COLO NiSATION OF ACYLIC DENTURE
FITTING SURFACES BY CANDIDA
SPECIES

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A thesis submitted in partial fulfilment of requirements for the degree of

Doctor of Clinical Dentistry (Prosthodontics)

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Dunedin
New Zealand
2014
ABSTRACT

Denture-associated candidal infection is usually painless and localised to a relatively small area of the mouth but may lead to serious complications such as systemic yeast infection in susceptible patients. This includes those who have a compromised immune system, particularly the elderly.

One of the factors predisposing to denture-associated candidal infection is denture-induced trauma, where an ill-fitting denture creates a more favourable environment for the growth of the yeast. In this research project, we investigated the patterns of the colonisation by Candida spp. of the oral mucosal and acrylic denture fitting surface in patients with ill-fitting dentures and following delivery of new dentures.

Sixteen edentulous patients attending the Faculty of Dentistry, University of Otago, for fabrication of new complete dentures were enrolled in the study; ten of these participants completed the study. Saliva and mucosal swab samples were collected from each participant and incubated on CHROMagar Candida plates. CHROMagar Candida impressions were captured from the fitting surfaces of boxed maxillary complete dentures. The numbers of colony forming units (CFU) of yeast present in the saliva samples and on the dentures were counted and the yeast species presumptively identified by their colony colour. Sampling was repeated following delivery and review of new dentures after 1 month, 3 months and 6 months.

Before delivery of new dentures, 8 of the 10 participants had yeast in their saliva at concentrations ranging from 20-4,280 CFU/ml. Half the participants had C. albicans in their saliva and 7 only had one species present, saliva from one participant contained 3 species. Only one participant had yeast in their saliva 1 month after delivery of their new denture, but this increased to 4 participants after 6 months. The old dentures of 6 of the participants were colonised with yeast, mostly C. albicans but C. krusei was detected on 4 of the old dentures. After 1 month only 2 dentures were colonised, but after 6 months 6 dentures were colonised with a variety of species. The new dentures were colonised by C. albicans and C. tropicalis, but after 6 months C. krusei was also present.
It was concluded that the provision of new dentures reduced the number of yeast in the saliva and on the denture fitting surface and reduced the *Candida* species diversity of the saliva and denture fitting surface for three months. These results suggest that *C. tropicalis* and *C. albicans* are early coloniser and *C. krusei* is a late coloniser of the denture fitting surface.
Writing the acknowledgements is perhaps the most difficult section of a thesis to write for an unemotional person such as myself. That being said, the right people need to be thanked.

My greatest thanks go to Professor Richard Cannon, without whom, this thesis would have never been completed. He got this project off the ground and helped turn some pretty average writing into something more than presentable. The truth is that without his awesome support, I would not be graduating as a prosthodontist.

The attention to detail of Professor Karl Lyons has been greatly appreciated and inspiring. I do not think anyone could have been more thorough with maintaining consistency and accuracy in writing. If I can achieve half his level of attention to detail, it will serve me greatly throughout my career.

My thanks go out to Jenine Upritchard for her tireless help in the laboratory. I probably owe her a drink or three. Doctor Vincent Bennani has to be thanked for teaching me technical skills and techniques that have made my clinical work a more enjoyable and predictable experience.

I would also like to thank the Ministry of Health, Dentsply and the participants, without whom the project would have been impossible.

Is limiting the number of additional degrees that your wife has over her husband a legitimate reason for undertaking a post-graduate doctorate? I think so. For this reason, my last thanks go to my wife Ellie, for inspiring me to undertake further studies, my working life will be forever better because of her.
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## Abbreviations and Symbols

<table>
<thead>
<tr>
<th>Abbreviation / Symbol</th>
<th>Meaning</th>
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<tbody>
<tr>
<td>%</td>
<td>Percent</td>
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<tr>
<td>+</td>
<td>Positive</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>µL</td>
<td>Microlitre</td>
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<td>µm</td>
<td>Micrometre</td>
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<tr>
<td>AD</td>
<td>Anno domino</td>
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<tr>
<td>BEC</td>
<td>Buccal epithelial cells</td>
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<td>C.</td>
<td>Candida</td>
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<td>CFU</td>
<td>Colony forming unit</td>
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<td>cm</td>
<td>Centimetre</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EC</td>
<td>Erythematous candidosis</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EPS</td>
<td>Extracellular polysaccharide</td>
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<td>g</td>
<td>Gram</td>
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<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HC</td>
<td>Hyperplastic candidosis</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>min</td>
<td>Minute</td>
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<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>NHANES III</td>
<td>National Health and Nutrition Examination Survey</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PC</td>
<td>Pseudomembranous candidosis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PMMA</td>
<td>Polymethyl methacrylate</td>
</tr>
<tr>
<td>PRP</td>
<td>Proline-rich protein</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinylchloride</td>
</tr>
<tr>
<td>RFLPs</td>
<td>Restriction fragment length polymorphisms</td>
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<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>SDA</td>
<td>Sabouraud’s dextrose agar</td>
</tr>
<tr>
<td>sIgA</td>
<td>Secretory immunoglobulin A</td>
</tr>
<tr>
<td>SOHO</td>
<td>Study of Oral Health Outcomes</td>
</tr>
<tr>
<td>U.S.A.</td>
<td>United States of America</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast extract peptone dextrose</td>
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1 LITERATURE REVIEW

1.1 COMPLETE DENTURE PROSTHODONTICS

1.1.1 AGING POPULATION

The proportion of older adults in our community is growing faster than any other age group. Worldwide, approximately 600 million people were aged 60 years and over in 2002, and this number is expected to double by the year 2025. By 2050, the elderly are expected to number 2 billion worldwide. This change in worldwide age demographics will place a great challenge on the health systems in many countries (Petersen and Yamamoto, 2005).

Statistics New Zealand project that while New Zealand’s population growth is expected to slow, due to a narrowing gap between births and deaths, the population will continue to age (Bascand, 2012). There will be significant changes in the age structure of the population. The median age of the New Zealand population increased from 26 years in 1971 to 37 years in 2012. It is likely that the median age will exceed 41 years by the late 2030s. Half the population could be older than 44 years by 2061. The gradual ageing reflects the combined impact of people having fewer children, people living longer, and the large number of people born between 1950 and the early 1970s moving into the older age group (65+) (Bascand, 2012).

The number of people aged 65+ in New Zealand has doubled since 1980, surpassing 600,000 in 2012. The number is likely to double again by 2036. It is highly likely that there will be 1.18-1.25 million people aged 65+ in 2036, and 1.44-1.66 million in 2061. The largest growth will occur between 2011 and 2036, as the baby boomer population moves into the 65+ age group (Bascand, 2012).

By 2031, it is expected that 20-22% of New Zealanders will be aged 65+, compared with 14% in 2012. By 2061, it is expected that 22-30% of the population will be aged 65+. Within the 65+ age group, the number of people aged 85 and over is expected to increase significantly. From 76,000 in 2012, it is highly likely that there will be 180,000-
210,000 people aged 85+ in 2036 and 290,000-430,000 in 2061. By 2061, about 1 in 4 people aged 65+ will be 85+, compared with 1 in 8 in 2012 (Bascand, 2012).

1.1.2 EDENTULISM

Although it is common to encounter an older adult who is experiencing oropharyngeal problems, many healthy geriatric persons can experience good oral health. The aging process is associated with many physiological changes in the oral cavity, however, many of these changes are directly connected to systemic problems and their treatment (medications, chemotherapy, radiotherapy), rather than the simple passage of time (Ship, 1999).

Dental caries remains a constant threat to the maintenance of dental health in individuals of all ages (Heft and Gilbert, 1991). The risk of periodontal disease is greater in older adults due to a multitude of oral and systemic factors (Kamen, 1997). Neurological diseases impair motor, sensory and cognitive function that can increase the risk of developing gingivitis, periodontitis and dental caries (Henry and Wekstein, 1997). Endocrinological diseases such as diabetes impair wound healing and can predispose to periodontal diseases. The vast majority of older adults are taking at least one prescription medication, and the use of many of these and other non-prescription drugs have oral sequelae. Therefore, dental and medical problems that are common in the elderly and their subsequent treatment with medications, radio- and chemotherapy can adversely affect oral health which is reflected in an increased incidence of tooth-loss and edentulism in the elderly (Ship, 1999).

Globally, poor oral health among older people manifests as a high level of tooth loss and edentulism. Indeed edentulism is prevalent among older people all over the world (Petersen, 2003). The use of professional dental health services is low among older people, particularly among the socio-economically disadvantaged. Barriers to oral health care among older people in industrialised countries are considerable. Impaired mobility impedes access to care, particularly for those who reside in rural areas with poor public transport. Given that some older people may experience financial hardship following
retirement, the cost or perceived cost of treatment, together with lack of dental care
tradition and negative attitudes to oral health, may deter them from visiting a dentist
(Petersen and Holst, 1995).

Based on the Third National Health and Nutrition Examination Survey (NHANES III)
(Marcus et al., 1996), even with substantial percentage declines in edentulism, an
increase in the actual number of adults will lead to an increase in the number of
dentures needed over the next two decades (Douglass et al., 2002). The total U.S.A.
adult population in need of one or two complete dentures will increase from 33.6
million in 1991 to nearly 37.9 million in 2020. A slight increase in total edentulism is
projected to occur every decade until 2020 when the estimated decline in percent
edentulism for the entire United States population will be more than offset by the
growth in the population aged 55 years and older (Douglass et al., 2002).

1.1.3 EDENTULISM IN NEW ZEALAND

For many years, New Zealand had the unenviable record of being a world leader in rates
of edentulism among adult populations despite a comprehensive child oral healthcare
programme (Hunter and Davis, 1982). For adults aged 35-44, edentulism prevalence
rates range from 0% in Yamanashi (Japan) in 1974 to 28% in New Zealand in 1976
(Sussex, 2008). The Canterbury study in 1973 showed that, at 36%, the prevalence of
edentulism among 35-44-year-old New Zealanders was almost three times that for any
other country taking part in the study (Arnljot et al., 1985). The second national survey
of oral health- the 1988 World Health Organisation Study of Oral Health Outcomes
(SOHO) indicated that the prevalence of edentulism in New Zealand had fallen
dramatically to 12% in 1988 (Hunter et al., 1992). Edentulism prevalence in New Zealand
projections indicate that this change should continue (Thomson, 1997; Sussex, 2008).

Substantial decreases in the prevalence of edentulism may have led to anticipation that
the complete denture service may have been rendered redundant. However, along with
declining edentulism prevalence, the change in population age structure due to the
aging of the baby boomer generation must be taken into account (Sussex, 2008). In
addition, the attitudes and preconceptions of New Zealand adolescents towards dental care may continue to supply the complete denture market with future customers (Fitzgerald et al., 2004).

1.1.4 PREVALENCE OF REMOVABLE COMPLETE DENTURES

To rehabilitate aesthetics, mastication and phonetics, most edentulous patients elect to replace their missing teeth with removable prostheses, such as polymethyl methacrylate (PMMA) dentures (de Baat et al., 1997). Removable dentures are particularly frequently used by older people in the industrialised world (Ettinger, 1993). Some countries report that one-third to one-half of their older people wear full dentures, the highest rates being among the socio-economically disadvantaged (Petersen and Yamamoto, 2005).
1.2 **CANDIDA**

Disease caused by endogenous fungal organisms such as *Candida albicans* in susceptible hosts is a major cause of morbidity and mortality. Some disease-causing fungi are of exogenous origin and are therefore considered to be strict pathogens. Others, such as *Candida* species can be found at significant levels on mucosal surfaces and the skin, even in healthy hosts, and it is from this position that they can cause disease, thus they are potential and often opportunistic pathogens (Huffnagle and Noverr, 2013).

Oral *Candida* infections appear to have been described as early as the 2nd century AD by Galen, who described *ad aphthas albus*. However, it was not until the mid-19th century that the clinical nature of oral candidosis was defined and the aetiologic agent identified (Samaranayake and MacFarlane, 1990; McCullough et al., 1996).

As a frequent component of the oral microbiota, and an opportunistic pathogen, *C. albicans* is poised to overgrow and penetrate tissue in response to an alteration in host physiology that compromises the immune functions which normally suppress their growth (Soll, 2002). The range of human infections caused by the dimorphic yeast *C. albicans* and related species is considerable; from relatively trivial conditions such as oral and vaginal thrush to fatal, systemic infections in the medically compromised (McCullough et al., 1996).

### 1.2.1 **CANDIDA COLONISATION**

When acrylic prostheses are inserted into the oral cavity, the PMMA of dentures is rapidly coated by salivary proteins to form a pellicle. This denture pellicle alters the surface properties of the PMMA and mediates subsequent adherence of microorganisms including *Candida* species (Edgerton and Levine, 1992).

The genus *Candida* is within the phylum Ascomycota which includes approximately 200 yeast species without a known sexual stage or other remarkable phenotypic character (McCullough et al., 1996). *Candida* biofilms are especially widespread and can form on most, if not all, medical devices in current use, such as stents, shunts, implants,
endotracheal tubes, pacemakers, and various types of catheters (Seneviratne et al., 2008). In the commensal state, a variety of Candida species live as benign members of the normal oral microflora of approximately 40% of healthy human individuals (Webb et al., 1998a).

Many Candida species are opportunistic pathogens associated with a variety of conditions ranging from superficial mucosal diseases, such as oral candidosis to life-threatening systemic infection, one of the most serious presentations being Candidal endocarditis in patients with valvular disease (Andriole et al., 1962). In hospitalised individuals, candidosis is the fourth leading cause of bloodstream infection in the United States (Edmond et al., 1999). Moreover, in the U.S.A., the crude mortality rate among patients with candidaemia has been increasing, and reported to be as high as 40% (Wenzel and Gennings, 2005). Candida colonisation of PMMA is associated with denture stomatitis and is the most common form of oral candidosis being reported in up to 65% of denture wearers (Budtz-Jørgensen, 1974b).

Candida biofilms found on PMMA form in a distinct pattern. Initially there is adherence of free floating (planktonic) Candida yeast cells in the oral cavity to the PMMA (1-2 h). This is followed by development into distinct microcolonies and then into a multilayered structure comprising a mixture of yeasts, germ tubes, and hyphae. Candida biofilm maturation is characterised by the excretion of an extracellular polysaccharide (EPS) layer (Chandra et al., 2001). Different Candida species, and different strains of the same Candida species, are dramatically different in their ability to form biofilms, indicating that ‘strong’ and ‘weak’ biofilm-forming species and strains exist (Thein et al., 2007). Candida cells in a biofilm are more difficult to eradicate and display increased antifungal resistance as compared to planktonic cells (Hawser and Douglas, 1995).

The physicochemical properties of the denture surface can influence Candida biofilm formation. For instance, there is greater C. albicans adhesion to, and penetration into, unpolished PMMA (Ramage et al., 2004) and tissue conditioner materials (Radford et al., 1998) compared with polished PMMA surfaces in vivo. The environmental conditions beneath a denture base appear to predispose for yeast colonisation and are different from those present on the outer polished surface. It is not known which factors are
conducive to increased adhesion, but the presence of serum components in inflammatory exudate, the presence of shed epithelial cells, and minimal salivary flow are likely factors (Samaranayake et al., 1980; Budtz-Jörgensen and Theilade, 1983).

It is generally assumed that because *Candida* species so commonly inhabit the oral cavities of healthy individuals, the commensal strain is the source of subsequent infection in susceptible individuals (Odds et al., 2006). This assumption, in turn, suggests that all, or most, commensal *Candida* species are also potentially pathogenic and all strains, by definition, are opportunistic. Although no genetic distinctions between commensal and pathogenic strains have been found (Hellstein et al., 1993), it is also possible that in transition from health to candidosis, there is species or strain substitution from less-adherent to more-adherent strains (Schmid et al., 1993).

### 1.2.2 COMMENSAL *CANDIDA* CARRIAGE

Colonisation depends on several factors: the acquisition of cells by the oral cavity, the attachment of cells in the oral cavity, the growth of those cells, the penetration of tissues and the removal of cells from the oral cavity (Cannon and Chaffin, 1999). At least 50% of the population are thought to be carriers of *Candida* species and, if sensitive enough tests were developed, more than 90% of healthy individuals may be shown to be carriers of the organism (Odds, 1987). A thorough analysis of the literature on *Candida* showed that the range of frequencies of commensal *Candida* carriage reported was extremely broad (3-48%), the median carriage frequency was 34.4% for all yeasts and 17% for *C. albicans* alone (Odds, 1988).

To obtain a more accurate assessment of commensal carriage, Soll and colleagues (Soll et al., 1991) sampled 17 body locations of 52 healthy women. Although *Candida* species were cultured from 73% of test individuals at one or more body locations, this was considered to be an underestimate as it was later discovered that *C. glabrata* did not grow on the defined medium used in that study. It was clear from these data that no single body location accurately reflected carriage prevalence and that the oral cavity was the most frequently colonised (56%). The data also revealed that apparently healthy
women were colonised by *Candida* species in body locations at levels also found in infections (Soll et al., 1991).

In the same study, it was demonstrated by DNA fingerprinting methods that the isolates from the vulvovaginal and oral regions of the same individual either represented different species, different strains of the same species or different substrains, and were in no case genetically identical (Soll et al., 1991). This suggested that commensal organisms may adapt to different body locations (Soll et al., 1991).

Previous studies have reported that the prevalence and density of *C. albicans* colonisation is greater in edentulous patients who wear dentures compared with fully dentate subjects, suggesting that dentures may encourage the growth of *Candida*. However, it is not possible to equate specific numbers of *Candida* with health and disease, as previously proposed (Arendorf and Walker, 1980).

### 1.2.3 Candida Taxonomy and Growth

Within the *Candida* genus, species are characterised primarily by colony morphology, carbon utilisation, and carbon fermentation (Shepherd et al., 1985). *Candida* species form soft cream-coloured colonies with a yeasty odour when grown under aerobic conditions in a pH range of 2.5-7.5 and a temperature range of 20-38°C. Growth is usually detected after 48-72 h incubation. The gross microscopic appearance of all the *Candida* species is similar: all yeasts are Gram-positive but sometimes the shapes of the yeast cells can vary from ovoid to spherical. The size of *C. albicans* yeast cells also varies from 2.9-7.2 x 2.9-14.4 μm (Odds, 1988).

### 1.2.4 Candida Virulence

The type of relationship that *Candida* has with its host depends on how it interacts with the epithelial surface it colonises. A subtle equilibrium exists between the potentially damaging *Candida* virulence factors and the host immune response. Frequently, it is
changes in host factors that lead to *Candida* becoming pathogenic. However, given the often reported morphological and physiological heterogeneity that exists between *Candida* species and certainly strains of *C. albicans*, it may also be that colonising strains are different and allow for persistence at mucosal surfaces (Williams et al., 2013).

Several virulence factors for *C. albicans* that promote successful colonisation and invasion of host tissues have been described, including the cell wall, cell surface ligands and receptors, secretion of extracellular proteinase enzymes, and morphological conversion (Macdonald and Odds, 1980; Calderone and Braun, 1991). The ability of *Candida* species to persist on the host surfaces of healthy individuals is an important factor contributing to its virulence. These surfaces may be the host’s mucosal surfaces or take the form of biomaterials of medical devices, for example, the PMMA of a denture. This ability to persist is particularly important in the mouth, where the organism has to resist the constant mechanical washing action and shear forces of saliva moving toward the oesophagus (Sitheeque and Samaranayake, 2003).

### 1.2.4.1 ADHESION AS A VIRULENCE FACTOR

In humans, *C. albicans* preferentially colonises mucosal surfaces, and the gastrointestinal tract is believed to be a major reservoir for infection (Odds, 1988; Cole et al., 1996). *C. albicans* survives better on moist surfaces than dry inanimate objects, but if the degree of contamination is high enough, viable cells will remain on dry surfaces for at least 24 h (Rangel-Frausto et al., 1994). In people whose mouths are colonised with *C. albicans*, the yeast can be found in saliva at an average concentration of 300 to 500 cells/mL (Arendorf and Walker, 1980).

The entry of *Candida* cells into the oral cavity is not sufficient for colonisation; they must be stably maintained. Yeast cells will be washed out by saliva and swallowed unless they adhere and replicate. Growth conditions in the oral cavity are considered poor and there is practically no growth in saliva unless it is supplemented with glucose (Samaranayake et al., 1986a). Adhesion is therefore of critical importance in colonisation and survival of
an oral Candida population (Cannon and Chaffin, 1999; Samaranayake and Ellepola, 2000).

There is a clear correlation between adhesion and virulence (Asakura et al., 1991). The species that are more commonly present on the human host, C. albicans and C. tropicalis, adhere to host cells in vitro to a greater extent than the less pathogenic species, such as C. krusei. Strains of C. albicans with a reduced ability to adhere in vitro have been shown to also have a reduced ability to cause infection in animal models (Calderone and Braun, 1991). Also, strains with an increased ability to adhere to BEC and produce larger amounts of extracellular proteinase have been shown to have increased host lethality in a mouse infection model (Ghannoum and Elteen, 1986).

