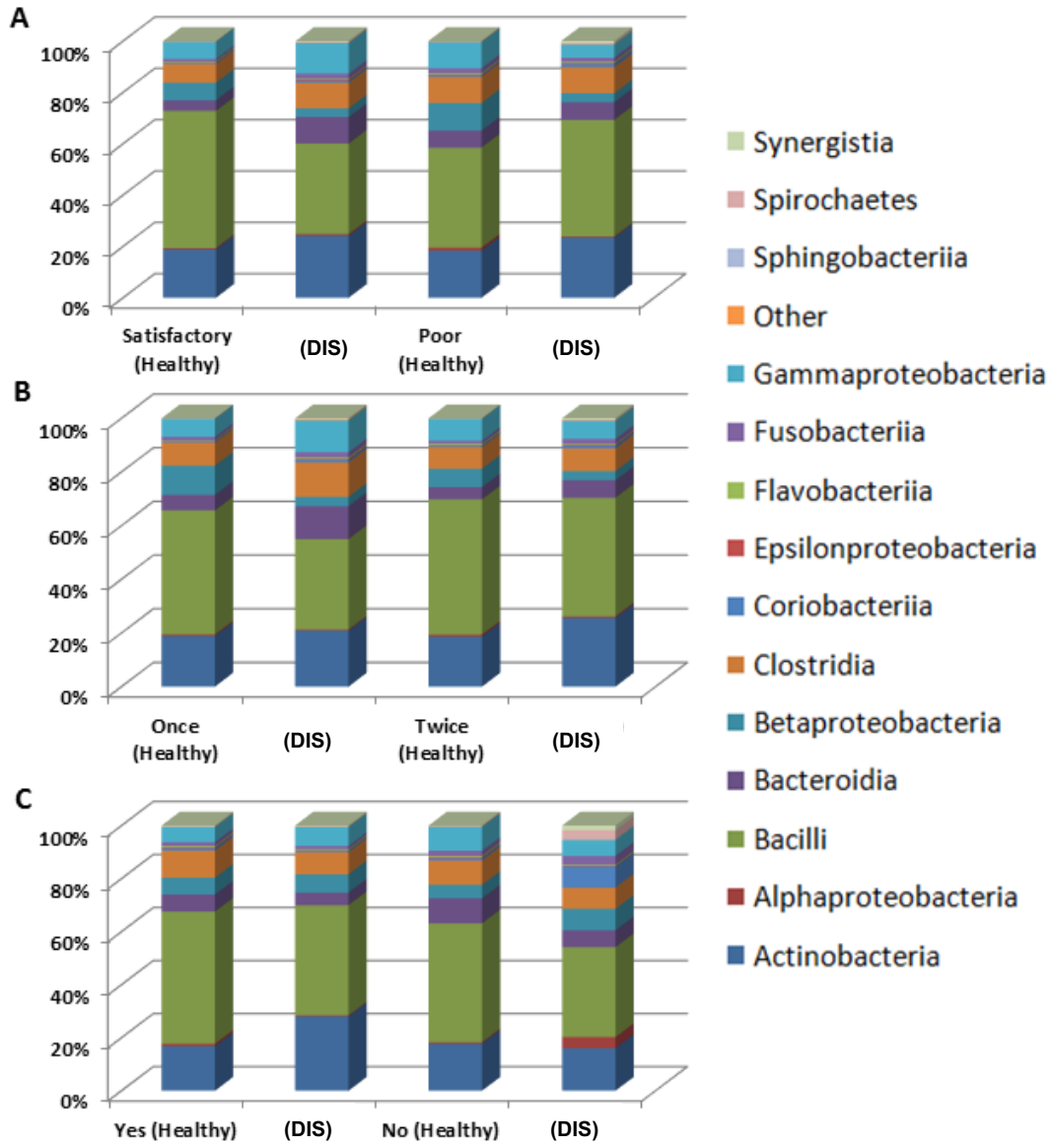

Figure 3.8: **The influence of disease status on the mucosal microbiome as a result of denture hygiene practices.** The microbiome was assessed for taxonomic differences in mucosal plaque, healthy and diseased patient groups were further split based on hygiene status (A), denture cleaning frequency (B) 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.

3.4.7 Influence of partial dentures and denture hygiene on the microbiome

The Shannon diversity index and dominance index were used to compare the denture microbiome for participants with partial dentures across the different hygiene practices (Figure 3.9). For hygiene status, no statistical significance was observed between satisfactory and poor hygiene groups in terms of diversity (Figure 3.9A) or dominance (Figure 3.9D). Whether dentures were cleaned once or twice per day also appeared to have no effect on the diversity or dominance of the denture microbiome (Figures 3.9B & 3.9E), and this also applied to sleeping with or without a denture *in situ* (Figures 3.9C & 3.9F).

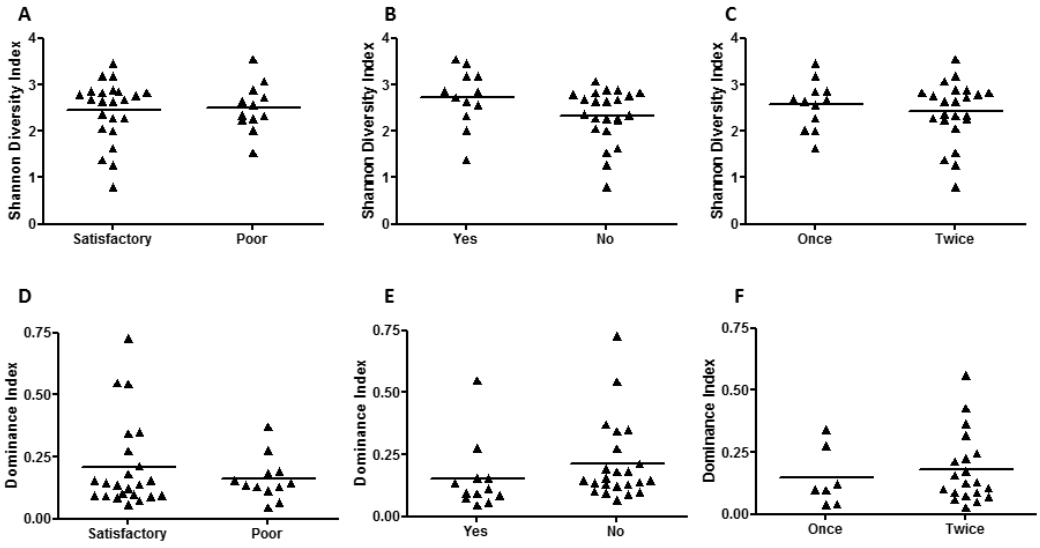

Figure 3.9: **Comparison of changes in the denture microbiome as a result of denture hygiene practices in partially dentate patients.** The microbiome was assessed for differences in taxonomic diversity and dominance of denture plaque for hygiene status (A) and (D), sleeping with a denture in situ (B) and (E), and denture cleaning frequency (C) and (F). Data represents mean. Statistical analysis was performed using a Mann Whitney test as data did not conform to a normal distribution.

Again, the Shannon diversity index and dominance index were next used to compare the diversity and dominance of the mucosal microbiome for participants with partial dentures across the different hygiene practices (Figure 3.10). Comparing hygiene status, no statistical significance was observed between satisfactory and poor hygiene groups in terms of diversity (Figure 3.10A) or dominance (Figure 3.10D). As for whether dentures were cleaned once or twice per day appeared to have no effect on the diversity or dominance of the denture microbiome (Figures 3.10B & 3.10D), respectively, and this was also true for sleeping with or without a denture *in situ* (Figures 3.10C & 3.10F).

