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THE PENETRATION AND DISINFECTION OF CANDIDA ALBICANS IN DENTURE BASE RESIN

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, in partial fulfilment of the requirements for the degree of Master of Science in Dentistry.

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Johannesburg, 2016

DECLARATION

I, Yasmin Osman Latib, declare that this research report is my own work. It has been submitted for the degree of Master of Science in Dentistry in the Faculty of Health Sciences at the University of the Witwatersrand, Parktown, Johannesburg, South Africa. It has not been submitted before for any other degree or examination at this or any other University.



…….
This 8th day of June 2016

RESEARCH OUTPUT

Conference Proceedings

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ABSTRACT

Purpose: *Candida albicans* is an oral commensal associated with denture stomatitis. Dentures become contaminated with this organism in yeast and hyphae forms, the latter having greater pathogenic potential. In addition, *C. albicans* can penetrate denture acrylic resin. The purpose of this study was to determine the extent of penetration of *C. albicans* into denture resin, and the ability of denture cleansers and disinfectants to eradicate the penetrated organism.

Method and materials: Heat-polymerising polymethyl methacrylate test plates (n=25) were prepared according to the manufacturer's instructions. Nine plates were inoculated with *C. albicans* and three each incubated at 37°C for 7, 14, and 21 days. In addition, 5 plates from a pilot study had been inoculated at 10 days. Each plate was fractured, processed and the sections viewed under SEM at 10 uniform intervals to measure the depth of penetration. One plate served as a control. Fourteen plates were inoculated with *C. albicans* and incubated at 37°C for 21 days after which they were removed and rinsed with normal saline. Three were immersed in sterile distilled water for 8 hours, 3 in a denture cleanser (Steradent®, Reckitt Benckiser, Slough, UK) for 10 minutes, 3 in 20 parts per million chlorine dioxide solution (SteriWright, Wright Millners, Cape Town, SA) for 8 hours, 3 air dried and lastly 1 plate was used as an unexposed control. Plates were fractured, processed and viewed under SEM for the presence or absence of *C. albicans*.

Results: All the plates were unevenly penetrated by *C. albicans* with the oval form of yeast as well as with hyphae. Biofilm of *C. albicans* on the surface was also noted. A significant

increase in penetration was observed on days 7, 10, 14 and 21 compared to the control plate ($p<0.01$) with no significant difference between days 14 and 21 ($p = >0.05$). The mean depth of penetration observed was 631 µm in sections inoculated for 21 days. All the contaminated plates showed the presence of *C. albicans* irrespective of cleanser or disinfectant with no significant difference between them ($p=0.69$).

Conclusions: *C. albicans* penetrated into the denture base resin over a period of time and none of the tested denture care methods were able to eradicate all cells *in situ*. This suggests that, if the *Candida* remains viable, recurrence of denture stomatitis may occur. It is therefore recommended that, until a proven disinfection procedure is developed, at least 1 mm of the intaglio surface of the dentures of patients with denture stomatitis be removed as part of the treatment regimen.

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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Denture stomatitis is a common disorder affecting denture wearers (Gendreau and Loewy, 2011) and *Candida albicans* is the predominant oral fungal infection associated with this (Buergers et al., 2008; Jose et al., 2010; Williams and Lewis, 2011; Jackson et al., 2014). Although the aetiology of denture stomatitis is multifactorial, including age, general health, oral hygiene, diet, gender, socio economic status, prosthetic use and trauma, the denture base itself would seem to be the main contributory factor (Gendreau and Loewy, 2011; Skupien et al., 2013). The predominant associated factors have been identified as the presence of *Candida* infection, poor denture hygiene as well as the continual wear of dentures (Gendreau and Loewy, 2011; Skupien et al., 2013). The fitting surface of the denture provides an ideal environment for the growth of *Candida* species, which are capable of adhering to denture base resin.

In spite of an improvement in access to dental care, the occurrence of edentulism remains significant, especially among the elderly (Gendreau and Loewy, 2011). In South Africa, the national oral health survey found that in 1988 the national mean of the edentulous population for age groups 35 to 44 years and 60 to 64 years was10.36%, and 26.76% respectively (National Policy for Oral Health in South Africa, 1988).

The prevalence of denture stomatitis ranged from 15% to over 70% (Gendreau and Loewy, 2011). Many authors have investigated the contribution of the surface roughness of the

denture base to the growth of *Candida* species (Verran and Maryan, 1997; Dua and Kashinath, 2008), but have generally only looked at the colonisation of *Candida* species on the surface of the denture base. It has been found that, in spite of 'successful' treatment with appropriate antimicrobial drugs for patients diagnosed with denture stomatitis, symptoms and the number of microorganisms returned to previous levels once drug therapy was completed (Glass et al., 2004). Studies have shown that *C. albicans* not only colonises the acrylic surface but it penetrates the acrylic (Wendt and Glass, 1987; Glass et al., 2004; Glass et al., 2010), probably as a result of water sorption by the acrylic resin (Richmond et al., 2004).

Since denture hygiene has been identified as a contributing factor to denture stomatitis, interest in the disinfection of dentures has increased, with a wide variety of disinfecting methods having been employed (Silva et al., 2006; Buergers et al., 2008). However, most studies have focused on the surface colonisation and disinfection of *Candida* (Silva et al., 2006; Buergers et al., 2008; Skupien et al., 2013).

1.2. Candida albicans

C. albicans is a dimorphic unicellular fungus, which typically grows as spherical to oval budding yeast cells but in certain conditions produces hyphae. Cultures grown on a commonly used culture medium, Sabouraud agar, appear as creamy white colonies with a beer-like aroma (Samaranayake, 2002).