1.2.4.2 Candida Adherence to Oral Surfaces

The mechanism of attachment between C. albicans and the host has been suggested to involve interactions between Candida cell adhesins and host cell receptors (Bouchara et al., 1990; Calderone and Braun, 1991). The adhesins of C. albicans are thought to be mannoproteins (Calderone and Braun, 1991). Investigations indicate that the adhesion of C. albicans to host cells is dependent on the type of host cell, the strain of the organism, and the host cell recognition systems (Calderone, 1993; 1994). These involve C. albicans adhesins recognising receptors on specific host epithelial and endothelial cells, and ECM. It was suggested that the spectrum of adhesins displayed by Candida may explain the wide range of body sites susceptible to invasion by Candida species. Perhaps colonisation and invasion of these different tissues is associated with the presence of particular cell surface adhesins that can recognise specific types of host cells (Calderone, 1993).

Proteins from the saliva absorb readily to oral surfaces, especially to tooth enamel, to form the acquired pellicle (Lamkin and Oppenheim, 1993). When these salivary components are absorbed to oral surfaces they act as ligands for C. albicans adhesion (Calderone and Braun, 1991). C. albicans cells have often been found in the subgingival dental plaque of individuals with periodontitis. An assay study by Cannon and colleagues
(Cannon et al., 1995b) showed that *C. albicans* adherence to hydroxyapatite spheres was saliva-mediated, as negligible attachment was observed to uncoated beads or to beads coated with serum compared to beads coated with whole saliva. It has been suggested that *C. albicans* itself binds poorly to the clean tooth surface and requires a pellicle of proteins to promote binding (Cannon et al., 1995a). An investigation by Şen and colleagues (Şen et al., 1997) added that, in the presence of calf serum, colonisation of enamel and cementum by *C. albicans* was greater than that seen on smear-layer-free dentine. Henriques and colleagues (Henriques et al., 2004) found no statistical difference when comparing the ability of *C. albicans* to adhere to acrylic and hydroxyapatite in the presence of artificial saliva, and postulated that both surfaces may be equally important reservoirs for *Candida* infections in dentate patients.

*C. albicans* cells bind *in vitro* to corneocytes (keratinized cells of stratum corneum) from medically compromised individuals at twice the rate as compared to corneocytes from healthy individuals (Srebrnik and Segal, 1990). Exfoliated buccal epithelial cells (BECs) are probably the best-investigated human cell type in *C. albicans* adherence studies. The binding capacity of BECs from new-born full-term infants for *Candida* is less than that of BECs from premature infants, school-age children, and adults, but increases over the infants’ first few days (Davidson et al., 1984; Cox, 1986; Polacheck et al., 1995).

### 1.2.4.3 EFFECT OF SALIVA ON CANDIDA ADHERENCE

All intra-oral surfaces are coated with salivary proteins, and a salivary pellicle forms within minutes of exposure. Thus, a biologically inert surface such as PMMA, commonly used in denture construction, when coated with salivary proteins acquires molecular groups that may serve as receptors for microbial adhesion (Radford et al., 1999). Salivary proteins also have been considered to serve as a source of nutrients for microorganisms (Kolenbrander and London, 1993).

The specific role of human saliva on the adherence and colonisation of *Candida* species to PMMA surfaces is not clear (Nikawa et al., 1997). It is known that innate defences, such as the flushing effect of saliva, and anti-*Candida* salivary components, such as
lysozyme, histatins, lactoferrin, calprotectin, and immunoglobulin-A, interact with the Candida species, decreasing its adherence to oral surfaces (Tanida et al., 2001; Dodds et al., 2005). Nevertheless, other components in whole saliva, including mucins (Elguezabal et al., 2004; Dodds et al., 2005), statherin (Johansson et al., 2000), and proline-rich-proteins (Tanida et al., 2001), have been reported to absorb to C. albicans, increasing its adherence to saliva-coated PMMA resins (Nikawa et al., 2000a; Nikawa et al., 2000b).

Studies regarding the influence of whole saliva on Candida adherence are contradictory. Koseki and colleagues (Koseki et al., 2004) found an increased prevalence of C. albicans, C. glabrata, C. tropicalis, and C. krusei in patients with reduced salivary flow rates. A reduction in the levels of C. albicans adherence to PMMA in the presence of saliva has been found by several authors (Samaranayake et al., 1980; Waters et al., 1997; Radford et al., 1999). Other authors have reported contrasting results, the in vitro studies of San Millán and colleagues (San Millán et al., 2000) and Ramage and colleagues (Ramage et al., 2004) affirmed that saliva increased C. albicans adherence to PMMA initially, but its effect was minimal or inhibitory after 24 h and depended on the morphological phase of C. albicans. The adherence of C. albicans increased in the presence of saliva, according to Vasilas and colleagues (Vasilas et al., 1992) and Millsap and colleagues (Millsap et al., 1999). Jin and colleagues (Jin et al., 2004) found no effect of saliva on adherence of C. albicans to surfaces.

These conflicting results may be due to the different strains and protocols used in each study, including different incubation periods, use of filtered or whole saliva, stimulated or unstimulated saliva, different saliva temperatures, and the presence or absence of nutrients that may interfere with cell viability and adherence capacity (Moura et al., 2006).

1.2.4.3.1 ORGANIC CONSTITUENTS OF SALIVA

Statherin and the proline-rich proteins (PRPs) provide attachment to tooth and other oral surfaces for a variety of oral microorganisms, including C. albicans (Johansson et al., 2000). In an adherence assay study by O’ Sullivan and colleagues (O’Sullivan et al., 1997),
strain-specific variation was evident in the ability of *C. albicans* to bind parotid saliva proteins. This ability to bind protein was correlated with the level of adhesion the *C. albicans* isolates had to saliva-coated hydroxyapatite beads (Cannon et al., 1995b). Thus, variations in these protein components provide possibilities for heterogeneities in microbial colonisation through their specific bacterial binding patterns (Dodds et al., 2005).

Histatins are another group of proteins that have received much interest. Of particular interest is the fact that the histatins have potent anti-*Candida* effects, and it has been suggested that these activities could be exploited as a natural defense against *Candida* (Tsai and Bobek, 1998). It is interesting that, paradoxically, concentrations of both lysozyme and histatins are elevated with *Candida* carriage, implying that there is a reactive increase in the production of these salivary proteins in response to mucosal infections (Yeh et al., 1997; Bercier et al., 1999).

### 1.2.4.3.2 SECRETORY IMMUNOGLOBULIN A AND MONOCLONAL ANTIBODIES

Elguezabal and colleagues investigated the inhibitory effect of human salivary components on the adherence of *C. albicans* to a resin composite restorative dental material (Elguezabal et al., 2004). Whole saliva significantly reduced the adhesion of *C. albicans* compared to the controls without saliva, however, secretory immunoglobulin-A (sIgA)-depleted saliva did not cause an inhibitory effect on adherence, showing an adhesion level similar to that of the controls (Elguezabal et al., 2004).

Although whole saliva has been shown to decrease adhesion of *C. albicans* to PMMA *in vitro* (Samaranayake and MacFarlane, 1980; McCourtie et al., 1986) a saliva-mediated enhancement of the adhesion of *Candida* to PMMA has also been described (Vasilas et al., 1992). One of the monoclonal antibodies used in this study, 21E6, enhanced adherence of all the strains to Herculite resin. This suggests that some antibodies, or antibody components, present in some of the saliva samples may enhance the adhesion of *Candida* to oral surfaces (Elguezabal et al., 2004). If this is the case, it would confirm the hypothesis of Casadevall and Scharff that the humoral immune response mounted
against a pathogen includes protective antibodies, non-protective antibodies and disease enhancing antibodies (Casadevall and Scharff, 1995).

1.2.4.3  EFFECT OF SERUM ON CANDIDA ADHERENCE

Trauma to the palatal mucosa is an important aetiological factor in chronic atrophic candidosis and it is likely that any inflammatory serous exudate would coat the fitting surface of the PMMA dentures. Studies by Samaranayake and colleagues (Samaranayake et al., 1980) and McCourtie and Douglas (McCourtie and Douglas, 1984) has shown that coating PMMA strips with serum enhanced adherence of *C. albicans*, while coating them with saliva inhibited adherence. Reports from the same group have confirmed similar results for *C. glabrata* and *C. tropicalis* (McCourtie et al., 1986).

1.2.4.4 EFFECT OF DENTURE-RELATED FACTORS ON CANDIDA ADHERENCE

An important factor when determining whether or not *Candida* colonisation will occur is the nature of the prosthetic material placed in the oral cavity (Edgerton and Levine, 1992; Göcke et al., 2002). Surface characteristics such as microporosities and roughness may cause the surface to harbour microorganisms that are difficult to remove by mechanical or chemical cleansing, thus increasing adherence *in vitro* (Verran and Maryan, 1997; Radford et al., 1999).

A reduction of denture plaque has been reported when the fitting surface of a denture base is either polished or glazed with a surface-finishing resin (Badawi et al., 1986). One study has shown that the application of a glaze (Perma Link, G.C. Corp., Tokyo, Japan) reduced the volume of denture plaque and colony forming units (CFUs) on the glazed side for one week. Despite these changes, the reduction in plaque levels was not sustained over the experimental period of one month (Budtz-Jörgensen and Kaaber, 1986).
Surface roughness is known to be a factor in the entrapment of microorganisms on surfaces and their protection from shear forces. Several studies show that the number of *C. albicans* cells adhering to smooth surfaces is relatively low when compared to rough surfaces (Verran and Maryan, 1997; Radford et al., 1999; Waltimo et al., 1999).

Radford and colleagues investigated the effect that the degree of roughness of PMMA surfaces and the type of denture-base material have on *C. albicans* adhesion (Radford et al., 1998). They observed greater adhesion of *C. albicans* to soft lining materials than to PMMA resin and greater adhesion to rough surfaces than to control surfaces for both the PMMA and soft lining materials (Radford et al., 1998).

Davenport showed that PMMA has little intrinsic roughness (Davenport, 1972). A rougher surface of PMMA specimens was found when PMMA was processed against plaster of Paris surfaces compared to PMMA processed against glass. A lack of diffusion of dye into the PMMA specimens indicated that if porosity existed, it was not open to the surface (Davenport, 1972).

**1.2.4.5 SITE-SPECIFIC INTRA-ORAL MUCOSAL COLONISATION**

Many authors (Lilienthal, 1950; Beare et al., 1968; Somerville, 1969) have sampled a single mucosal surface to ascertain the oral *Candida* carrier state, but Arendorf and Walker’s study showed that many carriers may be overlooked if certain sites, particularly the posterior dorsum of the tongue, are not examined (Arendorf and Walker, 1979).

In the dentate, *C. albicans* has been most frequently isolated from the tongue, particularly the posterior region. In almost one-third of dentate carriers, the tongue is the only positive oral site. The tongue and, additionally in denture wearers, the fitting surface of maxillary prostheses, seem to be the primary oral reservoir from which the rest of the mouth becomes secondarily colonised (Arendorf and Walker, 1979).

The distribution of *C. albicans* showed great variation in complete denture wearers but correlated well with areas of markedly inflamed mucosa in denture stomatitis patients. The presence and concentration of *C. albicans* at each site was significantly greater
(P<0.001) in subjects with denture stomatitis than in the normal denture wearers and dentate subjects (Arendorf and Walker, 1979).

1.2.5 CANDIDOSIS

Given that \( C.\ albicans \) colonises host surfaces at such a high prevalence, infections are unsurprisingly often endogenous in nature, frequently occurring subsequent to disruption of the host's immune response. Receipt of a broad-spectrum antibiotic, a high frequency intake of carbohydrates, hormonal imbalances, and poor nutrition may also be contributory factors (Williams et al., 2013).

1.2.5.1 CANDIDOSIS DEFINITION, HISTORICAL PERSPECTIVES, AND CLASSIFICATION

To minimise confusion, Samaranayake has proposed a classification system (Samaranayake, 1991) whereby oral candidosis lesions are subdivided into two main groups: Group I, or primary oral candidoses confined to lesions localized to the oral cavity with no involvement of skin or other mucosae; and Group II or secondary oral candidoses, where the lesions are present in the oral cavity as well as extra-oral sites such as the skin (Table 1). The group I lesions consist of the classic triad - pseudomembranous, erythematous, and hyperplastic variants - and some have suggested further subdivision of the latter into plaque-like and nodular types (Holmstrup and Axéll, 1990).
Table 1: Oral candidosis classification

<table>
<thead>
<tr>
<th>Primary oral candidosis (Group I)</th>
<th>Secondary oral candidosis (Group II)</th>
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<tbody>
<tr>
<td>The ‘primary triad’</td>
<td></td>
</tr>
<tr>
<td>• Pseudomembranous (mainly acute)</td>
<td>• Familial chronic mucocutaneous candidosis</td>
</tr>
<tr>
<td>• Erythematous (acute/chronic)</td>
<td>• Diffuse chronic mucocutaneous candidosis</td>
</tr>
<tr>
<td>• Hyperplastic (mainly chronic)</td>
<td>• Candidosis endocrinopathy syndrome</td>
</tr>
<tr>
<td>o Plaque-like</td>
<td>• Familial mucocutaneous candidosis</td>
</tr>
<tr>
<td>o Nodular/speckled</td>
<td>• Severe combined immunodeficiency</td>
</tr>
<tr>
<td>Candida-associated lesions</td>
<td>• Di George syndrome</td>
</tr>
<tr>
<td>• Denture stomatitis</td>
<td>• Chronic granulomatous disease</td>
</tr>
<tr>
<td>• Angular cheilitis</td>
<td>• Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>• Median rhomboid glossitis</td>
<td></td>
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<tr>
<td>• Linear gingival erythema</td>
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</table>

1.2.5.2 WHAT DETERMINES THE VARIATION IN THE DIFFERENT PRESENTATIONS OF CANDIDOSIS?

Although *C. albicans* is well recognised as the major aetiological agent of oral candidosis, it is not clear why several presentations of oral candidosis such as pseudomembranous (PC), erythematous candidosis (EC) and hyperplastic candidosis (HC) manifest in different individuals, sometimes singly and, on other occasions, in combination. The virulence attributes of *Candida* differ significantly within and between species and thereby play a contributory role in the manifestation of clinical presentations. However it is still unknown if different virulence attributes are involved in either a pseudomembranous or an erythematous response (Reichart et al., 2000). Why several variants of oral candidosis occur in the oral mucosa, sometimes in the same patient, remains elusive. Both the presence of organisms and the local host defences in the different tissues may play a significant role in modulating the clinical manifestations (Reichart et al., 2000).
1.2.5.3 CANDIDA COMMENSALISM OR PATHOGENESIS...

In the commensal state, Candida species live as benign members of the microflora of healthy individuals (Stenderup, 1986; Soll, 2002). Candida species are ‘carried’ in the oral cavity, gastrointestinal tract, anus and groin of healthy individuals, and also in the vaginal canal and vulva of healthy women. Even in health, Candida species can attain surprisingly high densities without symptoms of disease (Soll, 2002).

1.2.5.3.1 IS PATHOGENESIS ASSOCIATED WITH PARTICULAR STRAINS OF CERTAIN CANDIDA SPECIES?

It has been suggested that all strains have the capacity to modulate a variety of virulence factors like the yeast-hypha transition and high frequency phenotypic switching, or to differentially express a variety of virulence genes in response to environmental challenges. If so, each cell in a population is poised to up- or down-regulate genes in response to environmental cues (Soll, 2002).

Some Candida species, such as C. albicans and C. tropicalis, might be more pathogenic than others (Budtz-Jörgensen, 1974a), and different tissue responses to virulent and attenuated strains of C. albicans have been reported (Holbrook et al., 1983). Blignaut and colleagues found that the collections of isolates of C. albicans from patients who were HIV+ and had oral candidosis, those from patients who were HIV+ and did not have oral candidosis, and those from healthy individuals without oral candidosis were genetically indistinguishable (Blignaut et al., 2002). There may, however, be environmentally induced epigenetic changes that affect gene expression. No particular strain has been found that is responsible for oral candidosis (Pfaller et al., 1998; Marco et al., 1999; Soll, 2002) and there is no epidemiological evidence that particular strains have evolved with phenotypic characteristics that increase their pathogenic success (Soll, 2002).

A study by Hellstein and colleagues (Hellstein et al., 1993) used DNA fingerprinting to test whether a group of commensal strains was genetically distinct from a group of pathogenic strains of C. albicans isolated from the oral cavities of individuals from the
same geographical locale (Iowa, U.S.A.). Although the group of pathogenic isolates exhibited, on average, more phenotypic variability than commensal isolates, no genetic distinctions between commensal and pathogenic strains were found, suggesting a common clonal origin (Hellstein et al., 1993).

1.2.5.3.2 PHENOTYPIC SWITCHING COULD BE INVOLVED IN THE SHIFT FROM COMMENSALISM TO PATHOGENESIS

*C. albicans* is capable of colonizing and invading most cavities and tissues of the human body. One would expect such an effective pathogen to be capable of rapid variation in order to adapt to changing environments, and phenotypic switching may have evolved for this purpose (Soll, 1992).

In 1985, two reports (Pomés et al., 1985; Slutsky et al., 1985) demonstrated for the first time that common strains of *C. albicans* switched reversibly and at high frequency between a number of variant phenotypes. Although the molecular mechanism of the switch event has not been elucidated, it appears that switching regulates phenotype through the activation and deactivation of phase-specific genes at the level of transcription (Soll et al., 1993).

Switching has been demonstrated to affect a variety of virulence traits, including the yeast-hypha transition (Brown-Thomsen, 1968; Anderson et al., 1989), antigenicity (Anderson et al., 1990), adhesion (Kennedy et al., 1988; Vargas et al., 1994), sensitivity to neutrophils and oxidants (Kolotila and Diamond, 1990), secretion of proteinase (Soll et al., 1989; Morrow et al., 1992; Vargas et al., 2000), and drug susceptibility (Soll et al., 1989; Vargas et al., 2000). Switching provides *C. albicans* and related species, like *C. glabrata* (Lachke et al., 2000), with an extraordinary level of phenotypic variability. Every colonising population includes phenotypic variants poised for enrichment in response to selective pressures (Odds, 1997).
1.2.6 NON-ALBICANS CANDIDA SPECIES

Non-albicans Candida species are emerging as both colonisers and pathogens causing nosocomial fungal bloodstream infections (Thein et al., 2007). The proportion of non-albicans Candida species among clinical isolates is increasing: in 1970-1990, non-albicans Candida species represented 10-40% of isolates; in 1991-1998, they represented 35-65% of all strains causing candidaemias (Wingard, 1995; Viscoli et al., 1999). The most common species are C. tropicalis, C. glabrata, C. krusei and C. parapsilosis, which as a group represent about one half of all Candida strains isolated from blood cultures. Non-albicans Candida species are of special concern in medicine for two reasons. Firstly, the virulence and pathogenicity of some non-albicans Candida species, mainly in the immunocompromised host, is high resulting in significant mortality; and secondly, their resistance to currently available antifungal drugs (Krcmery and Barnes, 2002).

Reports have suggested differences in the virulence of non-albicans Candida species in comparison to C. albicans. Most non-albicans Candida species express virulence factors in vitro and are also pathogenic in animal models (Wingard, 1995). However, non-albicans Candida species have the ability to cause severe infections in humans, sometimes with a fatal outcome (Viscoli et al., 1999). For most isolates of C. albicans, antifungal susceptibility can be predicted with accuracy. In contrast, when the isolate is a non-albicans Candida species, species identification alone does not allow such prediction. C. krusei and C. glabrata are either inherently resistant to fluconazole or show secondary, acquired, resistance (Rex et al., 1995) and some isolates of C. krusei display resistance to amphotericin B (Nguyen et al., 1998).

C. albicans is the yeast most frequently isolated from the oral cavities of patients with denture stomatitis (McIntyre, 2001; Baena-Monroy et al., 2004). In recent years, various studies have isolated other Candida species alongside C. albicans, which may be involved in the pathogenesis for patients with oropharyngeal candidosis (Coleman et al., 1997; Bagg et al., 2003; Li et al., 2007). A wide variety of organisms have been shown to cause infection, with C. glabrata, C. dubliniensis, C. parapsilosis, C. krusei, and C. tropicalis being the most commonly reported (Lyon et al., 2006).
In an examination of 37 complete denture wearers, Coco and colleagues (Coco et al., 2008) found that, after *C. albicans* (75%), *C. glabrata* was the second most common yeast isolated (30%) from saliva and denture samples. This was followed by *C. krusei* (5%) and *C. parapsilosis* (3%) and *C. tropicalis* (3%). Several of the study participants had more than one species of yeast within their oral cavities (35%). Mixed species populations were observed in clinical health and in the presence of Newton’s Type I, II, and III candidosis (Coco et al., 2008). The combination of *C. albicans* and *C. glabrata* was the most prevalent of all yeast mixtures, isolated from 25% of study participants.