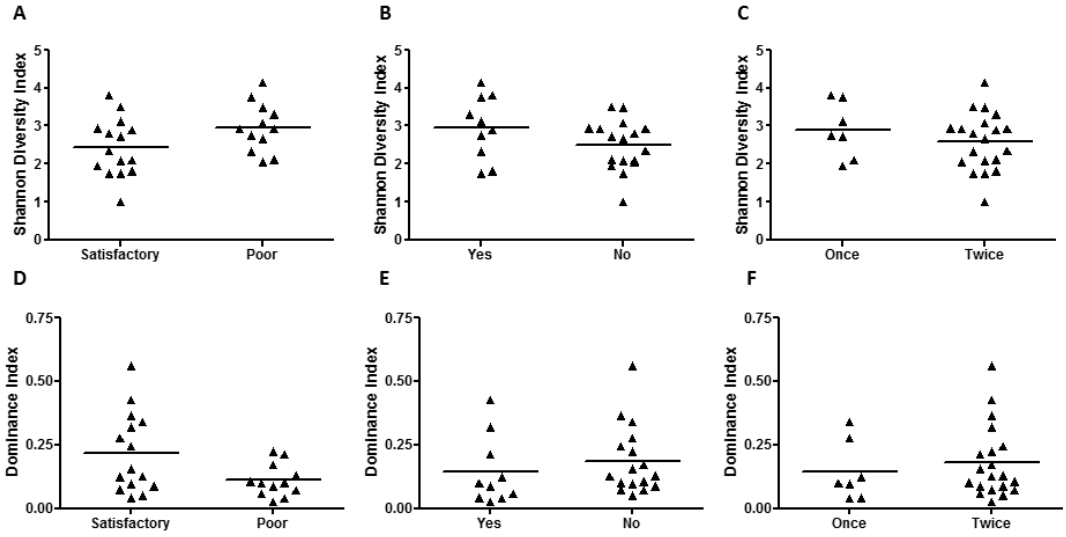

Figure 3.10: **Comparison of changes in the mucosal microbiome as a result of denture hygiene practices in partially dentate patients.** The microbiome was assessed for differences in taxonomic diversity and dominance of mucosal plaque for hygiene status (A) and (D), sleeping with a denture in situ (B) and (E), and denture cleaning frequency (C) and (F). Data represents mean. Statistical analysis was performed using a Mann Whitney test as data did not conform to a normal distribution.

3.5 Discussion

The oral cavity is a complex anatomical structure, composed of soft and hard biological tissues. The presence of prostheses, such as a denture, can influence oral health status, particularly in relation to the oral microbiome. Here the relationship between denture hygiene practices and the oral microbiome was investigated for the first time.

Historically, denture-related research has a disproportionate focus on yeast-associated infection (Coco et al. 2008, Pereira-Cenci et al. 2008, Dagistan et al. 2009), yet there is a distinct lack of understanding of the bacterial microbiome within the oral cavity of the denture wearer. *C. albicans* can grow either as mycelial or hyphal forms. A greater presence of *C. albicans* hyphae has been reported in patients with denture stomatitis (Webb 1998). The data shows *Candida* species were not significantly associated with the presence of DIS. In fact, the presence of *Candida* species was similar between both healthy and DIS groups. Suggesting that candidal colonisation is not solely the most important influence on denture microbiology and associated pathogenesis. Therefore, the contribution of bacterial microbiomes may be more influential than first appreciated. Indeed, there is increasing evidence that *Candida* and bacteria form polymicrobial biofilms, and that some bacterial species common to the oral cavity can enhance the pathogenicity of *C. albicans* (Diaz et al 2012, Falsetta 2014, Harriott 2009), and that the presence of specific oral bacteria is enough to transform a *C. albicans* biofilm forming ability (Arzmi et al. 2016).

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 causing a dysbiotic microbial composition, leading to the accumulation of fungi and bacteria associated with a diseased phenotype. Therefore, understanding how these factors can influence our microbiome and in turn impact on our general oral health should be taken into

account, as their impact (direct or indirect) may lead to more severe systemic effects (Maciag et al. 2014, O'Donnell et al. 2015, Maciag et al. 2016).

Poor denture hygiene unfortunately is commonplace amongst denture wearers, more often than not (de Castellucci Barbosa et al. 2008). This lack of knowledge may be the result of inconsistent recommendations from health care professionals, and in turn reflecting a lack of evidence of efficacy of treatments (Axe et al. 2016, Kiesow et al. 2016). Poor hygiene is thought to lead to the accumulation of plaque, subsequently increasing the complexity and diversity of the microbiome. Though, the data herein describe significant differences in diversity between healthy and diseased patients only at the mucosa, and not on the denture (O'Donnell et al. 2015). Therefore, compositional changes appear to relate to the site of inflammation rather than the prosthesis. Further investigation is required to determine if these changes are as result of poor hygiene. Initial culture analysis of the candidal denture colonisation showed that irrespective of denture hygiene habits, no differences in candidal colonisation was observed. Independent assessment of these *Candida albicans* isolates from this patient cohort was performed, and showed that there were no differences in their capacity to form biofilms (O'Donnell et al. 2017), suggesting that candidal colonisation is not solely the most important influence on denture microbiology and associated pathogenesis. It was hypothesised that the contribution of bacterial microbiomes may be more influential. However, there was a surprising lack of differences in microbial composition and diversity between those with satisfactory and those with poor denture hygiene, particularly given the abundant evidence demonstrating a link between poor denture hygiene and the development of oral diseases, e.g. gingivitis, periodontal disease, and candidiasis (Loe, 2000, Akpan & Morgan, 2002, Pihlstrom et al. 2005, Darwazeh et al. 2010). 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 (O'Donnell et al. 2015). Though, interestingly no microbiome differences were observed directly from the denture or indirectly on the mucosa in those partially dentate participants. However, the low number of samples may account for lack of observable significance. 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.

Also of interest was if denture hygiene influenced these microbiome changes in terms of denture related disease, so to determine if denture hygiene practices indeed influence mucosal inflammation. The results suggest having DIS indicated that any changes induced by poor denture hygiene were not influenced by microbiome changes *per se*. However, the interpretation of these data must be taken with caution, as it is not possible to definitively state that denture hygiene practices influence the composition of denture plaque opposed to the absolute quantity. Moreover, the influence of specific microbial interactions cannot be discerned, and a caveat to this study is that only DNA was investigated compositionally rather than the metatranscriptome, which would enable demonstration of how specific microbial interactions influences disease outcomes. Moreover, 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 due to colonization of streptococcal species, which are the main primary colonizers in the oral cavity (Syed & Loesche 1978, Moore et al. 1982). These would be more apparent on the denture surface of those that cleaned twice per day as they will likely have relatively immature plaque and 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). For gingival health, regular denture hygiene and maintenance of an ‘immature plaque’ is considered a useful target. However, it remains unclear whether the same applies to denture hygiene, and does beg the question, as to whether maintaining a strict hygiene regime is as important as we have been led to believe? Though, refraining from frequent denture hygiene habits cannot be condoned.

Sleeping with a denture *in situ* poses several risks, including choking and developing aspiration pneumonia (Iinuma et al. 2015, O'Donnell 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 a 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*, organisms of an anaerobic nature and often associated with oral disease. There is little information on the role of either *Bacteroidia* or *Epsilonproteobacteria* within the oral cavity, and whether they are pathogenic in nature. However, the role of *Fusobacteria* within the oral cavity is well documented (Aas et al. 2005). Whether the increasing levels of this microorganism can be associated with the development of disease remains unknown. Moreover, upon the denture surface the most notable change was the increase in another anaerobe, *Clostridia*, for those sleeping with their denture *in situ*. This pattern was apparent even when the groups were separated into healthy and DIS. This suggests 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.