C. albicans is an opportunistic pathogen that exists as a harmless commensal on moist mucosal surfaces. The frequency of *Candida* in individuals varies from 10% to 50% (Mathaba et al., 1995). Successful habitation of *Candida* species is due to its initial adherence to the host surface. This is accomplished through hydrophobic and electrostatic forces, as

well as the microorganism's virulence factors which include cell-surface adhesins (Williams and Lewis, 2011). Once adhesion has been established, colonisation and growth is required to maintain the presence of the organism at the host site (Williams et al., 2013). The extent of this colonisation determines whether eradication, growth or infection occurs.

1.3. *Candida albicans* and the colonisation of the oral cavity

The oral cavity harbours opportunistic pathogens including *C. albicans*, as well as beneficial and transient microorganisms. Colonisation is possible due to the presence of mucosal epithelial cells, saliva and exogenous nutrient. Many host and environmental factors affect the level of colonisation such as host immunity, quantity of saliva, oral bacteria, oral prostheses, oral hygiene, age and the use of medications. The prevalence of *Candida* colonisation is high in HIV positive patients (81.83%) compared to HIV negative patients (63%) (Patel et al., 2006). Similarly, the oral *Candida* carrier rate and the density of *Candida* were found to be high in diabetics compared with healthy individuals (Tapper-Jones et al., 1981). When Abu-Elteen and Abu-Alteen (1998) studied *Candida* carriage in denture wearers it was also found to be higher (78.3%) when compared with healthy dentate subjects (36.8%).

Contaminated dentures serve as a continuous source of infection. The predominant species of *Candida* carried by denture wearers is *C. albicans* (Cavaleiro et al., 2013) and it causes denture related stomatitis (Buergers et al., 2008; Jackson et al., 2014; Jose et al., 2010; Williams and Lewis, 2011).

Mathaba et al (1995) studied the genotypic relationship of different *C. albicans* strains in the oral cavity. The study concluded that denture stomatitis was due to the overgrowth of *C.*

albicans strains. Furthermore, re-infection following antifungal therapy was generally due to the re-emergence of the original Candidal strain.

1.4. Virulence of *Candida albicans*

The pathogenesis of *C. albicans* is due to its ability to express a number of virulence factors. These include adherence ability, biofilm and hyphae formation, the binding of complement and the production of hydrolytic enzymes (Williams and Lewis, 2011). Adherence to mucosal cells is the initial stage of pathogenesis which enables this fungus to colonise the host tissue and proliferate. In denture wearers, the next phase of candidal habitation is through its ability to generate a biofilm and hyphae, which has been shown to be a major predisposing factor for denture stomatitis (Yang, 2003; Williams and Lewis, 2011). This formation is further increased due to poor oral hygiene (Williams and Lewis, 2011). Oral bacteria, particularly the *viridans* group of Streptococcus are the major flora present in denture plaque and *C. albicans* is known to co-aggregate with this and with other oral bacteria.

In vitro studies by Ramage et al (2004) confirmed the presence of biofilms on denture surfaces, especially along cracks and imperfections. Ramage et al (2005) also showed the presence of yeast cells as early as 2 hours after inoculation, and attached budding yeast cells began to filament after 4 hours and form pseudohyphae. After 8 hours, true hyphae were found. Neighbouring cells, filaments and hyphae (pseudo and true) entwined to form an organised woven structure. After 24 to 48 hours, there was an increase in complexity of the biofilm which consisted of different layers as well as different morphological fungal forms. Therefore, the ability of *C. albicans* to alter its morphology has been considered as an important factor towards its virulence capability (Phan et al., 2000; Jackson et al., 2014).

Jackson et al (2014) assessed the development of hyphal and blastospore biofilm on abraded denture resin and found that the presence of hyphae increased biofilm mass and caused an increase in resistance on removal. Furthermore, an increase in surface roughness enhanced the retention of morphological forms of *Candida* as well as facilitated growth. Whilst this study highlighted the significance of surface roughness and denture abrasion to the growth of *C. albicans*, it did not examine the penetration of *C. albicans* in any morphological form into a denture base resin.

Germ tube formation has been suggested as an important virulence factor (Mathaba et al., 1995). Germ tubes are the initial projections found when *Candida* switches from yeast form to hyphal form (Yang, 2003). Hyphal formation was found to adhere better to surfaces and bind to several human proteins, including fibrinogen, c3d and lamanin (Trochin et al., 1991; Jackson et al., 2014). Furthermore, hyphae accommodate for host epithelium invasion, and this allows for dissemination of *Candida.* Whilst studies have proven the importance of hyphal formation as a virulence factor in denture stomatitis, the extent of hyphal formation has been found to not correlate with the infection (Mathaba et al., 1995). This has led to suggestions that other putative virulence factors (such as hydrolytic enzymes) may be more critical with denture stomatitis.

Finally, hydrolytic enzymes such as proteinases, phospholipases and lipases produced by *C. albicans* cause tissue damage. Secreted aspartyl proteinases degrade human proteins found at lesion sites and their proteolytic activity is important for the virulence of *C. albicans* (Yang, 2003). Phospholipase B is the major gene identified in *Candida* species, and has both hydrolase and lysophospholipase-transacylase activities. Although these enzymes are also

produced by the colonising strains, during infection, their production increases (Marco-Arias et al., 2009, Lie Tobouti et al., 2015).