### 1.2.6.1 *Candida krusei*

*C. krusei* is an emerging non-*albicans* *Candida* species, with a particular predilection for neutropenic adult cancer patients (Wingard, 1995). In a review of the literature by Krcmery and Barnes (Krcmery and Barnes, 2002), the overall mortality of *C. krusei* fungaemia was 30-70%, but the attributable mortality was about 40%, suggesting that the mortality of infections due to *C. krusei* may be of a similar order to *C. albicans*. Another view of virulence was given by Wingard, who postulated that *C. krusei* is of lower virulence than *C. albicans*. Many of the deaths reported in patients with *C. krusei* fungaemia were not due to fungal infection; giving weight to the idea that *C. krusei* simply accompanies the terminal stages of haematological malignancies (Wingard, 1995).

Generally, *C. krusei* is considered to be a transient commensal in humans and has been isolated only infrequently from the mucosal surfaces of various patient groups and as a mucosal inhabitant in healthy individuals (Odds, 1988). In his comprehensive review of the literature on oral carriage of *Candida* species, Odds (Odds, 1988) concluded that *C. krusei* is the fifth most dominant species (after *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. parapsilosis*) (Samaranayake and Samaranayake, 1994).

Relatively few studies have been conducted to determine the virulence of *C. krusei* in humans and laboratory animals (Samaranayake and Samaranayake, 1994). An early investigation by Hurley and Stanley (Hurley and Stanley, 1969) compared the cytopathic effect of *Candida* species in mouse cell cultures. The authors found that *C. krusei* and *C.
Parapsilosis were less destructive than C. albicans and C. tropicalis. Howlett (Howlett, 1976) later also found, using rat mucosa, that C. krusei was less invasive than C. albicans or C. tropicalis. C. albicans produced extensive epithelial invasion, penetrating all the layers of the epithelium but C. krusei did not penetrate the stratum corneum.

Similarly, few researchers have investigated the adhesion of C. krusei to host epithelial cells. King and colleagues (King et al., 1980) observed that C. krusei adhesion to buccal epithelial cells was far less than that of C. albicans. A related study by Samaranayake and colleagues (Samaranayake et al., 1994) compared the adhesion of C. krusei and C. albicans to buccal epithelial cells from healthy individuals and bone marrow transplant recipients. The adhesion of C. albicans to the buccal epithelial cells from the bone marrow transplant recipients was three-fold lower than to the control cells, but adhesion of C. krusei remained the same. It was postulated that this may reflect a selective colonisation process and may explain to some extent the frequent isolation of C. krusei from the mouth in compromised patient groups. (Shipman, 1979; Martin et al., 1981; Fisher et al., 1987).

A few investigators have examined the adherence of C. krusei to non-biological surfaces. Earlier researchers found that the adhesion of C. krusei to PMMA was the lowest in comparison with other Candida species, including C. albicans (Klotz et al., 1985; Miyake et al., 1985). Whereas, in a more recent investigation, it was demonstrated that the adhesion of C. krusei to PMMA is significantly greater than that of C. albicans (Samaranayake et al., 1994). Although the variations in these results from different research groups could be attributable to differences in the isolates utilised, techniques and culture media, it is fair to conclude that C. krusei is more adherent to inert surfaces than to buccal epithelial cells (Samaranayake and Samaranayake, 1994).

1.2.6.2 Candida glabrata

C. glabrata represents 8-37% of all Candida blood culture isolates and some centres report an increasing proportion of infections (Krcmery and Barnes, 2002). C. glabrata was initially regarded as a saprophyte with low virulence documented in animal models:
it grows without hyphae or pseudohyphae and lacks the virulence factors identified in *C. albicans* (Stenderup and Pedersen, 1962; Wingard, 1995). Despite this, *C. glabrata* can cause serious fungaemia in humans (Gumbo et al., 1999) but is generally regarded as less pathogenic than *C. albicans* (Nguyen et al., 1995). However, in the past two decades, as a consequence of the widespread use of immunosuppressive drugs and the emergence of the acquired immunodeficiency syndrome (AIDS), *C. glabrata* is increasingly implicated in human infection (Hajjeh et al., 2004). Indeed, depending on the site of infection, *C. glabrata* currently ranks as the second or third most frequently isolated *Candida* species from all reported cases of candidosis (Wingard, 1995; Pfaller, 1996; Fidel et al., 1999).

Some randomised studies have reported a much higher overall mortality associated with *C. glabrata* infection: around 50% in cancer patients, up to 100% in BMT patients, but this was crude rather than attributable mortality (Goodman et al., 1992; Anaissie et al., 1996; Krcmery et al., 1998). It seems reasonable to conclude that invasive *C. glabrata* infection occurs predominantly in the most ill patients - in the terminal stages of malignant disease, or those receiving intensive care - and this explains the somewhat higher overall mortality seen with *C. glabrata* than with other non-*albicans* *Candida* species (Goodman et al., 1992; Wingard, 1995; Krcmery, 1999a).

*C. glabrata* has been isolated from various oral sites, including buccal and palatal mucosa, dentures, tongue, dental plaque, and subgingival flora (Bagg et al., 2003; Portela et al., 2004; Rasool et al., 2005). It is frequently isolated from the oral cavity with other *Candida* species, the most common combination being *C. glabrata* and *C. albicans* (Li et al., 2007). Although *C. albicans* is still considered the major aetiologic agent in oral candidosis and accounts for 70-80% of organisms isolated from oral mucosal lesions, in recent years, *C. glabrata* has emerged as a notable pathogenic agent in the oral mucosa, either as a co-infecting agent with *C. albicans* (Redding et al., 1999; Redding et al., 2002; Redding et al., 2004) or as the sole detectable species from oral lesions (Canuto et al., 2000). Despite the fact that oral *Candida* infections are not associated with mortality, they are a significant source of morbidity, and trigger chronic pain or discomfort upon mastication, which may limit nutrition intake in immunocompromised or elderly individuals (Redding et al., 2000; Olmos et al., 2004).
Although *C. albicans* remains the primary *Candida* species isolated from the oral cavities in all age groups, several studies demonstrate that colonisation of the oral cavity by *C. glabrata* increases with increasing age (Lockhart et al., 1999; Qi et al., 2005). Qi and colleagues (Qi et al., 2005) noted an increase in the number of subjects with multiple *Candida* species co-isolated from their oral cavities with increasing age. As many as 71% of persons with *C. glabrata* oral infections have been shown to also carry other *Candida* species (Vazquez, 1999). Lockhart and colleagues (Lockhart et al., 1999) found that *C. glabrata* carriage in individuals ≥80-years of age wearing dentures was 58% but only 29% for those not wearing dentures in the same age group.

Similarly, although *C. albicans* remains the most common cause of oral candidosis, the proportion of cases in which *C. glabrata* has been isolated is increasing (Li et al., 2007). Compared with *C. albicans*, *C. glabrata* demonstrated a two-fold greater tendency to adhere to PMMA surfaces in an *in vitro* study performed by the Samaranayake group (Luo and Samaranayake, 2002). With this high propensity to adhere to denture surfaces, it is not surprising that *C. glabrata* has been identified as the predominant yeast isolated from dentures in elderly persons with chronic atrophic candidosis (Wilkieson et al., 1991). Studies on *Candida* colonisation on denture surfaces demonstrated that biofilm formation of some strains of *C. glabrata* was promoted in an environment with increased serum, implying that oral inflammation induced by denture plaque would facilitate *C. glabrata* colonisation on these surfaces (Nikawa et al., 2000c).

### 1.2.6.3 *CANDIDA PARAPSILOSIS*

*C. parapsilosis* is probably the only species to show an overall increasing incidence since 1990 in most studies of fungaemia (Krcmery and Barnes, 2002). In a review by Wingard, spanning 1952 to 1992, *C. parapsilosis* accounted for only 6% of fungaemia, and he states that ‘*C. parapsilosis* is an infrequent cause of infection among humans’ (Wingard, 1995). In contrast, more recent reports from North America and Europe show that *C. parapsilosis* is now the predominant cause of candidaemia in some centres (Levy et al., 1998).
In experimental models, the virulence of *C. parapsilosis* is lower than that of *C. albicans* and other non-*albicans* *Candida* species due to decreased adherence to epithelial cells (Wingard, 1995). On the other hand, *C. parapsilosis* adheres more efficiently to foreign body material but is associated with lower mortality than that documented for bloodstream infection with *C. albicans* or *C. tropicalis*. Several factors may give *C. parapsilosis* a selective advantage in the modern medical environment, including proliferation in high concentrations of glucose, adherence to prosthetic materials, colonisation of human hands, and possibly resistance to new antifungal agents (Weems, 1992).

1.2.6.4 **CANDIDA TROPICALIS**

*C. tropicalis* is one of the three most commonly isolated non-*albicans* *Candida* species (the others being *C. parapsilosis* and *C. krusei*). *C. tropicalis* accounts for 4 to 25% of all *Candida* isolates and a higher proportion (20-45%) of non-*albicans* *Candida* species isolated from blood cultures (Fraser et al., 1992; Wingard, 1995; Krcmery, 1999b; Viscoli et al., 1999). Disseminated *C. tropicalis* infections have been reported in patients with chronic mucocutaneous candidosis. No other species of *Candida* have been associated with disseminated fungal infections (Dixon et al., 2004).

With regard to virulence and pathogenicity, data from animal models suggest that *C. tropicalis* is at least as virulent as *C. albicans* (Wingard, 1995). *C. tropicalis* seems to display higher potential for dissemination in the neutropenic host than *C. albicans* and other non-*albicans* *Candida* species which may partially explain the reportedly relatively high mortality (33-90%) associated with *C. tropicalis*. Although relatively few studies have made the distinction between overall and attributable mortality, this was also higher for *C. tropicalis* than for *C. albicans* fungaemia (33-50%) (Powderly et al., 1988; Slavin et al., 1995; Krcmery, 1999b).

*C. tropicalis* was initially regarded as a species susceptible to fluconazole and amphotericin B, displaying decreased susceptibility to ketoconazole and miconazole (Blumberg et al., 2001). However, other reports have indicated the rapid development
of fluconazole and amphotericin B resistance (Powderly et al., 1988; Rex et al., 1995; Blumberg and Reboli, 1996; Nguyen et al., 1998). The reason for this rapid emergence of resistance is unclear but it is likely that some strains of *C. tropicalis* are inherently less susceptible to both azoles and amphotericin B, and prophylactic or therapeutic administration of those antifungals may select such naturally less susceptible variants (Wingard, 1995; Krcmery and Barnes, 2002).

Segel and colleagues (Segal et al., 1988) compared the adherence of various *Candida* species to PMMA surfaces and demonstrated that the six isolates of *C. albicans* used in the study were the most adherent yeasts. *C. parapsilosis* and *C. tropicalis* also showed a high degree of adherence, but less than *C. albicans*, whereas *C. krusei* showed lower adhesion.
1.3 DENTURE STOMATITIS

Denture stomatitis is a common oral mucosal lesion of clinical importance in the elderly. In many cases of denture stomatitis colonisation of the fitting surface of the prosthesis with yeast is observed (Jeganathan and Lin, 1992). The incidence of denture stomatitis correlates strongly to poor denture hygiene or to the amount of denture plaque (Vigild, 1987; Kulak-Ozkan et al., 2002). The use of defective and unsuitable dentures is also a risk factors for denture stomatitis (Fleishman et al., 1985).

The inflammatory changes seen beneath a maxillary denture was initially described as ‘denture sore mouth’ by Cahn (Cahn, 1936), but this term was replaced by ‘denture stomatitis’ as discomfort is often absent (Cawson, 1963). Other names used have been ‘chronic atrophic candidiasis’, which included angular cheilitis as well as denture stomatitis (Lehner, 1966), ‘denture-related candidiasis’ (Nairn, 1975), and ‘stomatitis prothetica’ and ‘stomatopathia prothetica’ (Nater et al., 1978). The term ‘denture stomatitis’ seems preferable since no predominant cause, other than the presence of a denture, has been universally accepted (Arendorf and Walker, 1987).

Denture-induced stomatitis is the most common form of oral candidosis and is usually found on the palatal mucosa beneath the fitting surface of the maxillary denture and both complete and partial denture wearers are affected. The condition is frequently symptomless but can show mucosal bleeding, swelling, burning and other painful sensations, halitosis, unpleasant taste and dryness in the mouth (Arendorf and Walker, 1987).

1.3.1 PREVALENCE

Arendorf and Walker reviewed the available literature and found that surveys of dental school prosthetic patients, or randomly selected populations of denture wearers, have often revealed a high incidence of palatal inflammation associated with the presence of a denture (Arendorf and Walker, 1987). The prevalence of denture stomatitis has been reported in 11-67% of complete denture wearers (Arendorf and Walker, 1987).
Budtz-Jörgensen and colleagues (Budtz-Jörgensen et al., 1975) assessed the prevalence of denture stomatitis and Candida infection in an elderly Danish population. Ten percent (463 individuals) of the population from three communities above the age of 65 were examined. Yeast samples were obtained from oral swabs of the palatal mucosa and denture fitting surface of the maxillary denture. Denture stomatitis was encountered in 65% of denture-wearers (Budtz-Jörgensen et al., 1975). Denture stomatitis is more common in females than males (Arendorf and Walker, 1987).

### 1.3.2 CLASSIFICATION

Denture stomatitis may be classified according to the clinical appearance of the inflamed mucosa under the maxillary complete denture, and includes three types (Newton, 1962). Type I refers to the initial stage of localised pin-point hyperaemia while type II, which is the most common form of denture stomatitis, is described as having diffuse erythema and oedema confined to the denture-bearing areas of the palatal mucosa and is demarcated at the margins of the denture. Type III refers to a hyperplastic reaction that results in a nodular lesion of the central palate often with associated atrophic areas; this is referred to as papillary hyperplasia (Newton, 1962). All three types may be found simultaneously and in varying combinations (Webb et al., 1998b).

Östlund (Östlund, 1958), Budtz-Jörgensen and Bertram (Budtz-Jörgensen and Bertram, 1970), and Bergendal and Isacsson (Bergendal and Isacsson, 1983) have composed similar classifications. These classifications also describe red spots usually found around the small palatal minor salivary glands (type I), diffuse reddening referred to as diffuse hyperaemic, smooth and atrophic mucosa extending over the entire denture area (type II) and hyperaemic mucosa with a nodular appearance in the central part of the palate (type III).
1.3.3 AETIOLOGY

General factors that have been proposed to predispose to denture stomatitis are: decreased salivary flow rate, various medications, various endocrinopathies, nutritional and metabolic factors, and defects in the host defense mechanisms. Suggested local predisposing factors are deficient denture hygiene, poor retention and instability of dentures (Könsberg and Axéll, 1994).

The majority of dental researchers believe that the aetiology of denture stomatitis is multifactorial, some stating that no primary aetiological factor exists (Ritchie et al., 1969; Webb et al., 1998b). However various factors have been thought to be significant, including denture trauma, Candida infection, bacterial infection, and the acquired denture pellicle (Arendorf and Walker, 1987). It is generally regarded that Candida species, in particular C. albicans, can be the causative agents of denture stomatitis, although the previously mentioned factors and denture plaque bacteria may be involved (Webb et al., 1998b).

1.3.3.1 DENTURE TRAUMA

Cawson found no convincing evidence that denture stomatitis was caused by denture trauma (Cawson, 1965). However, the literature seems to give ample evidence of the correlation between trauma of the mucous membrane, in the form of an unbalanced denture occlusion and poor denture stability and retention, with denture stomatitis (Hecht, 1939; Nyquist, 1952; Love et al., 1967; McKendrick, 1968). Nyquist appears to have been the first to suggest that trauma played a prominent role in the aetiology of this condition (Nyquist, 1952). A year later, Nyquist reported a good therapeutic effect after denture adjustment (Nyquist, 1953). Samaranayake and colleagues (Samaranayake et al., 1980) showed that the adherence of C. albicans to PMMA strips in vitro was enhanced if the plastic was pre-coated with serum.

Budtz-Jörgensen and Theilade assessed denture site-specific differences in the rate of plaque formation on dentures from patients with candidosis (Budtz-Jörgensen and
Theilade, 1983). They found the buccal surface of the buccal denture flange showed the lowest median yeast colonisation and was significantly lower than all sites on the fitting surfaces ($P<0.01$) (Budtz-Jørgensen and Theilade, 1983). The factors responsible for the almost selective colonisation of the fitting denture surface compared with the buccal denture flange by yeast cells are not known. The presence of serum components in the inflammatory exudate in denture stomatitis could favour yeasts under dentures (Samaranayake et al., 1980). Also, *C. albicans* is generally considered a keratophilic organism (Taschdjian et al., 1960; Howlett, 1976) and adherence of yeast cells to shed epithelial cells trapped in the space between the denture and the overlying mucosa may favour yeast colonisation (Budtz-Jørgensen and Theilade, 1983). Parotid sIgA could possibly reduce the adherence of yeast cells to the buccal denture flange which is continuously bathed in saliva as compared with the fitting surface where salivary flow is minimal (Budtz-Jørgensen and Theilade, 1983).

Budtz-Jørgensen and Bertram attempted to answer the question of whether denture stomatitis is due to a primary infection by *Candida* or whether the yeasts are secondary invaders of traumatic lesions (Budtz-Jørgensen and Bertram, 1970). Their investigation included 58 patients with complete dentures and denture stomatitis; and a control group of 58 patients with complete dentures and clinically normal palatal mucosa. The dentures were analysed and patients accordingly divided into two groups: those with traumatic dentures (unbalanced occlusion), and those with non-traumatic dentures. The investigators found the maxillomandibular occlusal relationships tended to be better in the control group than in the denture stomatitis group. This difference was not statistically significant. The study of the quality of the denture-bearing tissues revealed a statistically significant difference ($P<0.5$) between the denture stomatitis group and the control group which had a higher proportion of well-developed alveolar ridges covered by non-mobile alveolar mucosa (Budtz-Jørgensen and Bertram, 1970). The authors concluded that trauma alone may give rise to simple localised inflammation and that traumatic lesions of the oral mucosa predispose to *Candida* growth, yet *Candida* growth and infection were still seen under presumably non-traumatic dentures (Budtz-Jørgensen and Bertram, 1970). For this reason, Budtz-Jørgensen (Budtz-Jørgensen, 1974b) considered that construction of new properly-fitting dentures and the use of
tissue conditioners should be included in the treatment of the local simple lesion but was insufficient to ameliorate Candida-induced lesions.

1.3.3.2 **CANDIDA INFECTION**

In 1936, Cahn suggested that *C. albicans* might be responsible for denture stomatitis following an investigation of ‘denture sore mouth’ in four patients with compromised health (Cahn, 1936). A year later Bartels (Bartels, 1937) confirmed the presence of these organisms by culturing swabs from Cahn’s patients. There have been many subsequent reports of a significantly greater prevalence and concentration of Candida species in denture stomatitis patients compared with those in controls with healthy denture-supporting mucosa (Lyon and Chick, 1957; Cawson, 1963; Turrell, 1966; Budtz-Jørgensen, 1972; 1974b; Olsen and Birkeland, 1976; Arendorf and Walker, 1979).

Cawson observed that hyphae were present as the predominant, or only, form in most cases of denture stomatitis from which Candida had been isolated and it was concluded that hyphae were actively growing in the space between the denture and mucosa (Cawson, 1965). Striking evidence for the significance of *C. albicans* in the aetiology of denture stomatitis was produced by Lehner, who demonstrated statistically significant differences in the titres of antibody against *C. albicans* in controls, carriers and patients with denture stomatitis (Lehner, 1965).

Several investigators have demonstrated that *C. albicans* can be isolated more frequently from the tissue-fitting surfaces of PMMA resin dentures than from the corresponding mucosa (Lyon and Chick, 1957; Bahn et al., 1962; Cawson, 1965; Davenport, 1970; 1972; Santarpia III et al., 1988; Budtz-Jørgensen, 1990c). Arendorf and Walker found a higher percentage of Candida-positive subjects amongst healthy denture wearers, compared with dentate subjects (Arendorf and Walker, 1979). They observed a highly significant (*P*<0.001) increase in density of Candida colonisation of all mucosal sites in normal denture wearers, compared with dentate subjects. The increase in both the frequency of carriers and the density of Candida colonisation in denture wearers
compared with dentate subjects suggested that the prosthesis encourages the presence and growth of Candida species (Arendorf and Walker, 1979).

*C. albicans* can induce inflammatory changes within mucosal tissues (Budtz-Jörgensen, 1990b) that could be responsible for the erythema seen in denture stomatitis (Davenport, 1970; Budtz-Jörgensen, 1990a). An alternative view, however, has been proposed in which the condition may be related to a community of potentially pathogenic microorganisms. In two extensive studies (Theilade et al., 1983; Theilade and Budtz-Jörgensen, 1988) it was shown that the predominant cultivable flora of denture plaque comprised Gram-positive cocci (*Streptococci*) and rod-shaped bacteria. *C. albicans* represented only 0.3% of colony-forming units (CFUs) in the denture plaque of patients with denture-related stomatitis, however in the control groups; *C. albicans* comprised 0.002% CFUs. This equated to a 150-fold increase in the organism concentration in subjects with denture stomatitis.