It has been shown that denture microbiome composition development is a multifactorial process. Thus, this makes it difficult to define what the healthy oral microbiome of a denture wearer is exactly. However, the findings do suggest that maintaining satisfactory denture hygiene and hygiene practices do not appear to have a strong influence on altering the microbial composition as had been previously hypothesised. Therefore, modeling denture cleansing habits using an *in vitro* denture cleansing model (Sherry et al. 2016), or through carefully conducted randomized controlled clinical trials (Duyck et al. 2016), to evaluate longitudinal dynamic changes in the denture plaque will be more informative than existing cross-sectional study designs. Moreover, focusing solely on the microbiology of dentures does not completely represent all the problems denture wearers experience.

3.6 Summary

This study has specifically investigated the relationship between denture hygiene and the oral microbiome of denture wearers. Maintaining good denture hygiene and hygiene practices do not appear to have a strong influence on altering the denture microbial composition. Though, the absence of dental hygiene is not recommended.

As *Candida* and its colonisation on various denture base materials is still a contentious issue based on the available evidence, an *in vitro* experiment to further investage this relationship of denture base materials, *Candia* colonisation and denture cleansers was designed.

Chapter 4: Denture material and decontamination

4.1 Introduction

Budtz-Jorgensen and Bertram (1970) reported a significant association between inflammation and *Candida* colonization in patients with denture stomatitis (Budtz-Jorgensen and Bertram 1970). This association has been confirmed and over-emphasised in subsequent studies (Nanetti et al. 1993, Kulak-Ozkan et al. 2002, Dar-Odeh and Shehabi 2003, Baena-Monroy et al. 2005, Coco et al. 2008, Dagistan et al. 2009).

Scanning electron microscopy has been used to demonstrate the propensity of *Candida* biofilms to adhere along imperfections and cracks on denture surfaces (Ramage 2004). Further investigators have shown a rough surface to be more susceptible to biofilm formation, thereby increasing the risk of DIS development (Radford et al. 1998, Radford et al. 1999, Nevzatoglu et al. 2007, Pereira-Cenci et al. 2007, Webb BC 1998). Von Fraunhofer and Loewy (2009) reviewed factors involved in microbial attachment and colonization of denture surfaces, confirming that surface cracks and surface roughness facilitate attachment of microorganisms and development of the biofilm (von Fraunhofer and Loewy 2009). Filamentous cell forms of *Candida* were shown to become deeply embedded within surface deformities. Furthermore, these authors noted that prolonged brushing of denture PMMA with a toothbrush and abrasive dentifrices can create surface scratches that can enhance bacterial attachment and biofilm growth (Charman et al. 2009).

Differing denture materials have been suggested to play a role in promoting DIS (Budtz-Jorgensen 1970, Figueiral et al. 2007). Most (99%) of the dentures in the study were fabricated from PMMA, and/or metal. Macroscopically these materials have a different surface integrity, and it seems materials with a rougher surface exhibit higher yeast counts (von Fraunhofer and Loewy 2009). Though there are many alternative denture base materials available to clinicians. A group of denture materials collectively termed denture reline materials are a popular alternative to PMMA. Their use is dependent on the clinical situation usually to increase patient comfort. Studies conducted by Verran and colleagues (1997) indicated that *C. albicans* cell numbers were significantly higher on rough versus smooth acrylic and silicone surfaces following a one hour incubation (Verran and Maryan 1997). Likewise investigators have shown that chair-side addition soft denture liners have a higher surface roughness and the porous texture entraps yeast (Lefebvre et al.

2001, Pereira-Cenci et al. 2007, Tari et al. 2007). Though Moloplast B™ a silicone, however has not shown increased growth of *Candida* species and DIS over PMMA. (Wright et al. 1985, Nikawa et al. 2000).

A regular and thorough denture-cleaning regime is considered to be appropriate to prevent DIS development. Denture wearing patients can either mechanically and/or chemically clean their dentures. A denture cleanser is seen as an adjunctive chemical form of cleaning ones denture. 76% of dentists recommend denture cleansers as a method of denture decontamination (Dikbas, et al. 2006, Johnson 2013, Milward et al. 2013). A cross-sectional survey of denture hygiene in 1,000 edentulous patients from a University hospital concluded that 69% of denture wearers had inadequate denture hygiene measures. 35% of these patients used a denture cleansers (Milward et al. 2013). Of the current study population 44 (34%) claimed to use some variation of chemical disinfection as part of their denture hygiene practice. Whether denture cleansers are used alone or in combination with mechanical forms of cleaning, residual biofilm cells have been shown to persist on PMMA (Jose et al. 2010, Sherry et al 2016). Therefore, there is little known about how the various over the counter denture cleansers may affect the denture biofilm or indeed their effects on the denture materials themselves.

4.2 Aims

It is hypothesised that denture base material will affect the colonisation and subsequent disinfection of candidal biofilms. As only limited clinical data on candidal presence and denture materials were available, a series of *in vitro* experiments were designed to test if differing denture materials influenced the adhesion and growth of *C. albicans*.

Given the range of denture cleansers commercially available and the varying advice given by dental professionals in their use, the existing *in vitro* model was used to test the performance of 3 popular denture cleansers on candidal biomass and viability. Part of this work has been published in the Journal of Medical Microbiology (2017) by O'Donnell, L. E, Alalwan, H. K., Kean, R., **Calvert, G.,** Nile, C. J., Lappin, D. F., Robertson, D., Williams, C., Ramage, G. & Sherry, L (66, p54-60), in work entitled '*Candida albicans* biofilm heterogeneity does not influence denture stomatitis but strongly influences denture cleansing capacity'.

4.3 Methods

4.3.1 Microbial sampling

Ethical approval and clinical procedures are as described in chapter 2. Patient candidal culture isolates stored in chapter 2 were used for this series of *in vitro* experiments.

4.3.2 Denture materials

The three commonly used denture reline materials selected for investigation were:

Ufi gel SC® (Voco, Germany) is an addition cured silicone based non-hardening reline material. This comes in a cartridge with mixing tips so the correct mixing ratio is reproducible. It is commonly used chair side to reline a patient's denture and is self-cured in 5 min.

EverSoft® (Dentsply, Germany) is a plasticised methacrylate soft reline material. It comes in a powder and liquid form that can be mixed and added to the denture chair side. Its polymerisation is self-cured and accelerated by heat as the manufacturer recommends its submersion in warm water for 15 min prior to use by the patient.

Moloplast B® (Detax, Germany) is a single component silicone based non-hardening reline material. It is supplied by the manufacturer in a mono-phase consistency and requires processing with PMMA denture base at time of fabrication. This is a laboratory stage and therefore cannot be done chair side.

Conventional heat cured PMMA (Chaperlin & Jacobs Ltd) denture base material was used as the control material.

4.3.3 Bespoke flasks

Similar sized discs of each material were produced bespoke in order to comply with the dimensions of 24-well plates, which are used downstream for microbiological analyses. Customised 14mm diameter by 2mm height wax discs were produced using a preformed wax punch from sheets of 2mm thickness wax.

These were then flasked in 100% dental stone (Super yellow, John Winter & Co Ltd, UK) using a manufacturer recommended water to powder ratio of 100g to 20mL and vacuum mixed to avoid air entrapment. The stone mix was placed into the shallow half of the denture flask and the wax discs were evenly distributed across the surface. To avoid air blows, each disc was coated with a wetted film of the dental stone. Once this half of the flask was set, a separating medium (50% sodium silicate solution) was added to the stone surface and rinsed off. The mould was completed by filling the second part of the flask with dental stone covering the wax disc. During this process the denture flask was placed on a vibrating platform to ensure any air bubbles were removed from the surface of the wax. The completed flask was left to set over night. Once set the flask was opened and wax removed by steam cleaning and rinsing with a detergent to remove any residue from the mould pattern.