1.5. Denture stomatitis

Denture stomatitis is a chronic inflammatory condition affecting denture wearers. It has a characteristic erythematous oral mucosal area covered by a removable denture (complete or partial) (Gendreau and Loewy, 2011). The aetiology is multifactorial, with the predominant factors being poor denture hygiene, overgrowth of *C. albicans*, ill-fitting dentures as well as continuous wear of ill-fitting dentures (Gendreau and Loewy, 2011; Skupien et al., 2013). Cahn, in 1936, proposed that *C. albicans* was responsible for denture stomatitis (as cited by Arendorf and Walker, 1987). The denture base provides a niche environment for the overgrowth of *C. albicans*, due to the enhanced ability of *C. albicans* to adhere to the denture base, the reduced saliva flow under the surface of the denture and poor oral hygiene (de Souza et al., 2009).

Gendreau and Loewy's (2011) review on the prevalence of denture stomatitis found a high prevalence in Denmark (65%). This had been attributed to poor denture hygiene as well as the high prevalence of associated *Candida* infection in the elderly. Other local and systemic risk factors compound the clinical manifestation of denture stomatitis. These include diabetes mellitus, immunosuppression, medication, nutritional deficiencies as well as tobacco use (de Souza et al., 2009).

Pathogenesis of denture stomatitis begins with the development of a biofilm on the denture base resin. The contributory factors to this development include long term use of the denture, poor oral hygiene and overgrowth of the normal commensal of *C. albicans*. The biofilm

colonises the surface and penetrates into imperfections found along the denture base. The opposing mucosa in contact with the denture becomes infected, with the subsequent noticeable erythematous area (de Souza et al., 2009).

Clinical manifestations are best described using Newton's classification who proposed three stages (Newton 1962), which have since been referred to as types (Arendorf and Walker, 1987): Type 1 - associated with simple localised inflammation which manifests as pinpoint hyperaemic foci; Type 2 – simple diffuse (generalised) inflammation which manifest as diffuse hyperaemia of the denture – supporting tissues and; Type 3 – Granular inflammation which manifests as papillary hyperplasia (Newton 1962). Denture stomatitis is most often asymptomatic. However, some patients experience pain, itching or a burning sensation, mucosal bleeding and swelling, halitosis or an unpleasant taste and dryness in the mouth (Arendorf and Walker, 1987).

1.6. Management of denture stomatitis

Management of denture stomatitis begins with a thorough history and examination to obtain a correct diagnosis of predisposing factors as well as the type of stomatitis present. Treatment is aimed at improving hygiene (denture and oral), denture cleaning (mechanical and chemical) and antifungal agents (Felton et al., 2011), depending on the Newton type and severity.

Oral and denture hygiene is aimed at improving current habits. This entails brushing the oral mucosa with a soft brush, disinfection of the denture on a daily basis, and overnight soaking of dentures (Sharon and Fazel, 2010; Walsh et al., 2015). Felton et al (2011) described the

ideal denture cleanser as being antifungal and antibacterial, non-toxic, compatible with denture material, short acting (≤8 hours), easy to use and cost effective.

Pharmacological treatment includes the use of antifungal agents, locally or systemically (Walsh et al., 2015). Topical antifungal agents are the first line of drug therapy (Sharon and Fazel, 2010). Nystatin is one of the most widely used topical antifungals. Other antifungal agents include amphotericin B (topical and systemic), clotrimazole (topical and systemic), miconazole (topical and systemic), ketoconazole (topical and systemic) and fluconazole (systemic) (Sharon and Fazel, 2010).

1.7. Denture disinfection

Since denture hygiene has been identified as a contributing factor to denture related stomatitis, interest in the disinfection of dentures has increased. Various disinfecting methods have been employed, such as the use of glutaraldehyde, chlorhexidine, phenolic based and alcohol based disinfectants, microwave irradiation, sodium hypochlorite, hydrogen peroxide, vinegar, and exposure to oxygen through air drying (Silva et al., 2006; Buergers et al., 2008;).

Although many studies have found these various methods to be effective as disinfectants and useful in preventing *Candida* species colonisation (Silva et al., 2006; Buergers et al., 2008; Skupien et al., 2013), the disadvantages are that they may affect the hardness, flexural strength and colour stability of denture base resins (Silva et al., 2006).

Lee et al (2011) evaluated the efficacy of six different denture cleansing methods, including the chemical denture cleanser Polident® (GlaxoSmithKline) and found that the combination of mechanical cleansing and chemical immersion was more effective than either method

alone in the removal of *C. albicans*. A similar study by Paranhos et al (2009) contradicted those results, and reported that the mechanical method and combination methods were more effective than chemical methods alone.

The role of mouthwashes against *C. albicans* has also been investigated. Iseri et al (2011) studied a combination of mouthwashes and alkaline peroxidase chemical agents, such as CloySYSII (Poltola Plaza Dental Group, contains chlorine dioxide), Corsodyl (GlaxoSmithKline, contains 0.2% Chlorhexidine gluconate), Polident® (GlaxoSmithKline, contains carbon dioxide producers), Efferdent (Pfizer, contains carbon dioxide producers) and Fittydent (Mag.Hoeveler & Co, contains whitening power of baking soda and peroxide). The study revealed that CloSYSII and Corsodyl were found to be the most effective denture cleansers. This study also found differences in the efficacy of mouthwashes with different immersion times.

However, most studies have focused on the surface colonisation of *Candida* species. Glass et al (2011) tested an American and European denture cleanser (Polident®, GlaxoSmithKline) and its various delivery systems (microwave irradiation, sonication, vacuum and water temperature) and found significantly reduced numbers of microorganisms on the denture surface as well as the depth to which they penetrated.