Another study (Harding et al., 1991) has confirmed the complex microbiological picture of denture plaque. *C. albicans* was not consistently found in the denture plaque. The authors considered that bacteria (particularly Gram-negative anaerobic bacteria) as well as yeasts might be involved in the aetiology of denture stomatitis (Harding et al., 1991). From these results it is difficult to attribute the aetiology of the condition entirely to the presence of *C. albicans* in denture plaque, although the organism is widely considered the most significant pathogen (Radford et al., 1999).

### 1.3.3.2.1 MIXED CANDIDA SPECIES ON PMMA DENTURES

Simultaneous recovery of multiple *Candida* species has been most documented in *Candida*-associated denture stomatitis (Budtz-Jörgensen, 1990a), where the PMMA dentures provide an ideal environment for colonisation. Although oral carriage of more than one yeast species is relatively frequent (Samaranayake et al., 1987), there are surprisingly few data on the factors that influence this phenomenon (Thein et al., 2007). Thein and colleagues (Thein et al., 2007) developed a model to study dual species biofilm formation on PMMA surfaces using *C. albicans* and *C. krusei* as they are
commonly isolated from the oral cavity in both health and disease (Samaranayake et al., 1987). *C. albicans* and *C. krusei* isolates were selected and used at varying cell concentrations to inoculate PMMA strips treated with whole saliva and CFU counts were performed. The authors found that dual species biofilms showed consistently smaller cell populations compared with mono species biofilms. It was found that *C. krusei* suppressed the growth of *C. albicans* in the biofilm by over 80% when compared with mono species biofilms. Similarly, when compared with monospecies biofilm growth, the presence of *C. albicans* appeared to suppress the growth of *C. krusei* by approximately 60-65%, indicating that *C. albicans* and *C. krusei* are mutually antagonistic in community growth (Thein et al., 2007).

### 1.3.3.3 BACTERIA

Yeast cells adhere to, and colonise, oral surfaces including mucosa and PMMA dentures and have the ability to co-aggregate with, and co-adhere to, oral bacteria (Cannon and Chaffin, 1999). However, Nyquist observed that there was no significant difference between the numbers of bacteria counted in cases of denture stomatitis and clinically normal mucosa and also no difference in the types of bacteria (Nyquist, 1953).

In contrast to Nyquist’s findings (Nyquist, 1953), van Reenen’s studies (van Reenen, 1973) gave prominence to the role of bacteria in denture stomatitis. The numbers of Gram-positive cocci including streptococci, pneumococci and staphylococci were often larger from the palatal mucosa of patients with denture stomatitis than from mucosa of patients with clinically healthy palates. Nyquist suggested that no specific organism was associated with denture stomatitis lesions and that the infection was caused by a community of pathogens.

The response of denture stomatitis to antifungal therapy has been cited as evidence for the primary role of *Candida* (Quinn, 1985). However, nystatin and chlorhexidine have been shown to also inhibit bacterial populations (Budtz-Jörgensen and Löe, 1972; van Reenen, 1973), and this is complemented by the evidence that penicillin, with a known ability to enhance fungal growth (Seelig, 1966), has a beneficial effect on denture
stomatitis (van Reenen, 1973). Thus, the difficulty in distinguishing the role of yeasts and bacteria in denture stomatitis is plain (Arendorf and Walker, 1987).

### 1.3.3.4 ROLE OF THE ACQUIRED DENTURE PELLICLE IN DENTURE STOMATITIS

The ability of *C. albicans* cells to attach to PMMA have been correlated with cell surface hydrophobicity (Minagi et al., 1985), electrostatic charge (Mozes et al., 1987), and specific cell adhesin-like absorption to a plastic surface (Tronchin et al., 1988). In the oral cavity, however, prostheses are rapidly coated by a salivary pellicle that alters its surface properties. Bacteria and yeast cells have been found to adhere to the outermost layer of this pellicle instead of directly to the denture surface (Theilade and Budtz-Jørgensen, 1980; Walter and Frank, 1985; Edgerton and Levine, 1992). This denture pellicle layer is comparable to that found on enamel (Al-Hashimi and Levine, 1989) and oral mucosa (Bradway et al., 1989). It is composed of a layer of selectively absorbed salivary components and studies have found that pellicles from denture surfaces contain quantitative differences in total adsorbed proteins (Edgerton and Levine, 1992) relative to pellicles from native saliva (DiPaola et al., 1984).

The composition of the denture pellicle appears to be dependent on the tissue condition of the denture-bearing surface. Immunoblots of the acquired denture pellicle from patients with stomatitis have identified additional serum components, background polydisperse material (possibly representing pellicle degradation products), and *C. albicans* cell components that have not been detected in the acquired denture pellicle from healthy patients (Edgerton and Levine, 1992). These serum proteins may function to enhance *Candida* cell adhesion and growth on the denture surface. Therefore, pellicles formed on the maxillary denture tissue surface may be altered by the addition of serum or salivary components to create disease-promoting pellicles that permit increased and selective microbial colonisation (Edgerton and Levine, 1992).
1.4 EXPERIMENTAL APPROACHES TO ORAL CANDIDA RESEARCH

1.4.1 RECOVERY OF CANDIDA FROM THE ORAL CAVITY

Techniques available for the isolation of Candida from the oral cavity include the use of a smear, a plain swab (Silverman Jr et al., 1990), an imprint culture (Davenport, 1970), collection of whole saliva (Oliver and Shillitoe, 1984), the concentrated oral rinse (Samaranayake et al., 1986b), and mucosal biopsy. Each method has particular advantages and disadvantages as seen in Table 2 (Williams and Lewis, 2000).

<table>
<thead>
<tr>
<th>Method</th>
<th>Quantitative</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imprint</td>
<td>Yes</td>
<td>Targets infected site. Differentiates infected and carrier status</td>
<td>Can be difficult to apply when defined lesions are not evident</td>
</tr>
<tr>
<td>Oral rinse</td>
<td>Yes</td>
<td>Quantification of other microbes</td>
<td>Does not locate site of infection</td>
</tr>
<tr>
<td>Swab</td>
<td>No</td>
<td>Ease of use</td>
<td>Can remove surface epithelial layers and should be avoided in areas for biopsy</td>
</tr>
<tr>
<td>Whole saliva</td>
<td>Yes</td>
<td>Sensitive for assessing Candida carriage</td>
<td>Chair time required for sample collection may be lengthy</td>
</tr>
<tr>
<td>Biopsy</td>
<td>No</td>
<td>Important in the diagnosis of chronic hyperplastic candidosis</td>
<td>Invasive and inappropriate for the majority of infections</td>
</tr>
<tr>
<td>Smear</td>
<td>No</td>
<td>Widely applicable and demonstrates hyphae</td>
<td>Less sensitive than other methods</td>
</tr>
</tbody>
</table>

Table 2: Methods of sampling the oral cavity for the isolation of Candida
1.4.1.1 SMEAR

A smear taken from the oral lesion is fixed on to a microscope slide and then stained either by the Gram-stain or by the periodic acid Schiff technique. Using these methods, *Candida* hyphae and yeast appear either dark blue (Gram-stain) or red/purple periodic acid-Schiff (Williams and Lewis, 2000). A smear is of value in differentiating between yeast and hyphal forms but is less sensitive than culturing methods (Silverman Jr et al., 1990).

1.4.1.2 SWAB

A sterile cotton swab is gently rubbed over the tissue lesion and is then subsequently used to inoculate a primary isolation medium such as Sabouraud’s dextrose agar (SDA). Whilst being a relatively simple method of detecting growth, the procedure does not generally permit quantification of the infecting *Candida* (Silverman Jr et al., 1990).

1.4.1.3 ORAL RINSE AND CONCENTRATED ORAL RINSE

The oral rinse technique involves the patient holding 10 mL of sterile phosphate-buffered saline (0.01 M, pH 7.2) in their mouth for 60 s. The solution is then expectorated, concentrated (10-fold) by centrifugation and a known volume, usually 50 µL, is spread on an agar medium using a spiral plating system. After 24-48 h incubation at 37°C, growth is assessed by enumeration of colonies and expressed as yeast CFUs per mL of rinse (Samaranayake et al., 1986b). The oral rinse can also be plated without concentration. This is called the neat rinse culture. It is simpler than the concentrated oral rinse, and may be more accurate as yeast can be lost during the centrifugation concentration step, but is less sensitive than the concentrated oral rinse method.
1.4.1.4 IMPRINT CULTURE

The imprint method utilizes a sterile foam-pad of known size (typically 2.3 cm$^2$), previously dipped in an appropriate liquid medium, such as Sabouraud’s broth, immediately before use. The pad is then placed on the target site (mucosa or intra-oral prosthesis) for 30 seconds and then transferred to an agar plate for culturing. Imprint cultures can give a greater rate of detecting Candida carriers than either whole saliva or impression culture yields using sterile foam pads dipped in SDA (Arendorf and Walker, 1980).

1.4.1.5 WHOLE SALIVA

Whole saliva is a combination of secretions from major (parotid, submandibular, and sublingual) salivary glands and minor salivary glands. The collection of whole saliva is a simple, practical, inexpensive and reliable technique that can easily be used by all practitioners but cannot be utilised in patients with severe salivary gland hypofunction as is often seen in elderly patients. Patients are asked to expectorate into sterile test tubes over a constant time period. A known volume of this whole saliva is used to inoculate agar plates for culture to determine the presence of Candida and CFU/mL of whole saliva calculated (Navazesh et al., 1995).

1.4.1.6 EVALUATION OF CANDIDA ISOLATION TECHNIQUES

In summary, Candida can be detected qualitatively by taking a swab of a lesion for culture, or by direct microscopic examination of stained smears. Smears are of value in differentiating between yeast and hyphal forms, but tend to be less sensitive than culturing methods (Silverman Jr et al., 1990). The concentrated oral rinse and imprint culture methods of sampling both provide a quantification of Candida which is advantageous clinically since enumeration of yeast present can differentiate between accepted levels of commensal candidal carriage and presence of infection (Arendorf and Walker, 1979; Silverman Jr et al., 1990).
Methods which have been employed for quantifying oral carriage of yeast include microscopical assessment of epithelial smears (Budtz-Jørgensen, 1974b; Jenkins et al., 1977), impression cultures (Budtz-Jørgensen, 1974b), imprint cultures (Davenport, 1970; Arendorf and Walker, 1980), saliva cultures (Lilienthal, 1950; Lehner, 1967; Oliver and Shillitoe, 1984) and oral rinse cultures (Johnston et al., 1967). Samaranayake and colleagues examined the sensitivity of the impression culture, the neat rinse culture and the concentrated rinse culture methods (Samaranayake et al., 1986b). Each subject was requested to rinse their mouth thoroughly with 10 mL of sterile phosphate buffered saline for 60 seconds in the presence of the clinician and expectorate it into in a universal container. All oral rinses were analysed in two ways: first, by using the neat oral rinse per se and second, by concentrating the remainder by centrifugation at 1700 g for 10 min. The pellet was then resuspended in 1 mL of sterile phosphate buffered saline to obtain the concentrated mouth rinse. Samples were plated and the number of yeast CFUs on each plate was counted (Samaranayake et al., 1986b). The sensitivity of the imprint culture technique (94%) was similar to the concentrated rinse culture technique (92%), while the sensitivity of the neat rinse culture (88%) was slightly inferior. Enumerating the CFUs obtained from imprint cultures tended to be inaccurate when the density of yeasts exceeded ~50 CFU/cm\(^2\), due to confluent growth of the colonies. However, the upper limit of detection of the rinse technique was considerably higher and rinse samples with \(10^5\) CFU/mL could be estimated with ease, as these could be diluted with phosphate buffered saline to yield discrete and quantifiable numbers of colonies (Samaranayake et al., 1986b).

1.4.2 CANDIDA CULTURE MEDIA

The most frequently used primary isolation medium for Candida is Sabouraud’s dextrose agar (Odds, 1991) which, although permitting growth of Candida, suppresses the growth of many species of oral bacteria due to its low pH. Typically Sabouraud’s dextrose agar cultures are incubated aerobically at 37°C for 24-48 h. Candida cells form cream-coloured, convex colonies and differentiation between species is rarely possible (MacFarlane, 1990).
It is estimated that more than one *Candida* species is present in approximately 10% of oral samples (MacFarlane, 1990) and in recent years the ability to detect non-*albicans* species has become increasingly important. As a consequence it has been recommended that Sabouraud's dextrose agar be used in combination with a second differential medium, examples include Pagano-Levin agar or the commercially available chromogenic agars, namely, CHROMagar® *Candida* (CHROMagar, Paris, France), Albicans ID (bioMérieux, Basingstoke, UK), Fluroplate (Merck, Damstadt, Germany) or Candichrom® *albicans* (International Mycoplasma, Toulon, France) (Williams and Lewis, 2000).

### 1.4.3 **CANDIDA QUANTIFICATION**

Quantification of *Candida* cells on mucosal surfaces might be useful as it has been suggested that the pathogenicity of *C. albicans* depends upon the number of organisms present (Campbell and Heseltine, 1960). In a study by Epstein and colleagues, subjects expectorated unstimulated saliva into sterilised, wide-mouthed centrifuge tubes during a 15 min period. A 0.5 mL volume of uncentrifuged saliva was spread on Sabouraud's dextrose agar plates containing 50 mg of chloramphenicol/mL; plates were incubated at 37°C for 48 h, and the number of yeast CFU/mL of saliva was counted. The authors found a correlation between signs and symptoms of candidosis and concentrations > 400 CFU/mL of saliva. Subjects without evidence had *C. albicans* concentrations < 400 CFU/mL of saliva and were considered carriers. Statistical analysis showed significant differences between CFU concentrations in saliva from carriers and in saliva from patients with either chronic candidosis or acute candidosis, indicating that carriers and patients with disease can be distinguished within 95% confidence limits on the basis of quantitative cultures for *C. albicans* (Epstein et al., 1980).
1.4.4 CANDIDA SPECIES IDENTIFICATION

The methods used to detect and identify fungal colonies have evolved significantly in recent years. Historically, the ability to culture fungi was critical for its detection and identification via microscopy, biochemical assays, and selective media growth. Observation of pseudohyphae, germ tubes and chlamydospores (large thick-walled cells which develop at the tips of pseudohyphae) are helpful in identifying *C. albicans*. All pathogenic *Candida* species assimilate (take up and use) and ferment glucose as a carbon source, none assimilates nitrate as a nitrogen source, but they vary in their abilities to utilise other carbon and nitrogen sources (Odds, 1988). Thus *Candida* can be speciated according to its nitrogen and carbon source utilisation and fermentation pattern.

1.4.4.1 MORPHOLOGICAL CRITERIA

The germ-tube test (Taschdjian et al., 1960) is the standard laboratory method for identifying *C. albicans*. The test involves the induction of hyphal outgrowths (germ-tubes) from yeast cultured in serum for 2-4 h at 37°C. Approximately 95% of *C. albicans* isolates produce germ tubes (Perry and Miller, 1987), a property also shared by *C. stellatoidea* (re-classified as *C. albicans*) (Haley, 1971) and *C. dubliniensis* (Sullivan and Coleman, 1998), and rare isolates of *C. tropicalis* (Martin, 1979).

Chlamydospore (big, thick-walled resting/dormant growth forms) production is primarily associated with *C. albicans* but again is found with *C. dubliniensis* and rare isolates of *C. tropicalis* (Hasenclever, 1971; Sullivan and Coleman, 1998). Chlamydospores are induced *in vitro* by growth on corn-meal agar supplemented with Tween 80 at ~22°C for 72h (Hasenclever, 1971) and can aid in the identification of *Candida* species.
1.4.4.2 PHYSIOLOGICAL CRITERIA

Carbohydrate assimilation profiles for *Candida* species can be obtained by examining zones of growth around discs or wells impregnated with various sugars on basal agars (Di Menna, 1955). In contrast, fermentation tests are generally performed in liquid media and are based on demonstration of acid or gas production. These physiological tests are time-consuming and laborious to perform (Williams and Lewis, 2000).

1.4.4.3 COMMERCIALLY AVAILABLE IDENTIFICATION SYSTEMS

The introduction of commercially available identification systems has greatly reduced the laboratory time involved in the speciation of yeast isolates. The commercial systems presently available are based on growth or enzyme production profiles. Growth systems are relatively slow, typically requiring 72 h incubation, whilst enzyme systems can potentially provide results within a few hours. The Minitek system examines the ability of yeast to grow in the presence of discs impregnated with specific carbohydrates. The API-20 C system utilizes carbohydrate assimilations and since the API-20 C is 96-99% as accurate as conventional culturing procedures (Buesching et al., 1979) it is frequently used as a reference method for *Candida* identification (Sandven, 1990). Unfortunately, isolates of *C. dubliniensis* have atypical assimilation profiles that are not currently incorporated into the analytical profile indices for the API systems (Salkin et al., 1998).

The RapID Yeast Plus System is based on the enzymatic degradation of conventional and chromogenic substrates. Since this system detects *in situ* enzymes and does not require yeast growth, results can be obtained within 4 h of inoculation (Kitch et al., 1996).

1.4.4.4 INSTRUMENT-BASED IDENTIFICATION SYSTEMS

Semi-automated approaches are available for the identification of *Candida* species. With the Vitek-AMS system results are obtained after 4-8 h, based on the transmission of light through inoculated substrate (Liao et al., 1992).
The Abbott Quantum II (Sekhon et al., 1987) is a semi-automated system that identifies medically important yeasts based on biochemical tests and germ-tube formation. Similarly, the MicroScan Rapid Yeast identification panel tests for the presence of fungal enzymes. As growth is not a pre-requisite, the MicroScan system provides rapid identification after 4 h (Land et al., 1991; St Germain and Beauchesne, 1991). These systems are relatively expensive and are only cost-effective for mycology laboratories with large diagnostic throughputs.

1.4.4.5 GENETIC ANALYSIS

The phenotypic criteria used by the methods described above are subject to variable expression, strain-specific variation, and as such may lead to incorrect identification (Williams and Lewis, 2000). A potentially more accurate approach would be one based on the analysis of genetic variability (Maslow et al., 1993).

There are two traditional approaches for the identification of Candida based on genetic variation. These methods are analyses of electrophoretic karyotype differences (the distinctive migration of whole chromosomes through agarose in an electric field) (Monod et al., 1990) and restriction fragment length polymorphisms (RFLPs) using agarose gel electrophoresis or DNA-DNA hybridisation (Matthews and Burnie, 1989).

Electrophoretic karyotype analysis has been used to successfully differentiate isolates of C. albicans, C. tropicalis, C. parapsilosis, C. glabrata and C. krusei (Monod et al., 1990; Wickes et al., 1992). A sufficiently high level of species discrimination has been reported using pulsed field gel electrophoresis of whole chromosomes to suggest that the approach can be of value in the epidemiological typing of Candida strains (Williams and Lewis, 2000). It is a laborious method, however, and cannot readily distinguish between strains of the same species.

RFLP analysis provides an alternative to electrophoretic karyotyping. Extracted DNA is digested into fragments by DNA restriction enzymes prior to separation by gel electrophoresis. Analysis of the resulting banding profiles can be achieved by directly staining with ethidium bromide or labeling of specific fragments with DNA probes and
hybridizing them with blots of the DNA fragments. Although RFLP analysis offers a sensitive and reproducible approach for the discrimination of *Candida* strains, the technique requires computer-based systems and databases for comparisons of large numbers of strains (Wickes et al., 1992).

The polymerase chain reaction (PCR) has been used to identify *Candida* species based on detection of genes encoding chitin synthase (Jordan, 1994), actin (Kan, 1993) or cytochrome P$_{450\text{L}_1\text{A}_1}$ (Buchman et al., 1990). Ribosomal DNA contains genes encoding ribosomal RNA which are also a frequent target in PCR-based identification systems (Avni et al., 2011). RFLP studies have shown that rDNA sequences differ between *Candida* species (Niesters et al., 1993) and PCR amplification of 18S rDNA, enables discrimination of *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei* (Magee et al., 1987) and *C. dubliniensis* (Sullivan et al., 1995).