4.3.4 Preparation of denture materials

4.3.4.1 PMMA

The PMMA acrylic was mixed according to manufacturer's instructions. 24g of power was added to 10mL of denture base liquid (Chaperlin & Jacobs Ltd, UK) and thoroughly mixed for 30 sec. This was left for approximately 10 min until a snap-dough texture was present. A separating liquid (Metrodent, UK) was painted on the surface of the mould halves and allowed to dry. The acrylic dough was placed on one half of the mould and spread by hand. The opposing flask section was secured on top and compressed in a hydraulic press to 100 bar. The excess acrylic escaped from the sides and was trimmed away. The flask was then transferred to a vice to maintain continual pressure during the heat curing process. In accordance with the manufacturer's instructions the flask was submerged in a water bath to heat to a temperature of 70°C for a 7 h period and then maintained at 100°C for 3 h. The flasks were left in the water bath to cool overnight. Following polymerisation the flask was opened and the cured acrylic discs were removed. The edges of all the discs were polished with a rotary bur to ensure a smooth finish.

4.3.4.2 Reline materials

Between each fabrication cycle the mould pattern was steam cleaned and rinsed with a detergent ensuring a clean stone surface to apply another layer of separating liquid (Metrodent, UK). Ufi gel SC® (Voco, Germany) was expressed over the stone surface from the cartridge through a manufacturer recommended mixing tip. The flask was again closed, compressed, and maintained in a vice for 5 min until completely set as per manufacturer instructions. EverSoft® (Dentsply, Germany) powder and liquid was mixed in a ratio of 2:1 by volume as per manufacturer instructions and spread across the stone surface. The flask was closed, compressed, and maintained in a vice for 15 min submerged in water at 60°C until completely cured as per manufacturer instructions. Moloplast B® (Detax, Germany) silicone was taken straight from the container and spread across the stone surface. The flask was closed, compressed, and maintained in a vice while submerged in a water bath at 100ºC for 1 hour, and allowed to cool to room temperature as per manufacturer instructions to complete the curing process. All the reline material disc edges were polished with a rotary bur to ensure a smooth finish. To ensure no remaining free monomer could leach from the PMMA discs they were submerged in water for one week.

Figure 4.1: Example of the bespoke flasks used to fabricate discs of denture base materials.

4.3.4.3 Disc cleaning

Discs were added to 500mL of ddH_2O containing 1 Haz (Guest Medical) tablet for 24 h. Discs were then sonicated for 15 min in ddH_2O, treated with 100% ethanol for 2 h, sonicated once more in ddH_2O and each side of the disc UV treated for 15 min. This is a recognised method to eliminate contamination of the disc within our research group (Sheery et al. 2014).

4.3.5 *Candida albicans* growth

Selected strains of *C. albicans* were use for *in vitro* studies of microbial adhesion and biofilm formation on denture materials. A characterised laboratory strain of *C. albicans* (SC5314), and two clinical strains isolated form participants of this study were used (GSK90 and GSK106). These isolates were sub-cultured onto Sabouraud's dextrose agar (SAB [Sigma-Aldrich, Dorset, UK]). Plates were incubated at 30°C for 48 h. Isolates were propagated in yeast peptone dextrose (YPD) medium (Sigma-Aldrich, Dorset, UK), washed by centrifugation and standardised in the appropriate media (Sigma-Aldrich) to (1 × 107 cfu/mL). Cells were centrifuged at 3000rpm for 5 min and the resultant supernatant discarded. The remaining pellet was washed with 10mL of phosphate buffered saline (PBS [Sigma-Aldrich, Dorset, UK]) and centrifuged for 5 min at 3000rpm. The supernatant was discarded, the pellet resuspended in PBS, and the cells were counted using a Neubauer haemocytometer. *C. albicans* was standardized (1 × 10^7 cfu/mL) in artificial saliva (AS), which contained the following constituents: 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), and proteose peptone (0.5 w/v) in ddH2O (Sigma, Poole, UK). Urea was then added independently to a final concentration of 0.05% (v/v). For each of the 3 *C. albicans* strains used a 24 well plate contained 4 discs of each denture material. Three discs of each material were covered with 1ml of the *C. albicans*-AS inoculum. The remaining disc was covered with AS solution alone as a control. The plates were sealed and incubated for 4 and 24 h at 37^{o}C. The 4 h experiments were performed by Mr Hasanain Alalwan (PhD student, University of Glasgow), though data included in the analysis for comparison purposes.

4.3.5.1 DNA Extraction

Biofilms grown on each of the denture substrates were removed, disrupted and made into homogeneous suspensions by sonication at 35 kHz in PBS in a sonic bath for 10 min. These suspensions were transferred to RNase-free microfuge tubes. The sonicates were centrifuged for 10 min at 3000rpm, re-suspended in proteinase K extraction buffer and incubated at 55^{o}C for 20 min. DNA was extracted using the QIAmp mini DNA extraction kit (QIAGEN,) according to manufacturer's instructions, with a minor modification to include a mechanical disruption step with sterile acid-washed glass beads of 0.5 mm diameter (Thistle Scientific, Glasgow, UK). This was achieved by bead beating for 3 × 30 sec on a Mini-BeadBeater (Sigma-Aldrich, Gillingham, UK), while intermittently being placed on ice. DNA quality and quantity were then assessed using a NanoDrop® spectrophotometer (ThermoScientific, Loughborough, UK).

4.3.5.2 qPCR

Primers specific to the fungal 18S gene were used to carry out quantitative PCR. Primer sequences were checked for specificity to each target organism using the NIH-BLAST database. PCR amplification efficiency of the primer sets were optimised prior to gene expression analysis, with an efficiency of 96%. PCR was performed using the forward primer (F:CTCGTAGTTGAACCTTGGGC) and the reverse primer (R:GGCCTGCTTTGAACACTCTA). 200ng of DNA was used in a mastermix containing SYBR® GreenER™ (Life Technologies, Paisley, UK), UV-treated RNase-free water and forward/reverse primers (10 µM), following manufacturers instructions. qPCR was carried out using the step one plus real-time PCR unit (Applied Biosciences, UK), under the following conditions; 50^{o}C for 2 min, 95^{o}C for 2 min followed by 40 cycles of 95^{o}C for 3 sec and 60^{o}C for 30 sec. 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 colony forming equivalents (CFE's) based on standard curves created from serial ten-fold dilutions of *C. albicans*.

4.3.5.3 Quantification of biofilm biomass

For biomass quantification, the biofilms were air dried, 0.5% (w/v) crystal violet solution added for 5 min, and the solution then removed by carefully rinsing the biofilms under running water until excess stain was removed. These were de-stained by adding 1ml of 95% (v/v) ethanol into each well. The ethanol was gently pipetted to completely solubilize the crystal violet for 1 min.

4.3.5.4 Antimicrobial treatment of Candida albicans biofilms

Pre formed biofilms of *C. albicans* clinical isolates were selected to test a range of popular over the counter denture cleansers (Steradent, Milton and Poligrip). To minimise any effects of denture substrate, all isolates were grown in a 96-well flat-bottomed polystyrene plate for 4 h and 24 h, using standardised methodologies (Pierce et al 2008). Two hundred µl of these solutions were added to each biofilm and treated, as per defined by the manufacturer (10 min for Steradent; 15 min for Milton; 3 min for Poligrip). Untreated controls were included for comparison. Following treatment, biofilms were washed with PBS and standardised XTT and CV assays were carried out to assess biofilm metabolic activity and biomass respectively, as described previously (Sherry et al 2014). Scanning electron microscopy (SEM) was performed on the *C. albicans* biofilms grown on PMMA discs to visualise the effects of denture cleansing. Following 4 h and 24 h biofilm development, cells were treated with the various denture cleansers before carefully washed with PBS, fixed, processed and imaged, as previously described (Rajendran et al 2014).