1.7.1. Chlorine dioxide

Chlorine dioxide, used as a slow release tablet form is considered a reliable oral disinfectant due to its strong sterilising properties (Watamoto et al., 2013). It disinfects by oxidising the polysaccharide matrix of biofilm. In one study, the effectiveness of chlorine dioxide was

compared to sodium hypochlorite with the results indicating that chlorine dioxide achieved complete disinfection within 2 minutes, compared with sodium hypochlorite (Bell et al., 1989). In addition, 0.048% chlorine dioxide has been shown to be effective in sterilising alginate impression material in 90 seconds (Rweyendela et al., 2009). Chlorine dioxide as a mouthrinse was investigated (Iseri et al., 2011) where chlorine dioxide (CloSYSII) as well as Corsodyl showed the highest removal activity for all treatment times and completely removed *C. albicans* from the surface of an acrylic resin.

1.7.2. Steradent®

Steradent® (Reckitt Benckiser, Slough, UK) is a commonly used South African commercially available immersion denture cleaner. Steradent® releases oxygen radicals through its active ingredient, sodium bicarbonate. In one study, it was concluded that Steradent® was the most suitable cleaner of acrylic resin due to its low abrasivity as well as its effective removal of the initial *Candida albicans* biofilm (Harrison et al., 2004). Similar studies using other immersion denture cleaners found similar results. However, it was found that the use of an immersion denture cleaner on its own was not as effective in removing plaque compared with mechanical removal (Tarbet et al., 1984).

Whilst there have been numerous studies examining the surface contamination as well as disinfection of *C. albicans*, there is insufficient literature showing the penetration depth and the ability of disinfectants to penetrate into a denture resin base to remove this organism.

1.7.3. Other denture care procedures

Other disinfectant methods employed include leaving the denture exposed to air dry as well as immersion in water. Axe et al (2015) conducted a survey to provide data on recommendations dental health care professionals made to patients and the cleaning regimens of denture wearers in developed and developing countries. The study found that in developed countries, the use of denture cleansing tablets was the recommended choice by professionals, followed by regular toothpaste, fresh water and the use of soap and water. Results in developing countries noted the use of regular toothpaste as the recommended cleaning regimen by professionals, followed by soap and water, denture cleansing tablets and fresh water.

In contrast, the cleaning regimens of denture wearers found that the majority of wearers used regular toothpaste, followed by fresh water, mouthwash and denture cleansing tablets. The study also highlighted that a minority of denture wearers followed the denture hygiene recommendations made by dental health care professionals. This study reflects a lack of clear, systematic evidence upon which to base denture hygiene recommendations.

Duyck et al (2013) examined the overnight storage of dentures in water, being left dry, and water in combination with a denture cleaning tablet. They found that dentures stored in water in combination with a cleansing tablet had a significantly reduced bacterial count compared with storage in water alone and being left dry. Furthermore, the study also found no significant difference in the microbial colonisation of overnight storage in water alone and being left dry. Duyck et al (2016) expanded on this study by including a mechanical disinfecting method (brushing/ultrasonic cleanser) with a chemical method (water and a denture cleansing tablet / water alone). Both mechanical methods in combination with

overnight storage in water with a denture cleansing tablet were more effective than using a mechanical method with overnight storage in water alone (Duyck et al., 2016).

Stafford et al (1986) compared the overnight storage of dentures in water to air drying. The study found that the density of Candidal colonisation significantly decreased when the denture base was left to air dry for 8 hours. However, leaving the denture for the same period in water exhibited an increase in density of Candidal colonisation. These results focused on surface contamination and not the contamination of *C. albicans* within the denture base.

1.8. Purpose

Whilst there have been numerous studies examining the surface contamination as well as disinfection of *C. albicans*, there is insufficient literature reporting on the penetration depth or the ability of disinfectants to penetrate into a denture resin base to remove *C. albicans*. This study aimed to quantify the depth of penetration of *C. albicans* in a denture base and to investigate the ability of disinfectants to penetrate into the denture base to remove all *C. albicans* present.

CHAPTER 2
AIM AND OBJECTIVES

2.1. Aim

The aim of this *in vitro* study was to determine the depth of penetration of *Candida albicans* into a denture base resin over a period of time and to test whether denture care procedures (distilled water, air drying, chlorine dioxide and Steradent®) were able to eradicate all *Candida albicans in situ*.

2.2. Objectives

1. To expose heat polymerised denture base resin to *C. albicans* over different incubation times and then to determine the depth of penetration by means of Scanning Electron Microscope (SEM) observations.
2. To investigate the effect of the following denture care procedures on their ability to eradicate all *C. albicans* in situ:
   - Distilled water
   - Air drying
   - Chlorine dioxide
   - Steradent®

2.3. Null Hypotheses

2.3.1 There is no penetration of *Candida albicans* into the denture base resin.

2.3.2 There will be no differences between the different denture care procedures tested to eradicate *Candida albicans*.

CHAPTER 3
MATERIALS AND METHOD

An ethical waiver was issued by the Human Research Ethics Committee of the University of the Witwatersrand for the laboratory based study and the clearance certificate number is W-CJ-141205-1 (Appendix A).

3.1. Sample Size Calculation

The sample size estimation was based on the between-plate standard deviation of the penetration depth measurement, together with the minimum clinically meaningful between-group difference. The between-plate standard deviation of the penetration depth measurement was determined from a pilot study using five acrylic test plates inoculated with one strain of *C. albicans* over a period of 10 days.