### 1.4.4.6 CHROMOGENIC MEDIA

As discussed earlier, although *C. albicans* remains the most common cause of human candidosis, the frequency of infection attributed to other members of the genus is also increasing (Price et al., 1994; Wingard, 1995). The conventional methods of yeast identification, which mainly consist of assimilation and fermentation characteristics, are cumbersome (Nadeem, 2010). Therefore numerous isolation media have been developed that can identify these *Candida* pathogens within 4-72 h, depending upon the system used (Schuffenecker et al., 1993; Fenn et al., 1994; Kitch et al., 1996; Heelan et al., 1998). Several brands of chromogenic media are available for rapid identification of yeast. These special media yield microbial colonies with varying pigmentation due to the presence of chromogenic substrates in the agar that react with enzymes secreted by these microorganisms. These media are species-specific, allowing the organisms to be identified to the species level by their colour and colony characteristics (Nadeem, 2010).
1.4.4.6.1 CHROMagar Candida

CHROMagar Candida is a selective and differential medium that allows the isolation of yeasts and simultaneously presumptively identifies (by colour development and colony morphology) colonies of *C. albicans*, *C. tropicalis*, and *C. krusei* with a high degree of accuracy (Odds and Bernaerts, 1994; Beighton et al., 1995). It facilitates the detection and identification of yeasts from mixed cultures and can provide results 24 to 48 h sooner than standard isolation and identification procedures (Odds and Bernaerts, 1994; Louwagie et al., 1995).

The specificity of identification for CHROMagar® *Candida* is reported to be 95% (Pfaller et al., 1996). The manufacturer of CHROMagar Candida currently advertises its product as able to detect and differentiate three species, *C. albicans* by growth as light to medium green colonies, *C. tropicalis* by growth as steel blue colonies accompanied by purple pigmentation diffused into surrounding agar, and *C. krusei* by growth as large, fuzzy, rose coloured colonies with white edges, after incubation for 48 h at 37°C, as also reported in several studies (Odds and Bernaerts, 1994; Baumgartner et al., 1996; Bernal et al., 1996; Pfaller et al., 1996).

Nadeem and colleagues (Nadeem, 2010) and Odds and Bernaerts (Odds and Bernaerts, 1994) have concluded that when identifying species with CHROMagar Candida, it is not necessary to perform germ tube tests to confirm the presence of *C. albicans*. The colour of colonies on CHROMagar Candida was found to be similar to that given by the manufacturer. However, in heavily inoculated specimens, it was difficult to differentiate between mixtures of yeast species on a single agar plate because of the pale colour of the colonies (Nadeem, 2010). CHROMagar Candida correctly identified 99% of *C. albicans*, 98% of *C. tropicalis*, 100% of *C. krusei*, and 94% of *C. glabrata* but for other Candida species other tools of identification, for example, API 20C Aux along with CHROMagar Candida should also be used for correct identification (Nadeem, 2010).

Pfaller and colleagues examined 547 specimens by inoculating them in parallel onto CHROMagar Candida and Sabouraud’s dextrose agar (Pfaller et al., 1996). The authors were able to correctly identify to the species level 100% of the stock isolates of *C. albicans*, *C. tropicalis*, and *C. krusei* and 90% of the *C. glabrata* isolates. CHROMagar
Candida correctly identified 94% of C. glabrata isolates, 95% of C. albicans isolates, and 100% of C. tropicalis and C. krusei isolates. Agreement between observers reading the CHROMagar plates was 96% for C. albicans, 90% for C. glabrata, 66% for C. krusei, 100% for C. tropicalis and for other (non-chromogenic) Candida species. Over 95% of stock and clinical isolates of these species were correctly identified on the basis of colony morphology and pigmentation on CHROMagar (Pfaller et al., 1996).

1.4.5 PHOTOGRAPHIC DENTURE PLAQUE ASSESSMENT METHODS

For denture plaque assessment most methods assess plaque coverage by staining with a disclosing agent, most commonly erythrosine. Budtz-Jørgensen and Bertram proposed a simple index to grade plaque coverage on the denture fitting surface (Budtz-Jørgensen and Bertram, 1970). A slightly more sensitive method was described by the same group in 1978 (Budtz-Jørgensen and Knudsen, 1978). Augsberger and Elahi (Augsburger and Elahi, 1982) developed a well-used index for scoring denture plaque where the maxillary denture fitting surface was sectioned into four areas and the mean score calculated. In a more recent study by Paranhos and colleagues (Paranhos et al., 2007), the denture fitting surface was divided into 14 areas and the biofilm quantified in each area. This latter method appears to be a more time-consuming version of the Augsberger and Elahi index with no apparent advantages (Coulthwaite and Verran, 2009).

Plaque indices are most commonly used to assess denture plaque coverage without image capture. This is not ideal because the methods are subjective, examiner bias may occur, there is no reproducibility between studies and the methods have lower accuracy and sensitivity than image analysis (Coulthwaite and Verran, 2009). Digitised images can be stored and examined at a later time, allowing longitudinal comparisons, re-measuring for reproducibility and double-blind studies (Söder et al., 1993; Carter et al., 2004).

Coulthwaite and Verran compared the available visual plaque measurement techniques for ease of measurement, accuracy, and reproducibility, in order to recommend the ‘best’ method for standardisation (Coulthwaite and Verran, 2009). Dentures were rinsed to remove any loose food particles and stained with methylene blue disclosing solution.
for 1 min and then rinsed before visual scoring. The area of the denture fitting surface covered by stained plaque was measured and compared with the entire fitting surface area to give a percentage plaque cover. The authors found that conventional plaque scoring was not representative of the actual plaque area and demonstrated that visual scoring is, by definition, subjective. Based on the results of this study, the authors recommended blinded visual scoring of images as the gold standard using the method of Augsburger and Elahi (Augsburger and Elahi, 1982).

### 1.4.6 SITE-SPECIFIC DETECTION OF CANDIDA

Various techniques have been used to identify yeasts in investigations of the aetiology of candidosis. These include imprint cultures (Cawson, 1963), sampling and culture from the denture base (Budtz-Jørgensen, 1974b), and growth of denture plaque on sand-blasted adhesive tape (Budtz-Jørgensen et al., 1981). Santarpia and colleagues (Santarpia III et al., 1990) examined the complete dentures of 73 men and women who tested positive for *C. albicans*. One strip of beading wax was placed just below the height of the denture border and sealed using a hot spatula. A single strip of boxing wax was applied around the beading wax to create a mould to confine the growth medium (Santarpia III et al., 1988).

*C. albicans*-selective synthetic media (Difco Laboratories, Detroit, Mich.) was prepared and sterilised then cooled to 56°C and biotin added (Santarpia III et al., 1988). Fifty to 75 mL of cooled liquid synthetic media was pipetted into the deepest portion of the boxed denture. The agar was filled to 1 cm beyond the denture border and allowed to solidify for 10 min and chilled for an additional 5 min. The beading and boxing was removed and the replica gently freed from the denture and inverted into a sterile culture dish (Santarpia III et al., 1988). The replicas were incubated for a period of 3-4 days at room temperature (25°C) and documented photographically every 24 h for growth of *C. albicans* (Santarpia III et al., 1988).

The authors found that, in general, the severity of inflammation of the mucosal surfaces correlated with the number of *C. albicans* colonies on the agar replicas, but the
inflammation occasionally occurred at a site distant from the yeast colonisation. The largest number of \textit{C. albicans} colonies appeared in patients with a Newton Type III stomatitis diagnosis, as compared with patients with a Newton Type I or II diagnosis (Santarpia III et al., 1988).

Spiechowicz and colleagues (Spiechowicz et al., 1991) examined 22 complete denture wearers to compare the sensitivity of the standard technique of swabbing the denture to that of a cast agar replica technique for detecting \textit{C. albicans}. The majority of cultures obtained by swabbing failed to detect the presence of \textit{C. albicans}, while all cast agar replicas grew \textit{C. albicans}. The replica method for the detection of \textit{C. albicans} in edentulous patients seemed to be more sensitive than swabbing (Spiechowicz et al., 1991).
1.5 SUMMARY

The prevalence of complete tooth loss in New Zealand has been reported to be higher than in any other country, affecting a large proportion of the elderly. People who have lost all of their teeth will usually be restored with full dentures.

Once dentures are placed into the mouth, they become coated by a salivary pellicle. This pellicle facilitates subsequent colonisation of denture surfaces by various microorganisms including *Candida* species. Denture-associated *Candida* infection is usually painless and localised to a relatively small area of the mouth. However, it may lead to serious complications such as systemic yeast infection in susceptible patients, including those who have a compromised immune system, particularly the elderly.

Investigators believing mucosal trauma to be a significant aetiological factor in the development of *Candida*-associated denture stomatitis have supported the manufacture of new PMMA dentures (Nyquist, 1953). There is still much to learn, however, about the colonisation of dentures by yeast. It is not known where on the dentures the yeast adhere, or whether different yeast species are found in association with dentures as opposed to those present in saliva. Similarly it is also not know what effect new dentures will have on the colonisation of saliva and denture surfaces by *Candida* species.
1.6 AIMS OF THE INVESTIGATION

The aim of this research project was to investigate the patterns of the colonisation of saliva and the acrylic denture fitting surface by yeast species in patients with old ill-fitting dentures and following delivery of new dentures. It was postulated that the prescription of new dentures would affect the numbers and species of yeast colonising both the denture fitting surface and the saliva. A greater knowledge of the type, location and numbers of yeast colonising dentures and denture soft-liners will inform prosthodontic practice in order to reduce the incidence of Candida-induced denture stomatitis and its significant sequelae.

1.7 RESEARCH HYPOTHESES

1. The fitting surfaces of dentures are colonised in a distinctive pattern by Candida species.
2. The species of Candida adhering to the fitting surfaces of dentures are different to those present in saliva.
3. Candida colonisation of denture fitting surfaces is closely correlated with clinical signs of candidiasis.
4. Fabrication of new dentures will reduce Candida colonisation of denture surfaces and the saliva.

1.8 SPECIFIC OBJECTIVES

1. To examine the pattern of Candida colonisation of maxillary denture fitting surfaces and saliva in patients wearing complete acrylic dentures before and after receiving new prostheses.
2. To correlate Candida colonisation with clinical signs of inflammation.
3. To relate the number of yeast cells and the species of Candida colonising denture surfaces with those in the saliva.
1.9 SIGNIFICANCE OF THIS RESEARCH:

- To the best of the author’s knowledge, this will be the first in vivo study conducted to identify changes in the number and type of *Candida* species found adhering to denture acrylic and colonising the saliva following the delivery of new dentures.
- This research will endeavour to establish whether fabrication of new dentures could aid in the management of denture stomatitis.
- This research will inform prosthodontic practice and may lead to improved acrylic denture design, fabrication and deployment to minimise *Candida* colonisation and thereby ameliorate the effects of *Candida*-induced denture stomatitis.
## MATERIALS AND METHODS

### 2.1 EQUIPMENT

Table 3: Equipment used in this study

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water bath 3.5 L</td>
<td>Grant Instruments (Cambridge) Ltd., Cambridgeshire, Great Britain</td>
</tr>
<tr>
<td>Water bath 30 L</td>
<td>Julabo GmbH, Seelbach, Germany</td>
</tr>
<tr>
<td>Polypropylene tubes 50 mL</td>
<td>Greiner Bio-One GmbH, Frickenhausen, Germany</td>
</tr>
<tr>
<td>Polypropylene tubes 15 mL</td>
<td>Greiner Bio-One GmbH, Frickenhausen, Germany</td>
</tr>
<tr>
<td>Glass petri dishes (90 mm diameter)</td>
<td>Glassblowing unit, Division of Sciences, Zoology Annexe, University of Otago, Dunedin, New Zealand</td>
</tr>
<tr>
<td>Petri dish extenders</td>
<td>Fabricated from stainless steel pipe at the University of Otago</td>
</tr>
<tr>
<td>Plastic petri dishes (85 mm diameter)</td>
<td>Labserv, Auckland, NZ</td>
</tr>
<tr>
<td>250 mL Glass Schott bottles</td>
<td>Schott North America Inc., New York, U.S.A.</td>
</tr>
<tr>
<td>Glass universal bottles 23 mL</td>
<td>Glassblowing unit, Division of Sciences, Zoology Annexe, University of Otago, Dunedin, New Zealand</td>
</tr>
<tr>
<td>Glass spreader</td>
<td>Glassblowing unit, Division of Sciences, Zoology Annexe, University of Otago, Dunedin, New Zealand</td>
</tr>
<tr>
<td>Labnet P200 Pipettes</td>
<td>Labnet International Inc., New Jersey, U.S.A.</td>
</tr>
<tr>
<td>Test tube rack (Unwire half rack Resmer 30mm 9 tubes)</td>
<td>Thermo Fisher Scientific Inc., Massachusetts, U.S.A.</td>
</tr>
<tr>
<td>Canon EOS 1100D camera body</td>
<td>Canon Inc., Japan</td>
</tr>
<tr>
<td>Canon Macro Lens EF 100 mm 1:2.8</td>
<td>Canon Inc., Japan</td>
</tr>
<tr>
<td>UV/white light transilluminator</td>
<td>UVP, California, U.S.A.</td>
</tr>
<tr>
<td>Intraoral full arch mirror</td>
<td>Novus Dental Supplies, Colorado, U.S.A.</td>
</tr>
<tr>
<td>PVC caps and tubing</td>
<td>Marley New Zealand Ltd., Manurewa, Auckland</td>
</tr>
<tr>
<td>New Brunswick C660 Ultra-Low Temperature Freezer (-80°C)</td>
<td>Eppendorf AG, Hamburg, Germany</td>
</tr>
<tr>
<td>Incubator (30°C and 37°C)</td>
<td>Binder Gmbh, Tuttlingen, Germany</td>
</tr>
<tr>
<td>Cryotubes (1.5 mL)</td>
<td>Neptune Scientific, California, U.S.A.</td>
</tr>
</tbody>
</table>
### 2.2 MATERIALS

**Table 4: Materials used in this study**

<table>
<thead>
<tr>
<th>Materials</th>
<th>Producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Giroform® silicone putty</td>
<td>Amann Girrbach GmbH, Pforzheim, Germany</td>
</tr>
<tr>
<td>CHROMagar™ <em>Candida</em></td>
<td>CHROMagar Microbiology Inc., Paris, France</td>
</tr>
<tr>
<td></td>
<td>Supplier: Fort Richard Laboratories, Auckland, New Zealand</td>
</tr>
<tr>
<td>CHROMagar™ <em>Candida</em> plates</td>
<td>CHROMagar Microbiology Inc., Paris, France</td>
</tr>
<tr>
<td></td>
<td>Supplier: Fort Richard Laboratories, Auckland, New Zealand</td>
</tr>
<tr>
<td>Ufi Gel soft liner (SC)</td>
<td>Voco GmbH, Cuxhaven, Germany</td>
</tr>
<tr>
<td>Bacto Yeast Extract</td>
<td>Becton, Dickinson and Company, New Jersey, U.S.A.</td>
</tr>
<tr>
<td>Bacto™ Peptone</td>
<td>Becton, Dickinson and Company, New Jersey, U.S.A.</td>
</tr>
<tr>
<td>D(+)−Glucose</td>
<td>Merck Millipore, Merck KGaA, Darmstadt, Germany</td>
</tr>
<tr>
<td>Agar Bacteriological (LP0011)</td>
<td>Oxoid Ltd., Hants, U.K.</td>
</tr>
<tr>
<td>Distilled water (RiOs™ Essential 8)</td>
<td>Merck Millipore, Merck KGaA, Darmstadt, Germany</td>
</tr>
<tr>
<td>Glycerol (&gt;99% purity)</td>
<td>Merck Millipore, Merck KGaA, Darmstadt, Germany</td>
</tr>
</tbody>
</table>
2.3 STUDY PARTICIPANTS

2.3.1 ETHICAL APPROVAL

Ethical approval for the research project was gained from the University of Otago Human Ethics Committee, (Appendix 2: Ethical approval) prior to the collection of saliva, mucosal and denture samples. Consultation was undertaken with the Ngāi Tahu Research Consultation Committee (Te Komiti Rakahau ki Kāi Tahu), prior to the collection of saliva and denture samples, and the research was considered of importance to Māori health (Appendix 3: Ngāi Tahu Research Consultation committee response).

2.3.2 PARTICIPANT SELECTION AND INFORMED CONSENT

Participants were randomly selected from patients attending Postgraduate Diploma in Clinical Dental Technology (PGDipCDTech) clinics in the School of Dentistry, University of Otago, for the construction of complete dentures. The criteria for inclusion in the study were as follows: the participant was completely edentulous, was wearing acrylic complete dentures in the maxillary and mandibular dental arches, and was attending the clinic to have new acrylic complete dentures constructed. There were no established exclusion criteria. As this was a pilot study, 16 participants were included in the project. Due to a lack of data on denture colonisation by different Candida spp., a power analysis could not be used to calculate the number of participants required to answer the research questions.

An information sheet (Appendix 6: Information sheet for participants) was given to the participants, with a verbal explanation, prior to obtaining their consent to enter the study (Appendix 7: Consent form). After informed consent had been obtained, the participants’ personal information including their age, gender, smoking status, denture hygiene practices and brief medical history were recorded (Samaranayake et al., 1980) and it was confirmed that they accurately matched patient records from the School of Dentistry, University of Otago.
2.4 SAMPLE COLLECTION AND ANALYSIS

2.4.1 WHOLE SALIVA

The participant’s maxillary and mandibular complete dentures were removed and whole saliva samples were obtained. Whole saliva (5 mL) was collected into sterile 50 mL polypropylene tubes in the presence of the clinician over a period of up to 60 seconds (Samaranayake et al., 1986b). Saliva samples were transported to the Molecular Microbiology Laboratory, Faculty of Dentistry. The 50 mL tubes were vortexed for a few seconds to homogenise the sample and 100 µL of the saliva was dispensed onto CHROMagar Candida agar plates using an automatic pipette, spread evenly on the agar plates using a glass spreader and incubated at 37°C for 48 h.

The plates were then refrigerated for 48 h at 4°C, to enhance colony colour development. The colony forming units (CFUs) on the plates were counted and the species of yeast present presumptively identified from the colony colour (Samaranayake, 1986; Odds and Bernaerts, 1994). The agar plates were photographed and stored at 4°C.

2.4.2 MUCOSAL SWABS

Immediately after removal of the maxillary denture, a sterile cotton swab was gently rubbed over the maxillary primary denture bearing tissues on the patient’s left hand side in the region of the greater palatine foramen. The swabs were placed in 2 mL of sterile saline in a plastic tube and sealed for transport to the laboratory. The samples were then vortexed and 100 µl volumes used to inoculate CHROMagar Candida plates which were incubated at 37°C for 48 h. Species and colony forming units per mucosal swab were counted (Epstein et al., 1980; Beighton et al., 1995).
2.4.3 CLINICAL EXAMINATION AND INTRAORAL PHOTOGRAPHS

An oral examination was performed with the intention of identifying overt stomatitis according to the classification of Newton (Newton, 1962) and overt mechanical trauma of the denture-bearing tissues. Intraoral photographs were taken using cheek retractors, intraoral mirror and extraoral camera to obtain an unobstructed and clear view of the entire maxillary denture-bearing mucosa.

If overt stomatitis was evident, the participant’s maxillary denture received a GC Soft-Liner (GC Corporation, Japan) tissue conditioner to isolate the denture biofilm from the mucosa and minimise potential tissue trauma (Nyquist, 1953). The tissue conditioner was mixed and applied to the denture-fitting surface according to the manufacturer’s instructions.

2.4.4 CHROMAGAR CANDIDA DENTURE IMPRESSIONS:

Following optimisation of the denture fitting surface imprint culture methodology, CHROMagar Candida (CHROMagar Microbiology Inc., France) growth medium components were mixed with distilled water according to the manufacturer’s instructions in Schott bottles and microwaved for approximately 120 s until the solution reached boiling point and all solid components were dissolved. The molten agar was then cooled to 45°C in a water bath before transportation to the clinic. In the clinic, the molten agar was stored at 45°C in a water bath until use.

Participant’s maxillary complete dentures were removed from the mouth and immediately placed into sterilised polyvinyl chloride open-topped containers with the fitting surface facing outwards and boxed with plasticine. Molten agar was pipetted into the deepest portion of the boxed denture. Pipetting was carried out at a rate of approximately 100 mL/min so as not to disturb the microorganisms adhering to the denture surface. Pipetting was continued until the mould was filled 1 cm beyond the denture border but below the edge of the polyvinyl chloride container. The container
was covered in cling film and surrounded with ice for 30 min to accelerate agar solidification.

The cling film was then removed and the agar replica gently freed from the denture and boxing material using air from a sterilised air syringe, placed into a sterile culture dish and transported to the laboratory (Santarpia III et al., 1990). The replicas were incubated at 37°C for a period of 48 h.

The denture imprint cultures were then refrigerated for 48 h at 4°C, to enhance colony colour development. The CFUs on the imprints were counted and the species of yeast present presumptively identified from the colony colour (Samaranayake, 1986; Odds and Bernaerts, 1994). The agar plates were photographed and stored at 4°C.