4.3.6 Statistical analysis

Graph production and statistical analysis were performed using Prism 5.01 (Microsoft). Data were first assessed if they conformed to a normal distribution and were log transformed as necessary. T-Tests were used to compare cnadidal loads od healthy and diseased patients. A one-way ANOVA with Bonferroni correction was used to measure statistical differences between biofilms grown on the varios denture materials and antimicrobial viability testing of the isolates. A Kruskal-Wallis test with Dunn’s post test was used to measure differences in biofilm

biomass following treatment with different denture cleansers. All studies were performed alongside and in collaboration Mr Hasanain Alalwan (PhD student).

4.4 Results

4.4.1 Biofilm biomass on various denture substrata

The denture liner EverSoft® (Dentsply, Germany) was initially investigated, but the ability to produce discs routinely was not possible due to post-production distortion (Figure 3.4), and were therefore excluded from the study.

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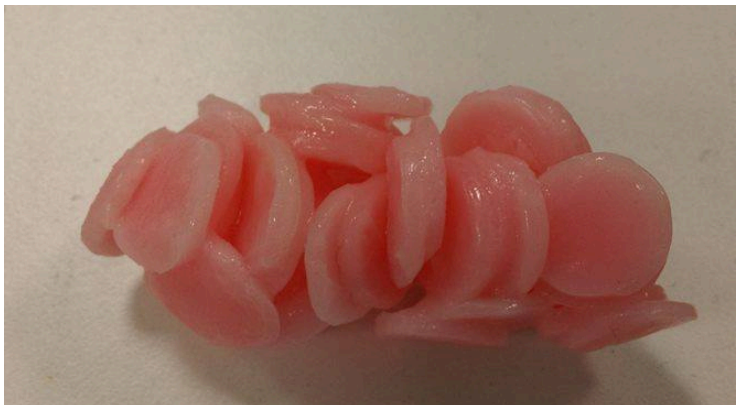


Figure 4.2: Example of damaged EverSoft® discs due to cleaning.

The impact of denture substratum upon *C. albicans* biofilm formation was assessed (Fig 4.3). At 24 h of biofilm formation, PMMA was the denture material with the most significant *C. albicans* burden (4.21×10^8 CFE), this being 2.8 times and 4.1 times greater than those of MOLLO ($p<0.05$) and UFI ($p<0.05$), respectively. Unsurprisingly, *C. albicans* biomass was significantly more abundant in 24 h biofilms than in their 4 h counterparts for each denture substratum; PMMA ($p<0.001$), MOLLO ($p<0.05$) and UFI ($p<0.001$). At early stages of biofilm development (4 h), no significant candidal burden was observed between the three substrata ($p>0.05$).

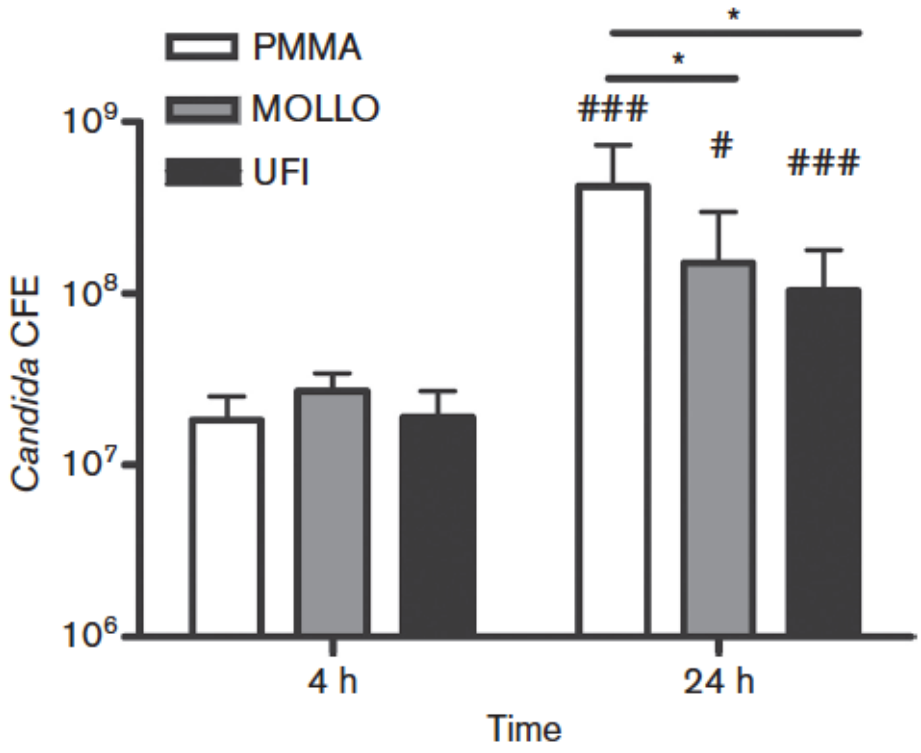


Figure 4.3: Impact of the denture substratum on *Candida* biofilm formation. *C. albicans* SC5314, and 2 clinical strains were grown as biofilms on PMMA, MOLLO and UFI denture materials, to assess biofilm formation at 4 h and 24 h. Data represents mean ± SD of thee isolates combined together. 24 h data was compared to 4 h counterparts (#$p<0.05$, ###$p<0.001$). Significant differences were also observed between different denture substratum at 24 h (*$p<0.05$).

4.4.2 Treatment of *C. albicans* biofilms with antimicrobial denture cleansers

4.4.2.1 Effect of denture cleansers on C. *albicans* biomass

Milton was the most effective cleanser against early biofilms (4 h), reducing biomass by 55%, compared to the untreated control ($p<0.05$), as shown in Figure 4.4. Poligrip was the second most active agent, reducing biomass by 28%. However, Steradent led to an increase in the denture biomass by 9%. When mature biofilms were considered (24 h versus 4 h), unsurprisingly, biomass increased by 3.3, 2.8, 5.2 and 5 times for the untreated, Steradent, Milton and Poligrip, respectively. However, no significant differences were observed when comparing the denture cleanser treatments to control biofilms.

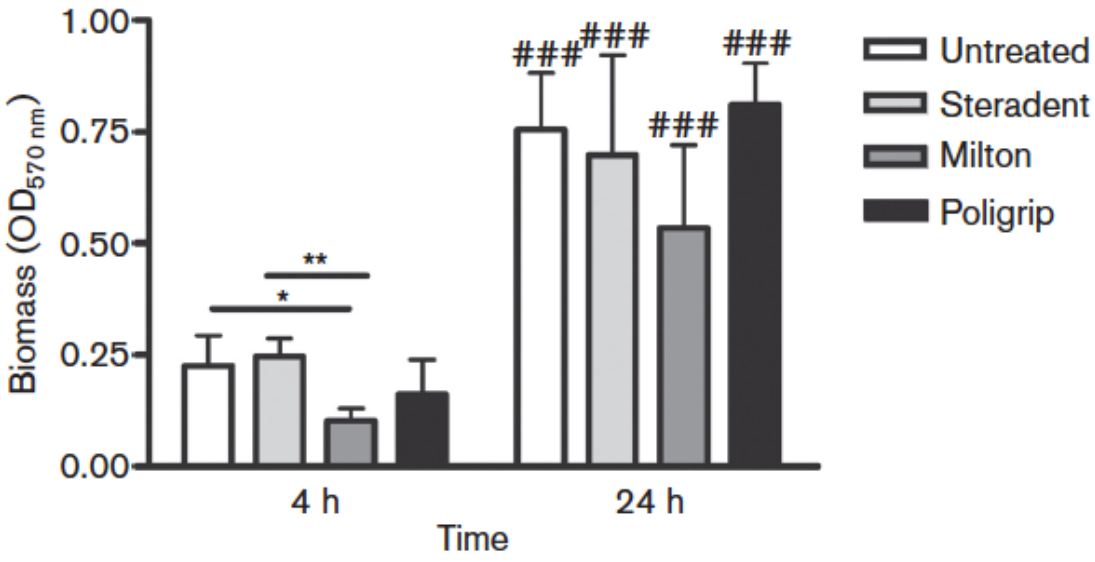


Figure 4.4: The effects of thee over-the-counter (OTC) denture cleansers on *C. albicans* biomass at 4 h and 24 h. Data represents mean ± SD. Significant differences were observed when comparing each cleanser at 4 h to their 24 h counterparts (##$p<0.01$ and ###$p<0.001$), as well as comparing cleansers to one another (*$p<0.05$, **$p<0.01$ and ***$p<0.001$).