The data from the pilot study were used to determine whether it was possible to reduce the number of measurements taken per plate. A Component of Variance analysis was conducted, where 100% of the variance was attributable to error variance (i.e. to between-measurement variability on the same fracture surface of the same plate) and none to fracture surface variance or plate variance. It was concluded that taking many measurements per plate was necessary to achieve a reliable estimate of the average penetration depth per plate.

The effect of time on penetration depth was determined by a one-way ANOVA with interaction. Sample size estimations were based on a significance level of 5%, a power of

80% and the effect sizes calculated from the pilot data. The minimum clinically meaningful between-group difference to be detected was set at 25 µm.

The calculated sample size was 6 (2 plates for each of the 3 time intervals). Ultimately, it was decided to use 9 plates (3 plates for each of the 3 time intervals).

3.2. Materials

3.2.1. Denture plates

Twenty-five unpolished heat-polymerising polymethyl methacrylate (PMMA) test plates measuring 4.0 x 1.0 x 0.5 cm were processed from the same manufacturing batch according to the manufacturer's instructions (Vertex-Dental BV, Netherlands). Each test plate was sterilised under ultraviolet light for 24 hours.

3.2.2. Disinfection procedures and disinfectants

Air drying, sterile distilled water, Steradent® (Reckitt Benckiser, Slough, UK) and chlorine dioxide (SteriWright, Wright Millners, Cape Town, SA) were used as disinfection procedures. One Steradent® effervescent tablet was placed in a 200 ml beaker of warm water. Immediately upon insertion, the tablet effervesced and dissolved in the water, with a colour change of the water from clear to blue indicating readiness for disinfection.

One tablet of chlorine dioxide was dissolved in 5.25*l* of distilled water, to make a 20 ppm solution of chlorine dioxide. The solution was covered and left standing still for 30 minutes, after which the solution was ready for disinfection.

3.2.3. *C. albicans* culture and inoculum

An ATCC 90028 culture of *C. albicans* was grown on Sabouraud agar aerobically at 37°C for 48 hours. The culture was suspended in 10 ml sterile distilled water and the turbidity adjusted to MacFarlane standard 1. This suspension was used as an inoculum. For each experiment, fresh cultures and inoculum were prepared and used.

3.2.4. Microbiological analysis

3.2.4.1. Pilot study

Five sterile acrylic plates were aseptically transferred into a beaker containing 200 ml of sterile Sabouraud broth containing 5 ml of horse serum. This medium was inoculated with 2 ml *C. albicans* inoculum and incubated aerobically at 37°C for 10 days. The medium was changed every alternate day. Plates were removed, gently rinsed with sterile normal saline and each plate was fractured in three pieces to expose a clean, untouched and unmodified surface, allowing for the penetration depth to be detected and measured. Each plate was fractured at two places to achieve 3 pieces per plate. This procedure generated 20 inner vertical surfaces. The fractured sections were sputter coated with a 5-10nm Gold-Palladium film, and mounted for SEM inspection. Each fractured surface was viewed at 10 uniform intervals and an average per surface was calculated. This was done four times along each acrylic test plate.

3.2.4.2. Penetration study

Eleven acrylic plates were used. Ten sterilised heat-polymerising PMMA test plates were placed in a beaker containing Sabouraud broth supplemented with 5 ml of horse serum. This medium was inoculated with 2 ml inoculum and incubated aerobically at 37 ºC for a period of

7, 14 and 21 days (Bell et al., 1989). The medium was changed on days 3, 7, 10, 14 and 17 respectively, where the inoculated medium was replaced with Sabouraud broth and further supplemented with 5 ml of horse serum. Three acrylic test plates were removed from the culture medium on day 7, 14 and 21 respectively, dipped gently in sterile saline to remove loosely attached cells, and left to dry (Chau et al., 1995; Lin et al., 1999). One plate was removed, transferred into 20 ml distilled water, vortexed, serially diluted and each dilution cultured for *C. albicans* to show viability and to be used as a positive control. One control plate with no *C. albicans* (day 0) was included in the test as a negative control. All 10 plates, excluding the positive control, were dried and processed for the SEM viewing. Plates were cut at two places creating 4 vertical surfaces. Ten evenly distributed areas on each of these vertical surfaces were examined for the presence or absence of *C. albicans* penetration and if there was a penetration, the depth of penetration was measured.

3.2.4.3. Disinfection study

A culture of *C. albicans* was grown on 14 sterile acrylic plates for 21 days as described in section 3.2.4.2. Three plates were removed, rinsed with sterile normal saline and air dried for 8 hours. Three plates were rinsed with sterile normal saline and immersed in sterile distilled water for 8 hours. Three plates were rinsed with normal saline and immersed in Steradent® for 10 minutes. Three plates were immersed in 20 ppm chlorine dioxide solution for 8 hours. One plate was removed, rinsed with sterile normal saline and used as an unexposed control. All the plates were dried and processed for SEM viewing. Plates were fractured at 2 places and viewed under SEM as described in 3.2.4.1. One plate was cultured on Sabouraud agar to test for viability.

3.3. Data Analysis

A descriptive analysis was performed on the results obtained from the penetration depth and disinfectant study.

A one-way ANOVA with interaction was used to determine the effect of time on the depth of penetration *C. albicans*. Data analysis was carried out using SAS software (SAS Institute Inc. USA). The 5% significance level was employed throughout the study.

3.4. Validity and Repeatability

One operator, trained in the use of SEM, fractured, evaluated and measured the depth of penetration of *C. albicans* as well as its presence or absence. Measurements were recorded at 10 uniform intervals on each fractured surface. Three test plates each were used for each incubation time interval. This allowed for repeatability of the study.