2.4.5 REPEAT SAMPLING AND ANALYSIS:

Saliva samples, mucosal swabs and agar denture impressions were also acquired from the participants 1 month, 3 months and 6 months post-insertion of the new acrylic complete dentures.
2.5 STORAGE OF *C. ALBICANS* STRAINS

Up to five representative *C. albicans* colonies (green in colour) from each CHROMagar *Candida* agar plate and denture replica, were picked with a sterile loop and plated on yeast extract peptone dextrose (YPD) agar plates divided into five segments (one segment for each individual colony). The YPD agar plates were then incubated at 37°C for 48 h.

The YPD agar was prepared by mixing 2 g yeast extract, 4 g bactopeptone, 4 g glucose and 4 g agar in 200 mL distilled water. The mixture was then autoclaved at 121°C with a holding time of 15 min to dissolve and sterilise the agar. After removal from the autoclave the mixture was poured into petri dishes and allowed to solidify for 12 h before refrigeration and use.

Colonies from the YPD agar plates were stored in YPD containing 20% glycerol. YPD broth was prepared by mixing 0.8 g yeast extract, 1.6 g bactopeptone and 1.6 g glucose with 80 mL distilled water and 20 g glycerol. The mixture was then autoclaved at 121°C with a holding time of 15 minutes. Portions (200 µL) of the prepared YPD + glycerol broth were pipetted into autoclaved cryotubes; one for each representative *C. albicans* colony from the saliva and denture impression samples (up to five cryotubes per sample). *C. albicans* from the YPD agar plates were suspended in the YPD + glycerol broth in the cryotubes until the solution was opaque. The cryotubes were then stored at -80°C for use in future studies.
3 RESULTS

3.1 STUDY PARTICIPANTS

Of the 20 participants screened, 16 were selected who met the inclusion criteria and were willing to participate in the research project. Denture imprint cultures from 4 participants (t=0) were contaminated due to researcher error before the cultures were photographed and colony counts performed. Of the 12 remaining participants, 11 continued to wear their new dentures for the duration of the project and of these, 10 were able to return for each sampling recall and were included in the data analysis (Table 5). The average age of the participants was 76.9 years. There were seven males and three females included and only one participant was a smoker. The average age of the participant’s old dentures was 8.2 years. Each participant suffered from at least one diagnosed medical condition.
<table>
<thead>
<tr>
<th>Participant</th>
<th>Age</th>
<th>Sex</th>
<th>Smoking status</th>
<th>Age of dentures</th>
<th>Diagnosed medical conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>71</td>
<td>Male</td>
<td>Non-smoker</td>
<td>13 years</td>
<td>Hypertension</td>
</tr>
<tr>
<td>8</td>
<td>59</td>
<td>Female</td>
<td>Smoker</td>
<td>5 years</td>
<td>Menopause</td>
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<tr>
<td>9</td>
<td>78</td>
<td>Male</td>
<td>Non-smoker</td>
<td>10 years</td>
<td>Hypertension, Hypercholesterolaemia, Gastric reflux</td>
</tr>
<tr>
<td>10</td>
<td>68</td>
<td>Female</td>
<td>Non-smoker</td>
<td>5 years</td>
<td>Hypertension, Hypercholesterolaemia, Allergic rhinitis, Osteoporosis, Arthritis</td>
</tr>
<tr>
<td>11</td>
<td>84</td>
<td>Female</td>
<td>Non-smoker</td>
<td>8 years</td>
<td>Hypertension, Congestive heart failure, Hypercholesterolaemia, Hypothyroidism</td>
</tr>
<tr>
<td>12</td>
<td>84</td>
<td>Male</td>
<td>Non-smoker</td>
<td>25 years</td>
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<tr>
<td>13</td>
<td>82</td>
<td>Male</td>
<td>Non-smoker</td>
<td>2 years</td>
<td>Gastric reflux, Hypertension, Prostate enlargement, Type 2 diabetes, Hyperuricemia, Congestive heart failure, Hypercholesterolaemia, Asthma, Chronic obstructive pulmonary disease, Vertigo</td>
</tr>
<tr>
<td>14</td>
<td>72</td>
<td>Male</td>
<td>Non-smoker</td>
<td>6 years</td>
<td>Hypertension, Arthritis</td>
</tr>
<tr>
<td>15</td>
<td>92</td>
<td>Male</td>
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<td>3 years</td>
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<tr>
<td>16</td>
<td>79</td>
<td>Male</td>
<td>Non-smoker</td>
<td>5 years</td>
<td>Hypertension, Hypercholesterolaemia</td>
</tr>
</tbody>
</table>
### 3.2 SALIVA CULTURES

The saliva and denture imprint samples obtained before the participants had new dentures fabricated (and they were still wearing their existing dentures), were designated “old dentures” in the tables and figures, and represent the samples at t = 0 months. Follow-up samples obtained following delivery of new dentures at 1 month post-insertion were designated “new dentures 1 month post-insertion”, 3 month recall samples were designated “new dentures 3 months post-insertion” and 6 month recall samples designated “new dentures 6 months post-insertion”.

#### 3.2.1 SALIVA CULTURE RESULTS OVERVIEW

Saliva samples were plated on CHROMagar Candida plates and the concentration of each yeast species present (presumptively identified by colony colour) calculated as CFU/ml saliva. Samples from participants 8, 12 and 15 were collected after alginate impressions had been taken of the maxillary and/or mandibular denture bearing surfaces (Table 6).
Table 6: Concentration of *Candida* species (CFU/mL) present in saliva samples before fabrication of new dentures and 1, 3, and 6 months post-delivery of new dentures

<table>
<thead>
<tr>
<th>Participant</th>
<th>Old dentures</th>
<th>New dentures 1 month-post insertion</th>
<th>New dentures 3 months-post insertion</th>
<th>New dentures 6 months-post insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yeast present</td>
<td>CFU/mL</td>
<td>Yeast present</td>
<td>CFU/mL</td>
</tr>
<tr>
<td>7</td>
<td>C. albicans 40</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>C. tropicalis 20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>C. albicans 20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>C. albicans 320*</td>
<td>C. albicans 70</td>
<td>C. albicans 80</td>
<td>C. albicans 780</td>
</tr>
<tr>
<td>13</td>
<td>C. tropicalis 330</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>C. krusei 3380</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>C. tropicalis 200</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>Other 1240</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>C. albicans 50*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>C. albicans 40</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* denotes no *Candida* found
* * denotes samples collected after alginate impressions
3.2.2 SALIVA CULTURES FROM PARTICIPANTS WITH OLD DENTURES

There was a large amount of variation in the yeast concentration obtained from participant saliva samples before denture fabrication (Figure 1). The total *Candida* concentration in saliva from participant 14 was over six times the next highest concentration at this time (Participant 13). Only saliva from Participant 14 contained multiple *Candida* species at this time point. This sample yielded a large number of *C. krusei* colonies, some *C. albicans* colonies and a large number of other yeast species that could not be readily identified as *C. albicans*, *C. krusei* or *C. tropicalis*. The saliva from two participants (8 and 10) did not result in any yeast growth, while the remaining participants’ saliva yielded single species: either *C. albicans* or *C. tropicalis*.

![Figure 1: Concentration of Candida in saliva from participants wearing old dentures (CFU/mL)](image-url)
3.2.3 SALIVA CULTURES FROM PARTICIPANTS WITH NEW DENTURES 1 MONTH POST-INSERTION

After delivery of new dentures, only one saliva sample (from participant 12) yielded *Candida* colony growth (presumptively identified as *C. albicans*); no *C. krusei*, *C. tropicalis* or other yeast colonies were obtained (Figure 2).

![Figure 2: Concentration of Candida in saliva of participants wearing new dentures for 1 month (CFU/mL)](image.png)
3.2.4 SALIVA CULTURES FROM PARTICIPANTS WITH NEW DENTURES 3 MONTHS POST-INSERTION

After three months of denture wear, the saliva from two participants was *Candida*-positive (Figure 3). Saliva from participant 14 yielded *C. tropicalis*, while *C. albicans* colonies were obtained from participant 12.

![Graph showing concentration of Candida in saliva of participants wearing new dentures for 3 months](image)

**Figure 3:** Concentration of *Candida* in saliva of participants wearing new dentures for 3 months (CFU/mL)
3.2.5 SALIVA CULTURES FROM NEW DENTURES 6 MONTHS POST-INSERTION

The six month recall revealed that two more participants provided *Candida*-positive saliva samples, both of which yielded mixed species - *C. albicans* and *C. krusei* (Figure 4).

![Figure 4: Concentration of *Candida* in saliva of participants wearing new dentures for 6 months (CFU/mL)](image-url)
3.2.6 CHANGES IN TOTAL SALIVARY CANDIDA CONCENTRATION

The individual participant’s total salivary Candida concentration (CFU/mL; i.e. C. albicans + C. tropicalis + C. krusei) and the mean total Candida CFU/mL of saliva of all participants at each sampling point are displayed in Table 7.
Table 7: Changes in participants’ salivary yeast concentrations (CFU/mL) with time

<table>
<thead>
<tr>
<th>Participant</th>
<th>Old dentures</th>
<th>New dentures (1 month post-insertion)</th>
<th>New dentures (3 months post-insertion)</th>
<th>New dentures (6 months post-insertion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>750</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>1370</td>
</tr>
<tr>
<td>12</td>
<td>320</td>
<td>70</td>
<td>80</td>
<td>780</td>
</tr>
<tr>
<td>13</td>
<td>330</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>4820</td>
<td>-</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>15</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mean</td>
<td>564</td>
<td>7</td>
<td>11</td>
<td>294</td>
</tr>
</tbody>
</table>

'-' denotes no *Candida* found
3.2.7 CHANGES IN PARTICIPANT SALIVARY CANDIDA CONCENTRATION

The changes in participant salivary Candida concentrations are displayed in Figure 5. Analysis of saliva samples from participants with old dentures (time = 0) revealed that the high Candida colony counts from participant 14 were an outlier. Following delivery of new dentures, salivary Candida concentrations remained low until the 6 month recall when colony counts began to rise for participants 7, 11 and 12. All other participant salivary Candida concentrations remained low.

![Figure 5: Changes in participant salivary Candida concentration with time](image-url)
3.2.8 CHANGE IN MEAN SALIVARY CANDIDA CONCENTRATION

The change in the mean salivary Candida concentration with time for all participants is presented in Figure 6. After delivery of new dentures, the mean total salivary Candida CFU dropped from 561 to 7 CFU/mL and remained low (at 10 CFU/mL) until the 3 month recall. At the 6 month recall, samples revealed a return towards baseline concentrations (294 CFU/mL).

Figure 6: Change in mean salivary Candida concentration with time
3.3 MUCOSAL SWAB CULTURES

Only three mucosal swab cultures yielded yeast colonies. Before delivery of new dentures, participant 14 provided a mucosal sample that yielded 5 *C. krusei* CFU. No mucosal swab samples from the 1 month recall were yeast positive. Participant 12 provided mucosal samples that yielded 3 *C. albicans* CFU and 2 *C. albicans* CFU at the 3 and 6 month recalls respectively.

3.4 DENTURE IMPRINT CULTURES

At each sampling time point, the participant’s maxillary dentures were boxed and molten CHROMagar *Candida* (cooled to 45°C) pipetted onto the fitting surface to form an imprint culture. After incubation for 48 h, the species present were presumptively identified by colony colour and CFU measured.

3.4.1 AGAR IMPRINT OF THE DENTURE FITTING SURFACE

A study was undertaken to optimise the method for acquiring agar imprints of the denture fitting surfaces. One strip of beading wax (Harareus Kulzer, USA) was applied just below the height of the denture border and sealed using a hot spatula. A single strip of adhesive duplicating tape (Enst Hinrichs GmbH, Germany) was applied around the beading wax to create a mould to confine the agar. Two percent [w/v] agar in water was mixed and sterilised at 121°C for 15 min then placed in a 55°C water bath. After 1 h in the water bath, the agar was poured into denture mould. After 5 min the system started leaking catastrophically (Figure 7).
Based on this result, an alternative method for confining the denture was developed. A sterilised piece of polyvinyl chloride (PVC) tube and a fitting PVC cap were used to confine the agar (Figure 8). Silicone plasticine was moulded around the denture to the border of the fitting surface (Figure 9). YPD agar 2% [w/v] at 55°C was poured into the mould, the agar took 25 min to set hard enough to be removed completely intact, but the mould did not leak (Figure 10). The denture agar replica was freed from the mould using an air syringe (Figure 11).
Figure 8: PVC tube and sealing cap

Figure 9: Denture bordered by plasticine and PVC tube
Figure 10: Mould filled with agar (45°C)

Figure 11: Agar imprint of denture fitting surface
3.4.2 *C. ALBICANS VIABILITY TEST*

A pilot study was undertaken to determine if *C. albicans* cells would remain viable when inoculated into liquid agar at 45°C or 50°C. YPD broth was autoclaved at 121°C for 15 min. A colony of *C. albicans* ATCC10261 was used to inoculate 2 mL YPD broth which was incubated at 30°C in a shaking incubator overnight. This culture was then used to inoculate 200 mL of YPD broth to give an OD<sub>540</sub> of 0.05. This culture was incubated at 30°C in a shaking incubator for 150 min after which it had reached an OD<sub>540</sub> of 0.234. Portions (1 mL) of this culture were used to inoculate 100 mL molten YPD agar, which had been kept in a water bath at either 45°C or 50°C, to give an OD<sub>540</sub> of ~0.2 which corresponded to approximately 0.4 x 10<sup>7</sup> *C. albicans* cells/mL. The molten inoculated YPD agar was then immediately poured into agar plates which took approximately 30 min to set, replicating the treatment of the clinical samples. Samples were incubated at either 30°C or 37°C.

After 24 h both 45°C samples (incubated at 30°C and 37°C) yielded confluent growth. Both 50°C samples yielded comparatively poor growth. After 48 h, the samples poured at 45°C were nearly 100% confluent (Figure 12), and the 50°C samples still had significantly less growth (Figure 13).
Figure 12: YPD agar (2%) @ 45°C inoculated with *C. albicans* ATCC10261 poured and incubated at 37°C for 48 hours

Figure 13: YPD agar (2%) @ 50°C inoculated with *C. albicans* ATCC10261 poured and incubated at 37°C for 48 hours
3.4.3 DENTURE IMPRINT CULTURE RESULTS OVERVIEW

The numbers of CFU for each species present after incubation of the CHROMagar Candida denture imprint at various time points are presented in Table 8.
Table 8: CFU of *Candida* species from denture imprint cultures before fabrication of new dentures and 1, 3, and 6 months post-delivery of new dentures

<table>
<thead>
<tr>
<th>Participant</th>
<th>Old dentures</th>
<th>New dentures 1 month-post insertion</th>
<th>New dentures 3 months-post insertion</th>
<th>New dentures 6 months-post insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yeast present</td>
<td>Total CFU</td>
<td>Yeast present</td>
<td>Total CFU</td>
</tr>
<tr>
<td>7</td>
<td>C. albicans 16</td>
<td>-</td>
<td>C. tropicalis 29</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C. krusei 8</td>
<td>-</td>
<td>C. krusei 1</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>C. krusei 152</td>
<td>-</td>
<td>C. krusei 10</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>C. albicans 15</td>
<td>-</td>
<td>C. albicans 341</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C. krusei</td>
<td>-</td>
<td>C. krusei</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>C. albicans 26</td>
<td>-</td>
<td>C. albicans 11</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C. tropicalis 93</td>
<td>-</td>
<td>C. tropicalis 73</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>C. albicans 7</td>
<td>-</td>
<td>C. tropicalis 15</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C. krusei 6</td>
<td>-</td>
<td>C. tropicalis 12</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>C. albicans 68</td>
<td>-</td>
<td>C. tropicalis 15</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C. krusei 1</td>
<td>-</td>
<td>C. tropicalis 12</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

' denotes no *Candida* found
3.4.4 DENTURE IMPRINT CULTURES FROM OLD DENTURES

Denture imprint cultures from participants’ old dentures revealed that only *C. albicans* and *C. krusei* CFUs were present. No *C. tropicalis* or other yeast species were obtained (Figure 14). The imprint from participant 10’s denture grew only *C. krusei* and the total CFU count was over double that from participant 14 who had the second largest CFU count. The denture imprint cultures from four participants (8, 9, 15 and 16) did not yield any yeast growth. The cultures from three participants (7, 13 and 14) showed mixed *Candida* species growth, all of which grew both *C. albicans* and *C. krusei*.

![Denture imprint culture total yeast CFU of participants wearing old dentures](image-url)

Figure 14: Denture imprint culture total yeast CFU of participants wearing old dentures
3.4.5 DENTURE IMPRINT CULTURES FROM NEW DENTURES 1 MONTH POST-INSERTION

After new dentures had been worn for one month, two denture imprint cultures yielded *Candida* colony growth (Figure 15). The imprint of the denture from participant 12 gave a large number of *C. tropicalis* and a small number of *C. albicans* colonies, while the denture imprint for participant 14 gave only a small number of *C. tropicalis* colonies.

![Figure 15: Denture imprint culture total yeast CFU of participants wearing new dentures for 1 month](image-url)
3.4.6 DENTURE IMPRINT CULTURES FROM NEW DENTURES 3 MONTHS POST-INSERTION

After 3 months of wear, four of the denture imprint cultures gave Candida colony growth (Figure 16). Participant 12 was again the only subject whose denture imprint yielded mixed Candida species growth (C. albicans and C. tropicalis). Denture imprint cultures from participants 7, 11 and 14 yielded single-species C. tropicalis CFUs.

Figure 16: Denture imprint culture total yeast CFU of participants wearing new dentures for 3 months
3.4.7 DENTURE IMPRINT CULTURES FROM NEW DENTURES 6 MONTHS POST-INSERTION

At the six month recall, two additional participants (10 and 15) yielded *Candida*-positive denture imprint cultures (Figure 17). Some *C. krusei* CFU were now obtained from the denture imprints of participants 10, 11 and 12. Mixed *C. albicans* and *C. krusei* CFU were obtained from the denture imprints from participant 11. Denture imprint cultures from participant 15 now yielded *C. tropicalis* growth.

![Figure 17: Denture imprint culture total yeast CFU of participants wearing new dentures for 6 months](image-url)
### 3.4.8 CHANGES IN TOTAL DENTURE YEAST COLONISATION

The individual participant's total denture imprint yeast CFU and the mean yeast CFU of denture imprint cultures of all participants at each sampling time point are displayed in Table 9.

#### Table 9: Changes in participant denture imprint culture yeast colonisation (CFU) with time

<table>
<thead>
<tr>
<th>Participant</th>
<th>Old dentures</th>
<th>New dentures (1 month post-insertion)</th>
<th>New dentures (3 months post-insertion)</th>
<th>New dentures (6 months post-insertion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>24</td>
<td>-</td>
<td>29</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>152</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>11</td>
<td>15</td>
<td>-</td>
<td>26</td>
<td>468</td>
</tr>
<tr>
<td>12</td>
<td>26</td>
<td>105</td>
<td>84</td>
<td>26</td>
</tr>
<tr>
<td>13</td>
<td>13</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>69</td>
<td>5</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mean</td>
<td>29.9</td>
<td>11</td>
<td>15.4</td>
<td>53.6</td>
</tr>
</tbody>
</table>

'-' denotes no *Candida* found
3.4.9 CHANGES IN PARTICIPANT DENTURE YEAST COLONISATION

The changes in participants’ denture imprint *Candida* colonisation are displayed in Figure 18. Imprint cultures from the old dentures (time = 0) and new dentures at 1 month and 3 months showed fairly similar levels of colonisation. However, at the six month recall, the denture from participant 11 gave a much greater CFU count than all the other participant denture imprint cultures.

![Figure 18: Changes in participant denture imprint Candida CFU with time](image.png)
3.4.10 CHANGES IN MEAN DENTURE YEAST COLONISATION

The change in the mean denture imprint culture *Candida* CFU counts for all participants is presented in Figure 19. After delivery of new dentures, the mean total denture imprint culture *Candida* count dropped from 30 to 10 CFU and remained low at the 3 month recall. The 6 month recall samples showed that the total *Candida* CFU counts exceeded baseline CFU counts from the participants’ old dentures.

![Figure 19: Change in mean denture imprint Candida CFU with time](image-url)
3.5 COMPARISONS OF SALIVA AND DENTURE IMPRINT CULTURES

3.5.1 CHANGES IN YEAST-POSITIVE SALIVA AND DENTURE IMPRINT CULTURES

A comparison of the number of *Candida*-positive saliva and *Candida*-positive denture imprint cultures (those containing at least one *Candida* species) is displayed in Figure 20. At baseline, 80% and 60% of the saliva and denture imprint cultures, respectively, were *Candida*-positive. After one month of new denture wear the percentage of both saliva and denture imprint cultures that were *Candida*-positive decreased markedly. At all sample collection points with new dentures, more denture imprint cultures were *Candida*-positive than the corresponding saliva cultures. At the six-month recall, the percentage of denture imprint cultures that were *Candida*-positive had returned to baseline.
Figure 20: Percentage of saliva and denture imprint cultures that were *Candida*-positive
3.5.2 CHANGES IN C. ALBICANS-POSITIVE SALIVA AND DENTURE IMPRINT CULTURES

The proportions of the saliva and denture imprint cultures that were C. albicans-positive mirrored one another at all time points, as displayed in Figure 21. Samples collected from participants with old dentures revealed that 50% of the saliva and denture imprint cultures were C. albicans-positive. This dropped to 10% of samples being C. albicans-positive after delivery of new dentures and remained at 10% until the three-month recall. At the six-month recall, the percentage of saliva and denture imprint cultures that were C. albicans-positive rose to 30%.