4.4.2.2 Effect of denture cleansers on *C. albicans* viability

Early (4 h) and late (24 h) candidal biofilm cell viability after treatment with each denture cleanser is seen in Figure 3.7. Milton reduced metabolic activity to 17% and 56% in 4 h ($p<0.001$) and 24 h ($p<0.05$) biofilms, respectively (Figure 4.5). The cellular viability at 4 h was less than that at 24 h. Poligrip had little impact on biofilm development, with 90% viable cells remaining following treatment of 4 h biofilms, and 78% at 24 h. Interestingly, Steradent was shown to have the greatest activity against mature biofilms, reducing cell viability to 67% at 24 h, compared to no reduction at 4 h ($p<0.001$). This being said, all denture-cleansing regimens were unable to reduced 24 h biofilm viability to less that 50%.

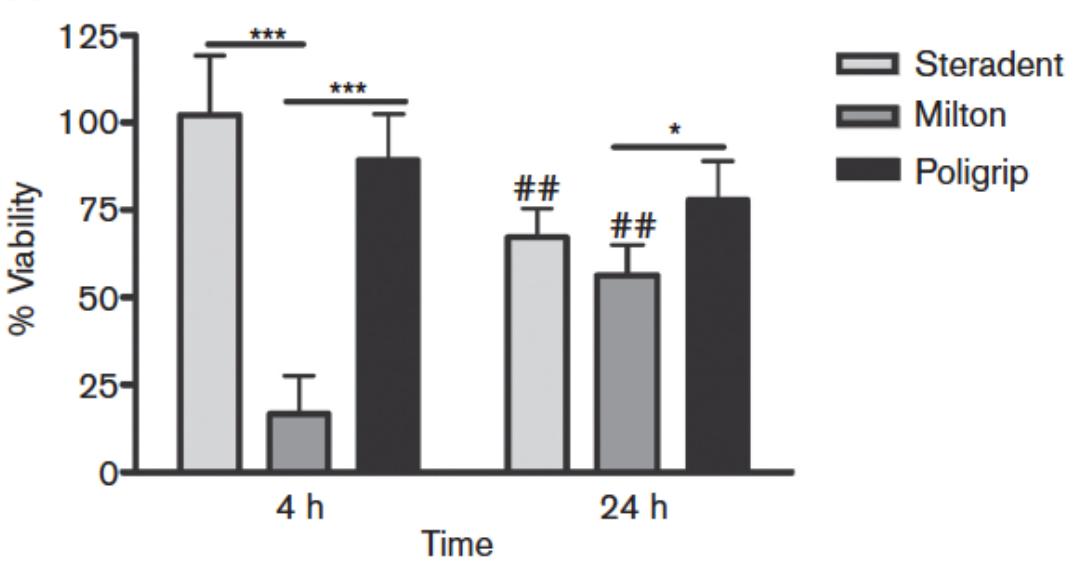


Figure 4.5: The effects of thee over-the-counter (OTC) denture cleansers on 4 h and 24 h *C. albicans* biofilm viability. Data represents mean ± SD. Significant differences were observed when comparing each cleanser at 4 h to their 24 h counterparts (##$p<0.01$ and ###$p<0.001$), as well as comparing cleansers to one another (*$p<0.05$, **$p<0.01$ and ***$p<0.001$).

4.4.2.3 Biofilm ultrastructural changes

Ultrastructural changes of the treated denture biofilms are illustrated in Figure 4.6. Steradent treated early biofilms exhibited minimal changes in appearance compared to the untreated control. However, at 24 h the biofilms were visually distinct, with a fibrous residue evident along the elongated hyphae. This may be a remnant of the denture cleanser retained within the biofilm as this was also observed in Milton and Poligrip mature biofilms.

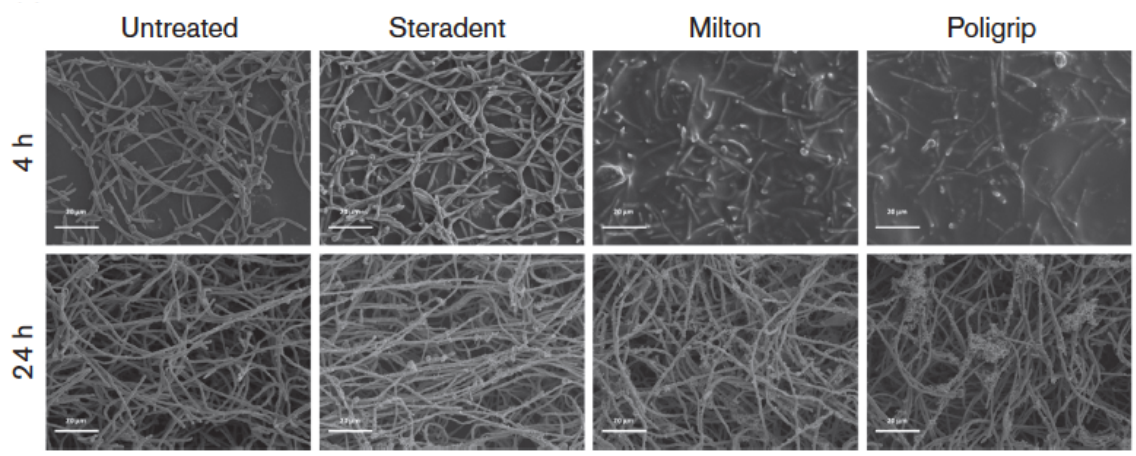


Figure 4.6: Assessing antimicrobial denture cleansers on *Candida albicans* biofilm formation at 4 h and 24 h. All images are shown at 1000× magnifications Scale bars represent 20 µm.

4.5 Discussion

One denture related factor that may dictate the microbiology of denture plaque is substrata. Changing this environment can vastly influence *C. albicans* biofilm formation and survival. Initial adhesion of microbes to dentures is key to their pathogenicity, yet denture substrata are highly variable, which may support differential adherence and biofilm formation. PMMA is classically used as a denture substratum, though its chemistry and topography create a preferential surface for biofilm formation (Park 2003). Alternative soft liner materials are increasing in popularity as they are softer against the mucosa (Palla et al. 2015). Studies comparing microbial growth on hard and soft denture liners have shown conflicting results, as one study comparing *Candida* biofilm growth on various soft denture liners found no significant differences in the quantity of cells recovered between materials (Mutluay et al. 2010). Unfortunately during cleaning of the denture reline materials the soft acrylic based material was damaged leaving only the two silicone based reline materials to examine. This study found that although there was no significant difference in microbial colonisation during early biofilm formation, PMMA became the most prone material to biofilm formation at later stages of development. A possible explanation of this greater colonisation on PMMA is because soft liners are even more porous in nature than hard acrylics (Wright et al. 1998) and thus higher numbers of microbes may become imbedded in these cracks and crevices. Therefore, even a powerful method such as sonication may not be sufficient enough to completely dislodge and fully appreciate the microorganisms hidden within these soft liners. There was no significant difference in the numbers of *Candida* counts between PMMA and the reline materials or between the individual reline materials at 4 h. This may be explained as the materials were only recently fabricated and not subject to wear and tear which can facilitate biofilm formation in surface defects as reline materials are more prone to degradation than PMMA. Yet the environment of the experiment was kept as similar to the oral environment as possible using whole saliva and *Candida* strains isolated from participants of the study. Other investigators have suggested that *C. albicans* adherence is affected by hydrophobicity such that materials with hydrophobic coatings or silicones may decrease the attachment and colonization by *C. albicans* (Yoshijima et al. 2010). This is the first study to use molecular methods such as qPCR to quantify biofilms on denture acrylics, in order to give more accurate quantitative microbial counts. The vast majority of studies in

this area have relied on culturing and staining techniques for quantification, and thus the methods used may account for the differences seen across studies (Bal 2008, Mutluay et al. 2010, Radford et al. 1998).