CHAPTER 4
RESULTS

4.1 Pilot study

Two hundred fractured surfaces of acrylic plates were examined for the depth of penetration of *C. albicans* to calculate the sample size. The results are shown in Appendix C. The mean depth was 44.4 μm and standard deviation 7.7, with a relative standard deviation (RSD) of 17.1%, and a mean range of 34-51 μm (Table 4.1). To determine if the number of measurements could be reduced, a components of variance analysis was carried out, which revealed the variance to be due to between- measurement variability on the same fracture surface of the same plate. No variance was seen to be due to either plate or fracture surface variance, and so it was concluded that all measurements per fracture surface were necessary. Based on a significance level of 5%, a power of 80% and a clinically meaningful between-group difference to be detected of 25 μm, the effect sizes calculated from these pilot data gave a sample size of 6: 2 plates for each of the 3 time groups (Faul et al, 2009). At a between-group difference of 20 μm this increased to 3 plates, and so it was recommended that at least 3 plates per time group be used, which would also allow for experimental problems or losses.

Table 4.1 Depth of penetration of *C. albicans* into acrylic plates – pilot study

Acrylic plate	Depth of penetration (μm) n=40
	Mean ± SD
1	38.7 ± 6.5
2	50.0 ± 8.2
3	34.0 ± 6.9
4	48.3 ± 8.0
5	50.8 ± 8.8
mean ± SD	**44.4 ± 7.7**

4.2. Depth of Penetration

As no changes were made to the analysis or experimental procedure between the pilot study and the main study, the results from the 5 plates used in the pilot study at 10 days' incubation were included in the depth of penetration analyses. The depths of penetration on days 7, 14 and 21 are also shown in Appendix C. The descriptive statistics for the plates and categorised by incubation time are shown in Table 4.2.

Table 4.2 The average depth of penetration of *C. albicans* into acrylic plates at various time intervals.

Average depth of penetration per plate								
Day	**N**	**Mean**	**Std Dev**	**Median**	**Interquartile range**		**Minimum**	**Maximum**
Overall	14	64	31	50	35	89	31	120
7	3	33	2	33	31	35	31	35
10	5	44	8	48	39	50	34	51
14	3	97	20	89	82	120	82	120
21	3	97	16	94	83	114	83	114

These results are plotted in Figure 4.1.

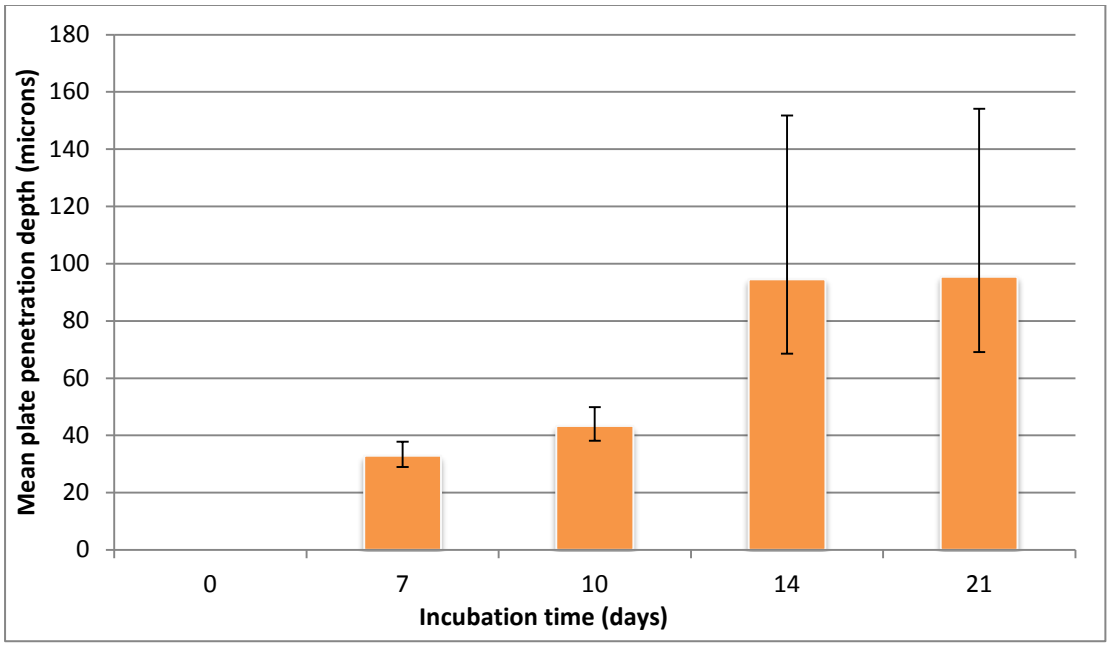

Figure 4.1 Mean penetration depths between the four incubation times. Error bars show the back-transformed confidence intervals.

The relative standard deviation within a fracture surface ranged from 83 to 316%, and within a plate, ranged from 130 to 202%. The overall ANOVA between the incubation times was significant ($p<0.0001$). Post-hoc analyses revealed the following:

- Mean penetration depth at 7 days was significantly lower that at 10 days ($p=0.038$)
- Mean penetration depth at 7 days was significantly lower that at 14 and 21 days (both $p<0.0001$)
- Mean penetration depth at 10 days was significantly lower than that at 14 and 21 days ($p=0.0012$ and 0.0011, respectively)
- There was no significant difference in the mean penetration between 14 and 21 days ($p>0.99$)

The variations observed within each fracture surface as well as within a plate, as evidenced by the relative standard deviations, necessitated an analysis of the maximum penetration at any given point on a fracture surface / plate at the different incubation times. The descriptive statistics are given in table 4.3.