Figure 21: Percentage of saliva and denture imprint cultures that were C. albicans-positive
3.5.3 CHANGES IN THE NUMBER OF SALIVA AND DENTURE IMPRINT CULTURES CONTAINING MULTIPLE CANDIDA SPECIES

A comparison of the number of saliva and denture imprint cultures containing multiple *Candida* species is presented in Figure 22. At baseline, 30% and 10% of the denture imprint and saliva cultures, respectively, contained multiple *Candida* species. The proportions decreased to 10% and 0% at both the one- and three-month recalls after the use of new dentures. At the six-month recall, 20% of both the denture imprint and saliva cultures contained multiple *Candida* species.

![Figure 22: Percentage of saliva and denture imprint cultures that contained multiple Candida species](image-url)
3.5.4 PROPORTION OF CFUS THAT WERE C. ALBICANS

The proportions of total Candida CFUs from saliva and denture imprint cultures at each time point that were C. albicans are presented in Figure 23. Samples obtained at t = 0 showed that 8.3% and 44.1% of total Candida CFUs from saliva and denture imprint cultures, respectively, were C. albicans. At the one-month review following delivery of new dentures, 100% and 10.9% of the CFUs from the saliva and denture imprint cultures respectively, were C. albicans. After three months, these values had dropped to 72.7% and 7.1% for the saliva and denture imprint cultures, respectively. At the six-month recall, there had been a minimal change for the saliva cultures, but the percentage of CFUs from the denture imprint cultures that were C. albicans had risen to 64.6%.

Figure 23: Percentage of Candida CFUs that were C. albicans
3.5.5 PROPORTION OF CFUS THAT WERE C. KRUSEI

The proportions of total Candida CFUs from saliva and denture imprint cultures at each time point that were C. krusei are presented in Figure 24. At all time points, unlike for C. albicans, the proportions of total CFUs that were C. krusei were similar for the saliva and denture imprint cultures. Samples collected at t = 0 showed that the proportions of total Candida CFUs that were C. krusei were 59.9% and 55.9% for the saliva and denture imprint cultures, respectively. At the one- and three-month reviews, none of the saliva and denture imprint cultures contained C. krusei colonies. At the six-month recall, the values rose to 27.5% and 30.4% for the saliva and denture imprint cultures, respectively.

Figure 24: Percentage of total Candida CFUs that were C. krusei
3.5.6 PROPORTION OF CFUS THAT WERE C. TROPICALIS

The proportions of total Candida CFUs from saliva and denture imprint cultures at each time point that were C. tropicalis are presented in Figure 25. The t = 0 sample showed that the proportion of total Candida CFUs that was C. tropicalis was 9.8% for the saliva cultures, while none of the denture imprint cultures contained C. tropicalis. At the one-month recall, 89.1% of the Candida colonies obtained from denture imprint cultures were C. tropicalis, whereas none of the saliva samples at this time point contained C. tropicalis. At the three-month recall, 27.3% and 92.9% of the total Candida CFUs from saliva and denture imprint cultures, respectively, were C. tropicalis. At the six-month recall, these values had returned towards the t = 0 values.

![Figure 25: Percentage of total Candida CFUs that were C. tropicalis](image)
3.6 CASE STUDIES:

3.6.1 CASE EXAMPLE 1

The initial 100 µL saliva sample from this participant yielded 2980 *C. krusei* and 200 *C. tropicalis* CFU/mL (Figure 26). Initial denture imprint culture of the 14 year old denture revealed a large number of colonies of multiple *Candida* species and a significant number of colonies that had washed out onto the boxing material (Figure 27). Despite the large number of colonies obtained from both the saliva and denture imprint cultures, the patient presented with clinically healthy maxillary denture-bearing tissues (Figure 28). This patient was unable to attend the 3-month recall appointment and could not be included in the data. However, even after 6 months wear of the new complete dentures, the saliva (Figure 29) and denture imprint cultures (Figure 30) showed a reduction in both colony counts and in the overall complexity of the yeast species obtained.

![Figure 26: Culture of saliva from participant with old denture present](image)

*(C. krusei 2980, C. tropicalis 200 CFU/mL)*
Figure 27: Denture imprint culture (old denture)

(C. albicans 82, C. krusei 74, C. tropicalis 318 CFU)

Figure 28: Denture-bearing mucosa at t = 0 (healthy)
Figure 29: Saliva culture (6 months post-delivery of new denture)

(C. tropicalis 40 CFU/mL)

Figure 30: Denture imprint culture (6 months post-delivery of new denture)

(C. tropicalis 19 CFU)
3.6.2 CASE EXAMPLE 2

The initial saliva sample from this participant yielded 2456 *C. krusei* colonies (Figure 31). In a similar manner, the imprint culture from the participant’s 7 year old denture revealed a near confluent carpet of *C. krusei* colonies (Figure 32). Despite the large number of yeast colonies obtained from the saliva and denture imprint cultures, the participant’s maxillary denture bearing tissues were clinically healthy (Figure 33). Unfortunately, this participant was unable to attend the 1 month and 3 month recall appointments and could not be included in the study. However, six-months after delivery of the new denture, many fewer *Candida* colonies were obtained from the saliva and denture imprint cultures. Additionally, a small number of *C. albicans* colonies could now also be obtained from both the saliva (Figure 34) and denture imprint cultures (Figure 35).

![Figure 31: Culture of saliva from participant with old denture present](image)

(*C. krusei* 24560 CFU/mL)
Figure 32: Denture imprint culture (old denture)

(C. krusei nearly confluent growth)

Figure 33: Denture-bearing mucosa at t = 0 (healthy)
Figure 34: Saliva culture (6 months post-delivery of new denture)

(C. albicans 160, C. krusei 1320 CFU/mL)

Figure 35: Denture imprint culture (6 months post-delivery of new denture)

(C. albicans 1, C. krusei 3 CFU)
3.6.3 CASE EXAMPLE 3

The saliva sample from this participant, while they were still wearing their old dentures, revealed a high concentration of *C. albicans* CFU (Figure 36). In contrast, the saliva samples from the 1- (Figure 37) and 3-month (Figure 38) recalls showed a significant reduction in the concentration of *C. albicans* CFU. However, at the 6-month recall, the saliva sample revealed a near return to baseline *C. albicans* CFU concentration (Figure 39). This was the only participant in this research project for whom *C. albicans* colonies could be obtained from each saliva sample over the 6-month data collection period.

![Figure 36: Culture of saliva from participant with old denture present](image)

*(C. albicans 320 CFU/mL)*
Figure 37: Saliva culture 1-month after delivery of new denture

*(C. albicans 70 CFU/mL)*

Figure 38: Saliva culture 3-months after delivery of new denture

*(C. albicans 80 CFU/mL)*
Figure 39: Saliva culture 6-months after delivery of new denture

(C. albicans 780 CFU/mL)
3.6.4 CASE EXAMPLE 4

Upon entry into the study, this patient presented with significant growth of *C. albicans* and other *Candida* species from the denture imprint of their existing 9 year old maxillary complete denture. Some CFU from the denture-fitting surface were washed onto the periphery of the boxing silicone (Figure 40). This patient was the only participant who presented with clinical signs of candidosis (diffuse chronic erythematous candidosis) as can be seen in Figure 41. The candidosis was managed with the use of a tissue conditioner and topical antifungal lozenges (Amphotericin B) before completing the fabrication of new complete dentures. After one month of wear of the new dentures, agar imprint culture revealed only a small number of *C. tropicalis* colonies on the new acrylic denture fitting surface (Figure 42). The patient experienced some issues with the new dentures and reverted back to wearing their old denture, after approximately 3 months, rather than returning for adjustments to the denture (due to denture sore spots). The old denture had been stored in a sealed plastic zip-lock bag with water for approximately 3 months before returning to the patient’s mouth. Although this participant could no longer be included in the research project, a final denture fitting surface imprint culture was obtained from their old denture that had been reinstated (Figure 43). Interestingly, the colonisation pattern of the denture fitting surface was similar to that seen originally. Although not intentional, this served as an impromptu control.
Figure 40: Denture imprint culture (old denture)

(*C. albicans* 80, *C. krusei* 18, *C. tropicalis* 14, Other 12 CFU)

Figure 41: Denture-bearing mucosa at t = 0 (diffuse chronic erythematous candidosis)
Figure 42: Denture imprint culture (1 month post-delivery of new denture)

(C. tropicalis 8 CFU)

Figure 43: Denture imprint culture after reverting back to using old dentures

(C. albicans 216, C. krusei 75, C. tropicalis 5 CFU)
4 DISCUSSION

The aim of this research project was to investigate the patterns of the colonisation of saliva and the acrylic denture fitting surface by yeast species in patients with old ill-fitting dentures and following delivery of new dentures. In particular, whether the prescription of new dentures would provide benefit in terms of the numbers and species of yeast colonising both the denture fitting surface and the saliva which have been suggested to be aetiological factors in the development of denture stomatitis (Arendorf and Walker, 1987; Webb et al., 1998b). The results of the saliva and denture imprint CHROMagar Candida cultures and select case studies will be discussed in this chapter in relation to the existing literature. The limitations of the study will also be addressed and comments made on possible future research directions.

4.1 STUDY PARTICIPANTS

A convenience sample was used to select participants for this research project. A number of participant factors make the results obtained from this sample population unrepresentative of the New Zealand complete denture-wearing population. All participants had attended a pre-screening appointment (not attended by the researcher) where people with overt candidosis were referred for treatment and excluded from the source population from which subjects were selected. This was likely the reason that only one participant had clinical signs of candidosis and possibly why all participants had satisfactory denture hygiene home practice. Participants were required to attend five 90 min clinical appointments in one week for the fabrication of their new complete dentures; the frequency of these long appointments is the likely reason that all of the participants were retired with an average age of 76.9 years. Examination of patients from a broader sample population including using a range of clinicians to enrol participants (including undergraduate dental students, post-graduate dental students, private general dental practitioners, and specialist prosthodontists) would be required to reduce the influence of institution-based sampling on the results obtained.
Of the 16 participants who entered the research project, six had to be eliminated from data analysis because of sample contamination, inability to tolerate their new prostheses, or an inability to return for recall sampling. A larger sample population would have reduced the impact that the loss of these participants to sampling had on the results obtained.

The mean age of the participants included in this research project was 76.9 years; 90% of whom were considered elderly (age over 60 years). It has been predicted that the elderly will represent 20-22% of the New Zealand population in the year 2061 (Bascand, 2012). It is difficult to know how representative the average denture age (8.2 years) was due to the difficulty in determining the typical longevity of removable complete dentures (Mazurat, 1992) and a lack of literature on the subject. A larger sample size would have been required to examine the effect of participant age and denture age on the results obtained.

4.2 SALIVA CULTURES

The use of whole saliva for saliva sample collection proved to be a simple and reliable technique in this research project. Every participant was taking at least one medication for which hyposalivation or xerostomia is a documented side-effect (Sreebny and Schwartz, 1986). This medication, combined with the age-related salivary gland structural changes (Drummond and Chisholm, 1984) may explain the difficulty that some participants had in producing a sufficient volume of saliva for plating. The use of a neat oral rinse or concentrated oral rinse may have proved useful in eliminating any possible effect that hyposalivation had on the saliva culture results, but sample sensitivity may have suffered (Samaranayake et al., 1986b).

Some of the baseline saliva samples were taken after alginate impressions of the patient’s maxillary and/or mandibular denture bearing surfaces had been taken. It is unknown what effect the impression procedure will have had on the saliva results obtained, but obtaining a sufficient volume of saliva for culturing may have been more
difficult for participants already suffering from hyposalivation. This was not a variable for the saliva samples collected after delivery of the new dentures.

At least 50% of the population are thought to be commensal carriers of *Candida* species and, if sensitive enough tests were developed, more than 90% of healthy individuals may be shown to be carriers of the organism (Odds, 1987). The range of frequencies reported of oral commensal *Candida* carriage is extremely broad (3-48%), the median carriage frequency has been reported at 34.4% for all yeasts and 17% for *C. albicans* alone (Odds, 1988). One study has found multiple yeast species from 35% of individual participants (Coco et al., 2008). If baseline saliva samples in this project were utilised alone, 80% of this studies participants were *Candida* positive, 50% were *C. albicans* positive and only 10% had multiple species present. There was one outlier participant sample that had over ten times the salivary yeast concentration of any other sample. This had a significant effect on the mean participant salivary *Candida* concentration at this time point. A greater number of participants would be required to reduce the effect of such outlier results.

Delivery of new dentures appeared to dramatically reduce the number of yeast positive salivary samples. Only 10% of participants were salivary yeast positive after one month of new denture wear. This increased to 40% after 6 months of wear. Dentures likely act as a reservoir for salivary yeast colonies. Surface characteristics such as microporosities and roughness may cause the surface to harbour microorganisms (Verran and Maryan, 1997; Radford et al., 1999). A reduction of denture plaque has been reported when the fitting surface of a denture base is either polished or glazed with a surface-finishing resin (Badawi et al., 1986). Such reductions in denture plaque levels are not sustained (Budtz-Jørgensen and Kaaber, 1986). Fabrication of new dentures is likely to have a similar effect and hence new dentures are likely to reduce salivary yeast counts. It is unlikely that new dentures will have eradicated all salivary yeast in the seven participants from whom no yeast could be cultured. What is more likely is that there was a reduction in the concentrations of salivary yeast to the point that they could not be detected using the whole saliva culture technique.
It might be expected that salivary yeast concentrations after six months of new denture wear would not be higher than that recorded at baseline. However three participants recorded salivary yeast concentrations after six months of new denture wear higher than that which was recorded at baseline. A possible reason for this is that the researcher obtaining the samples had improved their technique and because the participants were returning solely for sample collection, there was no risk of denture fabrication procedures altering the results. Alternative explanations include that these participants became lax in their oral and denture hygiene after the delivery of new dentures, or other underlying factors such as immunological health were compromised affecting their colonisation with yeast.

After six months of new denture wear, the average salivary yeast concentration had returned to over half that at t =0. If this trend were to continue, a return to baseline salivary yeast concentrations could be expected after approximately nine months of new denture wear. The increase in salivary yeast concentration after continued wear of the new dentures could be a result of biofilm maturity on the denture fitting surface providing a larger reservoir for salivary yeast. It could be also possible that with time the denture surfaces become degraded providing microporosities and roughness leading to greater adhesion of yeast species and a greater saliva reservoir.
4.3 MUCOSAL SWAB CULTURES

In the dentate, the posterior dorsum of the tongue is the most common intraoral site for \textit{C. albicans} colonisation (Arendorf and Walker, 1980) but in complete denture wearers, the denture-bearing mucosa is the main reservoir for intraoral yeasts (Budtz-Jörgensen, 1990a). Therefore one would expect similar numbers of \textit{Candida} positive biofilms to be present on the denture-bearing mucosa to the number of \textit{Candida}-positive saliva samples and denture imprint samples. In the present study, based on the saliva and denture imprint cultures, one would expect approximately 60-80% of the mucosal swab samples to be \textit{Candida} positive. However only 10% of the mucosal swab samples yielded yeast growth.

This could be the result of poor sensitivity of the particular method used for obtaining and culturing the mucosal swab samples. Perhaps if swabs were taken from a larger area of the denture-bearing mucosa or if the cotton swabs were placed directly into liquid medium for culture rather than dilution in sterile water, a larger rate of initial yeast carriage would have been obtained. Although liquid culture would identify yeast-positive swabs, it would not be quantitative (any number of yeast on the swab would give a positive liquid culture result), which was the objective of the study. Despite these concerns, in a study by Kulak and colleagues (Kulak et al., 1997), that placed their swabs directly into the chosen culture medium, the authors also found approximately 10% of clinically healthy subjects were positive for \textit{C. albicans}. Unfortunately no description of the swab technique was given, the method used to differentiate between \textit{Candida} species was not described, and \textit{C. albicans} was the only yeast species identified.
4.4 DENTURE IMPRINT CULTURES

Various techniques have been used to identify yeasts in investigations of the aetiology of denture stomatitis. These include imprint cultures, sampling and culture from the denture base, and growth of denture plaque on sand-blasted adhesive tape. Spiechowicz and colleagues (Spiechowicz et al., 1991) compared the sensitivity of a denture replica technique with direct swabbing of dentures and found that the replica method for the detection of *C. albicans* in edentulous patients seemed to be more sensitive than swabbing. Santarpia and colleagues described a technique using beading and boxing wax to create a mould to confine the growth medium in maxillary complete dentures (Santarpia III et al., 1988). *C. albicans*-selective agar was pipetted into the boxed denture at a temperature of 56°C.

In this investigation, a similar technique to that described by Santarpia (Santarpia III et al., 1990) was tested using beading wax and adhesive duplicating tape during experiments to optimise the method of acquiring agar imprints of the denture fitting surfaces. CHROMagar *Candida*, a chromogenic medium capable of differentiating different *Candida* species, at 55°C was poured into the denture mould but the system started leaking catastrophically after only 5 min. The low thermal conductivity of agar likely meant that the beading wax (which has a low melting temperature) was melted before the agar had cooled sufficiently to start solidifying.

Thus an alternative technique was developed using PVC tubing and caps with silicone-based plasticine boxing material and this worked well without leakage. All components used in obtaining the denture imprint cultures were sterile except for the plasticine boxing material. There was some concern that this boxing material was contaminated and may have been responsible for the yeast CFUs present on the periphery of some of the denture imprint cultures. These fears were alleviated by pouring and culturing an imprint culture with no denture present at each sample collection time and these gave no yeast growth. Applying the boxing plasticine to the periphery of the maxillary dentures was difficult without touching the fitting surface and buccal sulcus periphery of the dentures. Many colonies grew on the surrounding boxed areas of the denture imprint cultures. There is some risk that there could have been removal or
transportation of some of the yeast colonies during these boxing procedures and the pouring of the molten agar.

Another concern was that the temperature of the agar during pouring and setting (30 min) of the imprint cultures may have been sufficient to kill some of the CFU present on the fitting surface of the participant’s dentures. As was demonstrated in the *C. albicans* viability test (3.4.2), a difference of 5°C was enough to significantly affect the viability of one strain of *C. albicans*. Considering that only a single strain of a single species was tested, it cannot be concluded that from this one experiment that all *Candida* species and strains remain viable at 45°C, although three different species were detected on imprint cultures during the study. Despite this concern, a further reduction in temperature was not possible as this would be below the melting point of CHROMagar *Candida* and impractical for the denture imprint culture technique.

Imprint cultures from the participants’ old dentures, who completed the project and were included in the data analysis, yielded only *C. albicans* and *C. krusei* colonies, no *C. tropicalis* colonies grew. There was one particular outlier in this sample group that produced over double the CFU of the next highest sample (this was not the participant who had the high concentration of yeast in saliva). This may have had some impact on the mean denture yeast colonisation results obtained at this sampling point and a greater number of participants would be required to reduce the impact of such outliers.

When inserted into the oral cavity, the polymethylmethacrylate of new dentures are rapidly coated by salivary proteins to form a pellicle which mediates subsequent adherence of *Candida* species (Edgerton and Levine, 1992). Predictably, there was a general trend for a reduction in mean CFU cultured from denture imprint cultures after delivery of new dentures but there were two outlier participants. Participant 12 had a higher total colony count after delivery and wear of new dentures for one month. This reduced with time with a trend towards baseline values after six months. Participant 11 had a much higher colony count at the six month recall than either the baseline or one or three month sample collection times. No explanation is apparent for these two outlying results, but may reflect varying oral hygiene practices over the study period.
The mean denture imprint yeast CFU increased after three months of new denture wear. At the six month recall, the mean CFU had returned to, and exceeded, baseline values. A possible explanation for this unexpected result is the aforementioned participant 11 outlier which was over 5 times the next highest sample result and had an impact on the mean CFU at this time point.

Interestingly, *C. tropicalis* became a predominant species from the imprint cultures of new dentures at the one and three month recall sampling time points, whereas no *C. tropicalis* was detected at baseline from the samples included in the data. Over the six month recall period, for every participant from whom yeast was detected, *C. tropicalis* was the first species to be detected in varying amounts (although a small number of *C. albicans* CFU were detected from participant 12 at each sampling time). After six months of new denture wear, it appeared that the *C. tropicalis* colonies were being replaced by a mixture of *C. albicans* and *C. krusei*. A possible interpretation of these results could be that *C. tropicalis* colonies are early colonisers of new acrylic surfaces before the fitting surface has undergone minimal degradation and the biofilm later matures with the inclusion of *C. albicans* and *C. krusei* species. *C. tropicalis* has been shown to be more hydrophobic than other *Candida* species in contact angle studies (Minagi et al., 1986) and more adherent to denture PMMA with a lower a surface free energy than *C. albicans* (Minagi et al., 1985). These results could help explain the early colonisation of the PMMA fitting surface by *C. tropicalis* in the current study.