Currently, there is no universal method for cleaning dentures. 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). 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). There are numerous denture cleansers available. Therefore, three of the most popular brands - Steradent, Milton and Poligrip – were selected to assess their antifungal activity. Milton was the most effective treatment at reducing biomass and cell viability on early (4 h) biofilms. Yet, it did not completely eradicate the biofilm. Such results are interesting given previous findings showing that this treatment completely inhibited biofilm formation at 4 h (Ramage et al 2012). Most likely Milton was more efficient at reducing cellular viability of 4 h biofilms due to early biofilms not being as complex as their 24 h counterparts, meaning antimicrobials are able to penetrate more easily, elicit their activity and reduce viability more readily than they can in mature biofilms. Interestingly, at 4 h, the hyphal cells appeared to have become embedded with the denture acrylic, which may be due to the cleansers becoming incorporated within the material and changing the surface topography, as suggested elsewhere (Koroglu et al. 2016, Peracini 2010).

Methodological constraints due to conventional CFU quantification may explain the difference in biomass and viability, since it has been recently demonstrated counting CFU can give false negative results. By using live/dead qPCR on the same experiment, ~ 1 x 10^5 cells/ml viable cells were detected, where CFU counting detected no live cells (Sherry et al. 2016). Moreover, as the biofilms matured, Milton lost its effect, most likely because of the thicker biofilm, which would be more difficult to penetrate and disrupt. Yet, other work has shown denture cleansers and antimicrobial mouthwashes can significantly reduce the viability of mature *C. albicans* denture isolates (Jose et al. 2010, Ramage et al. 2011). These studies do not confirm the biofilm forming capacity of the assayed isolates, whereas this study used known HBFs, which could explain why our

isolates were more resistant to treatment, a phenomenon reported elsewhere with these phenotypes (Ramage et al. 2012). Moreover, previous studies have shown that *C. albicans* is not completely eradicated from the denture, even when using various denture cleansing treatments (Ramage et al. 2012). The collection of SEM images supports this as they show that *C. albicans* cells remain on the denture acrylic irrespective of treatment. It cannot be guaranteed that all these cells were dead and therefore it is likely that some are still alive and could begin to recolonise the denture when placed back into the mouth. Furthermore, full penetration of the biofilms is unlikely due to the protective effects against antimicrobials, such as efflux pumps and extracellular glucans, provided by the extra cellular matrix (Rajendran et al. 2014, Taff et al. 2012).

4.6 Summary

From the data it appears that both PMMA and silicone substrates had a significant increase in candidal colonisation between 4 h and 24 h time periods. At 24 h PMMA had a significantly greater *candida* load than the silicone reline materials. Neither Steradent, Milton or Poligrip completely removed the biofilm or eliminated candida viability. Milton, however, appeared to perform better on 4 h biofilm growth than on 24 h biofilm and out perform its two commercial counterparts. Though whether this is clinically significant is another matter. The data does however provide insights into the importance of denture materials as reservoirs for adhesion of important pathogenic species.

Chapter 5. Final Discussion

5.1 Discussion

5.1.1 Denture induced stomatitis and systemic disease

DIS is a multi-factorial disease that has a high prevalence in the aging denture wearing population. While patients may be unaware of the disease process going on in their mouths, it is becoming better understood how this local infection can impact negatively on their systemic health. Therefore, the importance of prevention, or early detection, and a subsequent timely resolution could be of key importance for patients' systemic health. This is particularly highlighted from a recent study where 65% of individuals wearing a denture harboured significant quantities of pathogens capable of causing respiratory infections, regardless of existing oral disease status (O'Donnell, et al 2015). This suggests that even denture wearers with healthy palatal mucosa and good oral hygiene carry an increased systemic risk. With aspiration pneumonia being the leading cause of death of individuals over 65 years old, denture wearing individuals could be at greater risk of developing life-threatening infections due to putative respiratory pathogens residing upon their denture (Guest and Morris 1997).

5.1.2 Oral health and clinical implications of denture induced stomatitis

Denture wearers may encounter various health care workers in dental, general medicine, or indeed residential environments. With the shift in ageing population demographics and number in residential care, a consistent, effective, and most importantly, simple message regarding prevention of oral disease is important (Burnett et al 1993, Foltyn 2017). As previously discussed, DIS is common-place in the ageing population and this body of work has confirmed the prevalence of DIS in Glasgow is no different to other UK regions (Barbeau 2003, Shulman and Rivera-Hidalgo 2005). Therefore, what advice should health care professionals be providing to denture wearers and their family/carers to prevent the development of DIS or systemic illness? For example gender and age have been quoted repeatedly as being risk factors for DIS, but pragmatically, are these risk factors actually modifiable? The data has confirmed smoking, poor denture hygiene and wearing a denture while sleeping increased the odds of DIS by 3.7, 3.5, and 2.8

times, respectively. While it maybe considered easy to inform a patient not to wear a denture while sleeping, their emotional state and social acceptance to appear toothless even while sleeping may not tolerate this advice. This is supported by Burnett and colleagues (1993) who concluded that verbal and written information on denture care given to individuals may need to be supplemented to be effective in implementing change (Burnett et al 1993). Rather controversially, the data show denture age and cleaning frequency habits, seem to have little influence on the presence of DIS. Therefore, remaking a patients denture because it is old will have little effect on the presence of DIS. This message would potentially save the NHS time and resources, and prevent patients investing time and finance into fabrication of a prosthesis that would have little to no oral health benefits. However, informing patients that the number of times they clean their denture is not important in the development of oral disease could be misinterpreted by patients and be counter productive. What could be more clinically relevant is to stress that the quality of denture cleaning is more important than quantity, i.e. meticulously cleaning their denture once daily is better than poorly cleaning the denture three time a day. Though, what constitutes a meticulous clean, macroscopically or indeed microscopically is still to be determined.

As previously stated, the majority of the denture wearers are in the aging population, and as such their physical dexterity for mechanical cleaning of a complex dental prosthesis maybe lacking. The importance of chemical disinfection may therefore be of greater relevance. There are many different denture-cleaning aids available to people that wear a removable prosthesis. These include a variety of over-the-counter denture cleansing solutions and antimicrobial mouth washes. While health care professional rely on industry to develop new products for patients to use, their efficacy must be justified, as they have financial implications for patients and potentially negative effects on the materials they are designed for. However, the data present herein would suggest some of the most popular denture cleansers have a little effect on mature biofilm viability. When you consider the previous findings that the majority of participants wore a denture while sleeping, the development of a mature biofilm is inevitable. Therefore clinically, the importance of removing the denture while sleeping may have a two-fold advantage, helping to reduce the risk of DIS, but furthermore it may be beneficial for the effectiveness of denture cleansers to reduce the viability of the denture microbiome.

It is recognised that participants were recruited to the study by convenience sampling, a non probability based sampling technique which suffers from bias. The generalizability of the results herein has a limit to populations out with the sample studied. However, the measured prevalence of DIS (37.4%) was within the average range quoted for UK and American populations (Burnett et al 1993, Foltyn 2017). Furthermore, when considering the numerous patient and denture related factors, there is the potential for these subgroups to be underrepresented in the sample. Further analysis to examine if these risk factors were independent of each other would require statistical regression analysis, something this study was not powered for.