Table 4.3 The maximum depth of penetration at any point on a plate (at different times).

Depth of penetration: individual measurements								
Day	**N**	**Mean**	**Std Dev**	**Median**	**Interquartile range**		**Minimum**	**Maximum**
7	120	32.9	50.9	0	0	62.5	0	225.0
10	200	44.4	76.6	0	0	54.4	0	300.0
14	120	96.9	141.9	0	0	139.1	0	588.2
21	120	97.0	166.4	0	0	134.1	0	630.6

Figure 4.2 shows an SEM micrograph of the control on day 0 (no exposure to *C. albicans*) highlighting the anatomy of the fracture surface. An uneven surface is noticed, with a large crevice extending through the body of the acrylic test plate. When compared with the inoculated acrylic test plates, there was no biofilm extending on the surface of the plate.

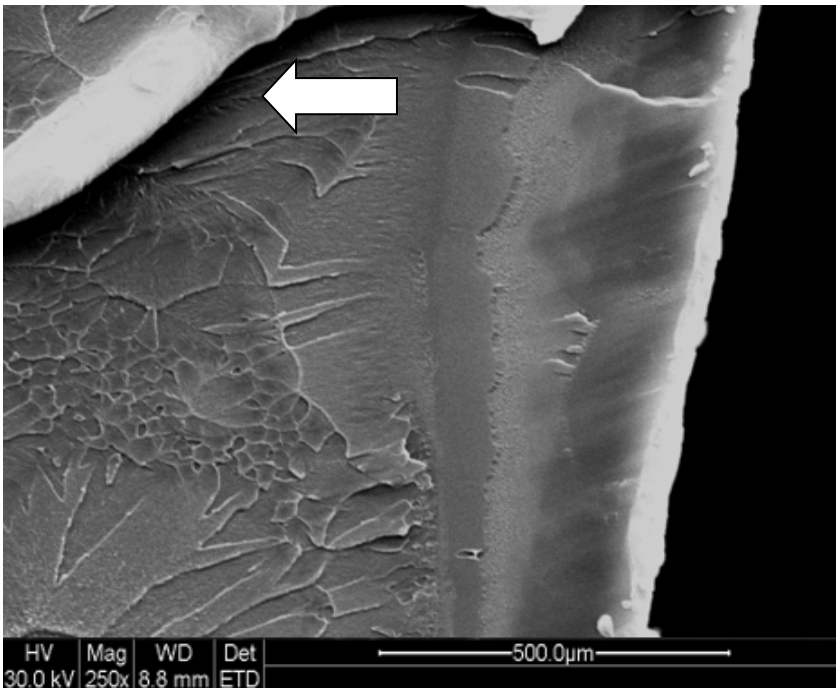

Figure 4.2 An SEM photomicrograph of an unexposed acrylic test plate, highlighting an uneven surface and crevice (white arrow).

Microscopic examination of the acrylic plates exposed to *C. albicans* for 7, 14 and 21 days revealed the presence of *C. albicans* in some areas of the acrylic test plate, as shown in Figures 4.3 to 4.5.

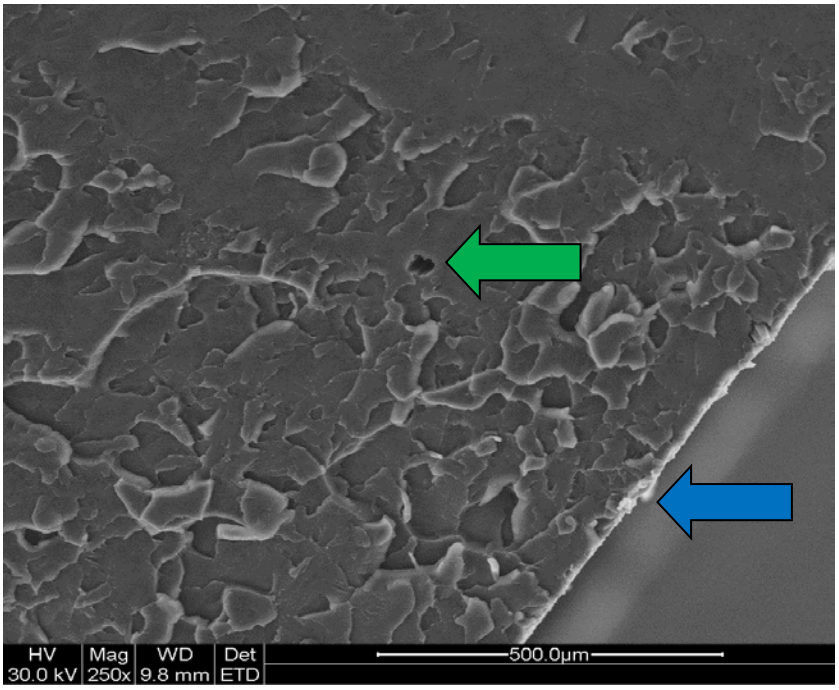

Figure 4.3 An SEM photomicrograph, at magnification of 250x, of exposed acrylic plate on day 7, indicating no candida cells. A thin layer of biofilm is evident on the surface (blue arrow). A 'pore' is visible (green arrow)