It was initially hypothesised that the maxillary denture fitting surface would be colonised in a distinct pattern. In this study, there was no evidence to suggest that particular areas of the denture imprint cultures were more frequently or more densely colonised than others. A greater number of participants may be required to explore if any such pattern exists.
4.5 COMPARISONS OF SALIVA AND DENTURE IMPRINT CULTURES

At baseline, 20% more saliva cultures were yeast positive than denture imprint cultures. Following delivery of new dentures at each recall interval there was a higher percentage of yeast positive denture imprint cultures than saliva cultures. With continued wear of new dentures there was a trend for the mean salivary and denture imprint CFUs to return towards baseline. The mean CFUs on yeast-positive denture imprints returned to baseline after six months of new denture wear. If these trends were to continue in a similar a manner, saliva values would return to baseline after approximately 13 months of new denture wear. The difference in saliva and denture imprint culture results could be explained by a greater number of yeast present on the denture fitting surface than is present in the saliva and that new denture surfaces are attractive surfaces for colonization and deplete the saliva of yeast. Also imprint cultures may be better at detecting the presence of yeast as seen in the study by Spiechowicz (Spiechowicz et al., 1991).

There was a significant reduction of \textit{C. albicans}-positive saliva and denture imprint cultures following delivery of new dentures which remained low until the 3 month recall. After six months there was a return towards baseline, and if this trend were to continue, a return to baseline values for both saliva and denture imprint cultures could be predicted to occur after nine months of new denture wear. The mirroring of the percentage \textit{C. albicans}-positive saliva and denture imprint cultures across all time points was interesting and could be explained as that in edentulous patients, the PMMA surfaces are the primary reservoir for \textit{C. albicans} from which it enters the saliva.

Delivery of new dentures leads to a significant reduction in the number of saliva and denture imprint cultures containing multiple \textit{Candida} species. For up to three months of new denture wear, more denture imprint cultures than saliva cultures contained multiple \textit{Candida} species. This could indicate that the complete dentures of edentulous patients are the reservoir for multiple yeast species. After six months of new denture wear the mean CFU concentrations for saliva had returned to exceeded those found at baseline. If the trends for the denture imprint cultures were to continue, a return to baseline could be expected after nine months of new denture wear.
Delivery of new dentures resulted in a large reduction in the proportions of total *Candida* CFUs from denture imprint cultures that were *C. albicans*, while all of the yeast colonies isolated from saliva cultures after one month of new denture wear were *C. albicans*. With continued wear the denture imprint values tended to decrease after the one month recall, while the saliva values began to climb back to and exceed baseline values after 6 months. The lower percentage of denture imprint CFUs being *C. albicans* than the percentage of saliva culture CFUs would tend to favour the hypothesis that *C. albicans* is a relatively late coloniser of the denture fitting surface and that the dentures are not necessarily the primary reservoir for salivary *C. albicans* CFU.

Unlike *C. albicans*, the proportions of total *Candida* CFU that were *C. krusei* from saliva and denture imprint cultures nearly mirrored one another at baseline and following delivery of new dentures. Based on these results, a return to baseline could be expected after nine months of new denture wear for both saliva and denture imprint cultures. The similar values for saliva and denture imprint cultures could indicate that the dentures are the primary reservoir for oral *C. krusei*.

The patterns seen for the proportions of total *Candida* CFUs from saliva and denture imprint cultures that were *C. tropicalis* was in direct contrast to that seen for *C. albicans*. Approximately 90% of the colonies from the denture imprint cultures were *C. tropicalis* for up to three months of new denture wear. However, after six months of wear, baseline values for denture imprint cultures were re-established for *C. tropicalis*. There was comparably little variation in the proportions of total *Candida* CFUs from saliva cultures that were *C. tropicalis*. These results suggest that *C. tropicalis* is a species comparably well suited to the early colonisation of new denture fitting surfaces. However, with continued wear the growth of other species, particularly *C. albicans*, is selected.
4.6 RELATION OF INTRAORAL PHOTOS TO DENTURE IMPRINT CULTURES

Of the 20 subjects screened for entry into the project, only one presented with clinical signs of denture-induced stomatitis (Figure 41). There was no corresponding Candida colonisation of the denture imprint cultures from this participant while they were wearing their old denture (Figure 40). There was no sign of inflammation of the maxillary denture-bearing mucosa after 1 month of new denture wear. The denture imprint culture revealed C. tropicalis colony growth in the area of the posterior palatal seal, which approximately matched an area of inflammation seen at t=0. A greater number of participants, particularly those with clinical signs of denture stomatitis, would be required to reveal if site-specific denture colonisation corresponds with areas of mucosal inflammation. Although some authors have stated that the distribution of C. albicans correlates well with areas of markedly inflamed mucosa in denture stomatitis patients (Arendorf and Walker, 1979), issues remain over the risk of colony transportation during pouring of denture imprint cultures making relation of denture colonisation and mucosal irritation illusive (Spiechowicz et al., 1991).

4.7 HYPOTHESES

The results of this research have supported and/or refuted the following hypotheses:

1. The fitting surfaces of dentures are colonised in a distinctive pattern by Candida species: Refuted
2. The species of Candida adhering to the fitting surfaces of dentures are different to those present in saliva: Confirmed
3. Candida colonisation of denture fitting surfaces is closely correlated with clinical signs of candidiasis: Refuted
4. Fabrication of new dentures will reduce Candida colonisation of denture surfaces and the saliva: Confirmed (but only for a limited time period)
4.8 CONCLUSIONS

Within the aforementioned limitations of the present study, the following conclusions can be made:

1. The provision of new dentures reduces the number of yeast in the patient’s saliva and on the denture fitting surface for up to 3 months.
2. The provision of new dentures reduces the overall Candida species diversity of the saliva and denture fitting surface for three months. After six months there is greater species diversity in the saliva and on the denture fitting surface.
3. C. tropicalis is not present on the fitting surface of old dentures but is an early coloniser of new dentures. C. krusei only colonises the denture fitting surface after 6 months of wear.

4.9 FUTURE RESEARCH AND RECOMMENDATIONS

It is recommended that future research includes a larger sample size to confirm the changes seen in denture colonisation and the salivary yeast concentrations following delivery of new dentures. Ideally the samples would be collected from subjects being treated with complete dentures by a range of practitioners including clinical dental technician post-graduate students, post-graduate students, general dentists and specialist prosthodontists.

C. albicans was the Candida species most often found in saliva and associated with denture fitting surfaces. It would be interesting to see if the strains of C. albicans in saliva are the same as those found on denture fitting surfaces and whether the same strains are retained after new denture fabrication. Further in vitro studies should be performed on representative C. albicans colonies from the denture imprints, mucosal swabs and saliva so they can be characterised at the molecular level. By using MLST analysis which compares the DNA sequences of seven house-keeping genes, the genetic relatedness of the strains from the various samples before and after new denture fabrication could be measured.
5 REFERENCES


Hajjeh RA, Sofair AN, Harrison LH, Lyon GM, Arthington-Skaggs BA, Mirza SA et al. (2004). Incidence of bloodstream infections due to *Candida* species and in vitro susceptibilities


APPENDICES

6.1 APPENDIX 1: PRESENTATIONS

The Australian and New Zealand Academy of Prosthodontics Biennial Scientific Meeting in Melbourne, Australia on 20th June 2014

Title of Oral Presentation: Colonisation of acrylic denture fitting surfaces by Candida species

Authors: N.J. Knight, R.D. Cannon, K.M. Lyons, V. Bennani

Departments: Oral Rehabilitation and Oral Sciences, University of Otago, Dunedin, New Zealand (Aotearoa)

Research Day Presentation, Faculty of Dentistry – Sir John Walsh Research Institute on 31st July 2014

Title of Oral Presentation: Colonisation of acrylic denture fitting surfaces by Candida species

Authors: N.J. Knight, R.D. Cannon, K.M. Lyons, V. Bennani

Departments: Oral Rehabilitation and Oral Sciences, University of Otago, Dunedin, New Zealand (Aotearoa)
6.2 APPENDIX 2: ETHICAL APPROVAL

Professor R Cannon  
Department of Oral Sciences  
Faculty of Dentistry

Dear Professor Cannon,

I am writing to let you know that, at its recent meeting, the Ethics Committee considered your proposal entitled 'Colonisation of acrylic denture fitting surfaces by Candida species'.

As a result of that consideration, the current status of your proposal is: Approved

For your future reference, the Ethics Committee's reference code for this project is: 12/229

The comments and views expressed by the Ethics Committee concerning your proposal are as follows:

While approving the application, the Committee would be grateful if you would respond to the following:

The Committee recommends that you elaborate on how your research findings will be used in the Information Sheet (i.e. the benefits of the research).

Please provide the Committee with a copy of the updated Information Sheet, if changes have been necessary.

Approval is for up to three years from the date of this letter. If this project has not been completed within three years from the date of this letter, re-approval must be requested. If the nature, consent, location, procedures or personnel of your approved application change, please advise me in writing.
Yours sincerely,

Mr Gary Whitt
Manager, Academic Committees
Tel: 473 8256
Email: gary.whitt@otago.ac.nz

cc. Professor R D Cannon Head Department of Oral Sciences

Figure 44: Ethical approval confirmation from the University of Otago Human Ethics Committee
6.3 APPENDIX 3: NGĀI TAHU RESEARCH CONSULTATION COMMITTEE RESPONSE

Ngāi Tahu Research Consultation Committee

Te Komiti Rakahau ki Kāi Tahu

19/06/2012 - 03
Tuesday, 19 June 2012

Professor Cannon
Oral Sciences
Dunedin

Tēnā koe Professor Cannon

Title: Colonisation of acrylic denture fitting surfaces by Candida species.

The Ngāi Tahu Research Consultation Committee (The Committee) met on Tuesday, 19 June 2012 to discuss your research proposition.

By way of introduction, this response from the Committee is provided as part of the Memorandum of Understanding between Te Rūnanga o Ngāi Tahu and the University. In the statement of principles of the memorandum, it states "Ngāi Tahu acknowledges that the consultation process outlined in this policy provides no power of veto by Ngāi Tahu to research undertaken at the University of Otago". As such, this response is not "approval" or "mandate" for the research, rather it is a mandated response from a Ngāi Tahu appointed committee. This process is part of a number of requirements for researchers to undertake and does not cover other issues relating to ethics, including methodology; they are separate requirements with other committees, for example the Human Ethics Committee, etc.

Within the context of the Policy for Research Consultation with Māori, the Committee base consultation on that defined by Justice McGeachan:

"Consultation does not mean negotiation or agreement. It means: setting out a proposal not fully decided upon; adequately informing a party about relevant information upon which the proposal is based; listening to what the others have to say with an open mind (in that there is room to be persuaded against the proposal); undertaking that task in a genuine and not cosmetic manner. Reaching a decision that may or may not alter the original proposal."

The Committee considers the research to be of importance to Māori health.

As this study involves human participants, the Committee strongly encourage that ethnicity data be collected as part of the research project. That is the questions on self-identified ethnicity and descent, these questions are contained in the 2006 census.

The Ngāi Tahu Research Consultation Committee has membership from:

Te Rūnanga o Ōtautahi Incorporated
Kāti Huirapa Rūnaka ki Puketeraki
Te Rūnanga o Moeraki

Figure 45: Letter from the Ngāi Tahu Research Consultation Committee
## 6.4 APPENDIX 4: MINISTRY OF HEALTH ORAL HEALTH RESEARCH FUND

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**New Zealand Dental Association**

**NZDA House**
Building 1, 196 Main Highway
Eleroa, Auckland 1061
PO Box 28064, Remuera 1541
Ph: +64 9 579 8001
Fx: +64 9 570 0010
Email: research@nzda.org.nz

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### ADVICE OF RESEARCH FUNDING GRANT APPLICATION AS ASSESSED BY THE PANEL OF THE MINISTRY OF HEALTH ORAL HEALTH RESEARCH FUND

**WEDNESDAY 18 JULY 2012**

<table>
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<td>Name of Applicants</td>
<td>Cannon RD, Knight NJ, Lyons K, Bennani V</td>
</tr>
<tr>
<td>Title of Research</td>
<td>Colonisation of acrylic denture fitting surfaces by Candida species</td>
</tr>
<tr>
<td>Amount Awarded</td>
<td>$8,646</td>
</tr>
<tr>
<td>Conditions of Award</td>
<td>Funding of this project is subject to ethics approval being obtained and is made on the condition that the Assessment Panel receives a satisfactory Progress Report by 01 June 2013 and annually by 1 June each year beyond 2013. A Final Report and copy of any publications / reports are required at the completion of the study. Note: The format and content requirements of Progress and Final Reports are currently being reviewed. You will be advised of the revised requirements in due course.</td>
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**General Comments**

The Panel congratulates the recipients on the award of this grant and looks forward to a progress report by 01 June 2013.

**Signed:** Malcolm D. McMillan (Chair, Ministry of Health Oral Health Research Fund Panel)

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**YOU ARE REQUIRED TO SUBMIT A PROGRESS REPORT ON YOUR RESEARCH BY 1 JUNE 2013**

(please email your report to research@nzda.org.nz)

The Principal Researcher should sign, date and return a COPY of this advice notice (in the panel below) to acknowledge the conditions and enable receipt of the Award. If the Principal Researcher is a post-graduate student then the student’s supervisor should sign and return this form. Thank you.

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**Principals Researcher OR Student Supervisor**

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**Figure 46: Acceptance letter from the Ministry of Health Oral Health Research Fund Committee**
Thursday 28th June 2012

Dr Nicholas James Knight
Postgraduate DClinDent Pros
Otago University
P.O. Box 647
Dunedin, NZ 9554

Dear Dr Knight,

DENTSPLY Research Fund 2012

Congratulations on being a Research Grant recipient for the DENTSPLY Research Fund 2012.

Your research topic and application has been reviewed, and DENTSPLY has decided to offer support for the project.

We are pleased to provide you with a grant of NZD$1250 (exclusive of GST).

Please note that by accepting this grant, you are agreeing to:

- speak and/or present a poster at an agreed nominated conference with recognised acknowledgement to DENTSPLY;
- provide an informal progress report 12 months after commencement of the project; and
- provide DENTSPLY with a copy of the final research document.

To confirm your agreement to these obligations, please sign the enclosed copy of this letter and return to us as soon as possible.

In order to receive payment of the grant, please note the following steps:

- Forward a tax invoice to DENTSPLY in the amount of NZD$1250 (exclusive of GST) and include the following details:

  Name of the institution
  Address, telephone number and fax number of the institution
  ABN number of the institution

  Please ensure that you use the following words on the invoice:

  "Grant to be paid to (name of organisation/institution) for NZD$1250"
DENTSPLY will then forward a cheque.

- If you wish for the money to be placed directly into an institution's bank account, please advise of the following:
  
  Name of Bank account
  Address of the Bank
  Account number including BSB details

DENTSPLY will send a remittance advice to confirm the payment has been made.

Please direct your tax invoice to Emily Gray at the DENTSPLY, Mount Waverley address as listed on page 1.

Once again, congratulations. We look forward to seeing the outcomes of your valuable research.

Yours sincerely

Dr George Alexopoulos BDSc MBA

Cc Professor Richard Cannon

On behalf of The University of Otago (institution), I accept this research grant and agree to the obligations outlined in this letter.

Name Nicholas James Knight

Signature

Date 10/7/12

Figure 47: Letter from the Dentsply Research Fund Committee
Colonisation of acrylic denture fitting surfaces by *Candida* species

INFORMATION SHEET FOR PARTICIPANTS

Thank you for showing an interest in this project. Please read this information sheet carefully before deciding whether or not to participate. If you decide to participate we thank you. If you decide not to take part there will be no disadvantage to you and we thank you for considering our request.

The yeast *Candida* is often found in people's mouths and is usually harmless but can cause infections in people with impaired immune systems.

In this research project, we wish to investigate the changes that occur in the types of *Candida* yeast species in those patients:

- who have ill-fitting dentures;
- after their dentures are cushioned with a tissue conditioner; and
- when they are supplied with new dentures.

This project is being undertaken as part of the requirements for the Postgraduate Doctor of Clinical Dentistry (in Prosthodontics) degree.
Patients eligible to participate in this study are those:

- without teeth;
- who currently have full upper dentures; and
- are attending the clinical dental technology and 4th year dentistry clinics in the School of Dentistry, Otago University, for construction of new acrylic dentures.

A total number of 40 participants are to be recruited. A $20 petrol voucher will be given to participants taking part in the second phase of the project to compensate them for costs involved in attending review appointments.

Should you agree to take part in this project, you will be asked to:

- Dribble some saliva into a sterile bottle; this will only take about 5 minutes.

If your saliva is found to contain yeast you will be asked to take part in the second phase of the project in which you will be asked to:

- Answer a medical and dental history questionnaire.
- Return for repeat swabs, impressions and photographs at 1 week, 1 month, and 3 months after receiving your new denture.
- Allow a simple mouth examination and photographs of the roof of your mouth beneath your denture. There may be some slight discomfort associated with taking the photographs.

This will take about one hour in total.

Please be aware that you may decide not to take part in the project without any disadvantage to yourself of any kind.
What samples will be collected?

- A small sample of your saliva.
- A photograph of the roof of your mouth.
- Swabs of the tissues underneath your upper denture.
- Impressions of your denture (not your mouth).

What personal information will be collected?

- Date of birth.
- Gender.
- Ethnicity.
- A history of current and previous medical problems and medications.
- Age of your denture and methods of denture cleaning used.

What are the purposes for which the data or information is being collected?

- Initial samples will be used to determine if Candida yeasts are present in your saliva, on the roof of your mouth and on your old denture.
- Subsequent samples will be used to observe changes in these yeast species in your saliva, on the roof of your mouth and on your new denture.

This information will be used to gain knowledge on the patterns of yeast growth which are responsible for denture-associated infection. Only the researcher and supervisors will have access to this information. Your samples will be identified by a unique number not your name.

How will data or information be securely managed, stored and destroyed?

All participants will be assigned a unique number which will be used to label samples for analysis. Samples will not be readily identified as belonging to a named individual. Upon completion of the study, all samples will be destroyed. The data collected will be securely stored in such a way that only those mentioned below will be able to gain access to it. At the end of the project, any personal information will be destroyed immediately. Raw data on which the results of the project depend will be retained in secure storage for five years, after which it will be destroyed.

What data or information will be reflected in the completed research?

The results of the project may be published and will be available in the University of Otago Library (Dunedin, New Zealand) but every attempt will be made to preserve your anonymity.
Will the participants have the opportunity to correct or withdraw the data/information?

The completed medical and dental history will be reviewed with participants to ensure its accuracy.

Will participants be given the opportunity to view the data or information that relates to them?

Participants can request copies of photographs and reports from saliva, mucosal and dentures samples. This opportunity will be offered after completion of yeast growth.

Please note: you may withdraw from participation in the project at any time and without any disadvantage to yourself of any kind.

If you have any questions about the project, either now or in the future, please feel free to contact either:

Nicholas James Knight and/or Professor Richard Cannon
Department of Oral Rehabilitation Department of Oral Sciences
(03) 479 7125 (03) 479 7081
nicholas.james.knight@hotmail.com richard.cannon@otago.ac.nz

This study has been approved by the University of Otago Human Ethics Committee. If you have any concerns about the ethical conduct of the research you may contact the Human Ethics Committee through the administrator (ph (03) 479 8256). Any issues you raise will be treated in confidence and investigated and you will be informed of the outcome.

Figure 48: Participant information sheet
6.7 APPENDIX 7: CONSENT FORM

Colonisation of acrylic denture fitting surfaces by Candida species

CONSENT FORM FOR PARTICIPANTS

I have read the Information Sheet concerning this project and understand what it is about. All my questions have been answered to my satisfaction. I understand that I am free to request further information at any stage.

I know that:

1. My participation in the project is entirely voluntary;
2. I am free to withdraw from the project at any time without any disadvantage;
3. Personal identifying information will be destroyed at the conclusion of the project but any raw data (on which the results of the project depend) will be retained in secure storage for at least five years;
4. There may be some minor discomfort during the taking of photographs of my mouth;
5. A $20 petrol voucher will be offered as remuneration for sample collection visits following delivery of your new denture if you take part in the second phase of this project;
6. The results of the project may be published and available in the University of Otago Library (Dunedin, New Zealand) but every attempt will be made to preserve my anonymity.

I agree to take part in this project.

(Signature of participant) .............................................. (Date) ..............................................

Figure 49: Participant consent form