5.1.3 Denture induced stomatitis and *Candida* species

Historically, denture-related research has a disproportionate focus on yeast-associated infection (Coco et al. 2008), yet there is a distinct lack of understanding of the bacterial microbiome within the oral cavity of the denture wearer. The development of the denture microbiome composition has many environmental determinants. Thus, this makes it difficult to define what the healthy oral microbiome of denture wearer is exactly. Unlike the distinct health and disease-associated microbiota reported for other oral diseases such as periodontitis, the bacterial communities residing on dentures in health and disease have been shown to be rather similar to each other (O'Donnell et al. 2017). Initial culture analysis of the candidal denture colonisation showed that irrespective of denture hygiene habits, no differences in candidal colonisation was observed. It was suggested that candidal colonisation is not solely the most important influence on denture microbiology and associated pathogenesis (O'Donnell et al 2015). Therefore, the overall microbial load may have a greater impact on stomatitis development than the actual microbial composition of the denture plaque (Shi et al. 2016). Similarly, other ecological niches in the mouth may have a synergistic or inhibitor relevance on the denture microbiome. For example, bacterial plaque that collect around teeth. While it is wildly reported complete dentures carry a greater risk for patients to develop DIS because they cover a greater surface area of the mucosa, the current data suggests in fact partial dentures were more associated with DIS (Emami et al. 2008, Jainkittivong 2010, Salerno, et al. 2011). This may be

explained because the biological load of a patient with both teeth and dentures have potentially twice the microbial burden.

In contrast, it has been shown in other host environments, for example the in the intestinal and female reproductive tracts, bacteria compete with *C. albicans* for adhesion sites and secrete substances that inhibit fungal attachment to control *C. albicans* invasion and disease (Boris and Barbes 2000). Treatment with broad-spectrum antibiotics is a risk for imbalance in the normal microbiome, and is a predisposing factor associated with *C. albicans* colonization while bacterial suppression occurs to minimise selective competition (Hogenauer et al 1998). Thus, antibiotic therapies that specifically target pathogens, in contrast to broad-spectrum antibiotics, may help prevent secondary problems that arise upon bacterial-fungal interactions. These adhesive interactions between *C. albicans* and other indigenous oral microbes have been identified as being important for co-adhesion to mixed microbial communities in biofilms. Biofilms of *C. albicans* and oral streptococci are similarly more resistant to antibiotics than their single species counterparts (Morals and Hogan 2010). Though, when considering DIS, the prescription of antifungal agents is clinically effective at resolving the oral lesion. So while bacteria and fungi interactions likely occur in the denture microbiome, yeasts do have an important role in the clinical presentation of DIS and so cannot be dismissed completely. However, at the population level, an oral health message reliant on antifungal medication alone for the resolution of DIS would have significant detrimental ramifications i.e. increased fungal resistance (Wong et al. 2014.

The *in vitro* model used to test denture substrates and denture cleansers were based on clinical isolates. In light of the emerging evidence, a polymicrobial model would be a more clinically representative design, rather than mono-species *C. albicans* experimental design. Other factors to consider are the physical washing and antimicrobial effects of saliva. One factor not taken into consideration was the potential impact of denture adhesives. It is common-place for patients to rely on denture adhesive products for better function and comfort of their denture (Grasso 2004). Denture adhesives can be in form of creams, powders, sheets and cushion adhesives and can leave a residue on the denture surface, which is in contact with the mucosa. Therefore, it could be extrapolated that denture adhesives provide another reservoir for colonisation and could inhibit the action of denture cleansers.

Clinically, this may further implicate chemical disinfection is not adequate and compound the importance of mechanical cleaning. Though, this would require further investigation to confirm.

5.1 Further research

It is unlikely some of the patient and denture related factors identified here that contribute to DIS are mutually exclusive. With this in mind, a further study could be powered for multiple regression analysis of these factors to further understand their associations.

The oral microbiome in health and disease of patients wearing dentures requires further investigation to understand not only the longitudinal composition changes but also interactions between bacteria species and *Candida* which can be used to inform further *in vitro* modeling.

Using freshly made denture materials does not reflect the majority of patients wearing dentures. Therefore, either artificially aging the denture material or sampling patients' own worn dentures may provide a more representative surface to test candidal growth.

Antimicrobial denture cleansing treatments do not fully eradicate *C. albicans* isolates with an enhanced capacity to form biofilms, leaving behind live cells that can disperse and recolonise. An alternative to *in vitro* modeling could be carefully conducted randomized controlled clinical trials, to evaluate longitudinal dynamic changes in the denture plaque that will be more informative than existing cross-sectional study designs. Moreover, focusing solely on the microbiology of dentures does not completely represent all the problems denture wearers experience.

Appendices

Appendix 1. Participant demographic data collection sheet

University of Glasgow

Denture Biofilm Study

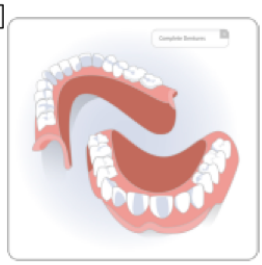
Study Code

Summary Clinical Report

Gender Male ☐ Female ☐ **Date of Birth** ___/___/___

COMPLETE DENTURES

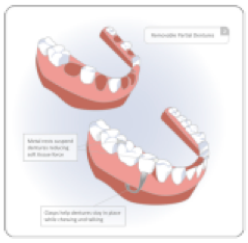
☐



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 ☐

18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38

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

Denture stomatitis (see Photos):

No Inflammation ☐ Grade 1 ☐ Grade 2 ☐ Grade3 ☐

Comment:

Soft tissue: Please comment on any other soft tissue lesions including obvious periodontal 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 ☐ **D** Plaque sample ☐ tooth number_____

B Sonicate ☐ **E** Saliva ☐

C Intra oral swab ☐ Specify area_____

Appendix 2. Participant consent

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	
7. I agree that the research team can access my clinical records	
8. 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 3. Ethical approval

WoSRES
West of Scotland Research Ethics Service

NHS
Greater Glasgow and Clyde

West of Scotland REC 3
Ground Floor – The Tennent Institute
Western Infirmary
38 Church Street
Glasgow G11 6NT
www.nhsggc.org.uk

Dr Douglas Robertson
Clinical Lecturer in Restorative Dentistry
University of Glasgow
D17 Glasgow Dental School
378 Sauchiehall Street
Glasgow
G2 3JZ

Date 17th January 2013
Your Ref
Our Ref
Direct line 0141 211 2123
Fax 0141 211 1847
E-mail Liz.Jamieson@ggc.scot.nhs.uk

Dear Dr Robertson

Study title:	**The influence of denture microbiota on oral and systemic disease**
REC reference:	**12/WS/0212**
IRAS project ID:	**101624**

Thank you for your recent email. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 29 August 2012

Documents received

The documents received were as follows:

Document	*Version*	*Date*
Participant Consent Form	2	10 December 2012
Participant Information Sheet	3	04 January 2013

Approved documents

The final list of approved documentation for the study is therefore as follows:

Document	*Version*	*Date*
Covering Letter		07 August 2012
Investigator CV		
Other: CV Key Investigator		
Other: CV Student - Lindsay O'Donnell		
Other: Grant Letter		14 June 2012

Participant Consent Form	2	10 December 2012
Participant Information Sheet	3	04 January 2013
Protocol	1	29 June 2012
Questionnaire: Oral Health	1	07 August 2012
REC application		08 August 2012
Summary/Synopsis		07 August 2012

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all participating sites.

12/WS/0212 **Please quote this number on all correspondence**

Yours sincerely

Mrs Liz Jamieson
Committee Co-ordinator

Copy to: *Dr Erica Packard, NHS Greater Glasgow and Clyde*

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