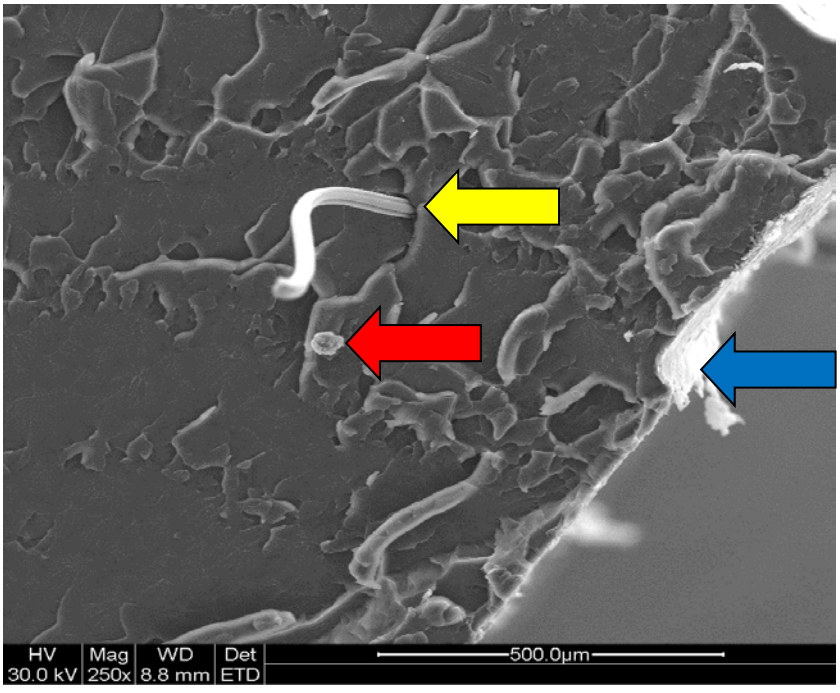

Figure 4.4 An SEM photomicrograph, at magnification of 250x, of exposed acrylic plate on day 14, indicating the presence of a candidal cell (red arrow) and a fibril (yellow arrow). The biofilm is more evident on the surface (blue arrow).

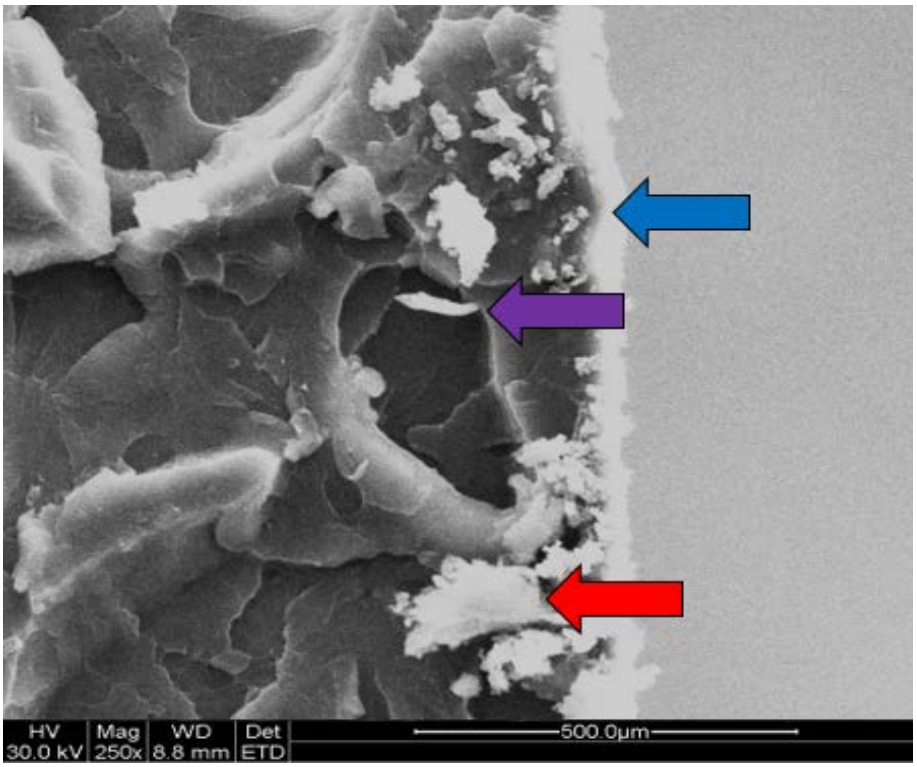

Figure 4.5 An SEM photomicrograph, at magnification of x250, of exposed acrylic plate on day 21, indicating the presence of abundant candida colonies scattered within the denture base resin (red arrow), hypha (purple arrow) as well as a thick biofilm mass on the surface (blue arrow).

4.3. Disinfection of *C. albicans*

Three plates per disinfectant and one plate for the control were tested. Plates were fractured at 2 places creating 4 inner surfaces which were viewed at 10 places under SEM. The results were recorded as presence or absence of *C. albicans*. This procedure produced 120 readings per disinfectant and 40 readings for the control (Appendix D).

All the plates had some evidence of the presence of *C. albicans* even after disinfection. The percentage of sites with evidence of *C. albicans* is shown in Table 4.4 and Figure 4.6. The analysis of the differences in the percentage of sites with evidence of *C. albicans* between the four disinfection methods was carried out by a one-way ANOVA. The overall ANOVA was not significant: p=0.69.

Table 4.4 The percentage of sites with evidence of *Candida*

Disinfectant	Percentage of sites with evidence of Candida			
	N Plates	N Obs	Mean	Std Dev
Control D21	**1**	40	40.0	
Overall (4 disinfectants)	**12**	480	35.8	8.8
Air Dry	**3**	120	35.0	10.9
ClO2	**3**	120	30.8	10.1
D Water	**3**	120	40.0	10.9
Steradent	**3**	120	37.5	4.3

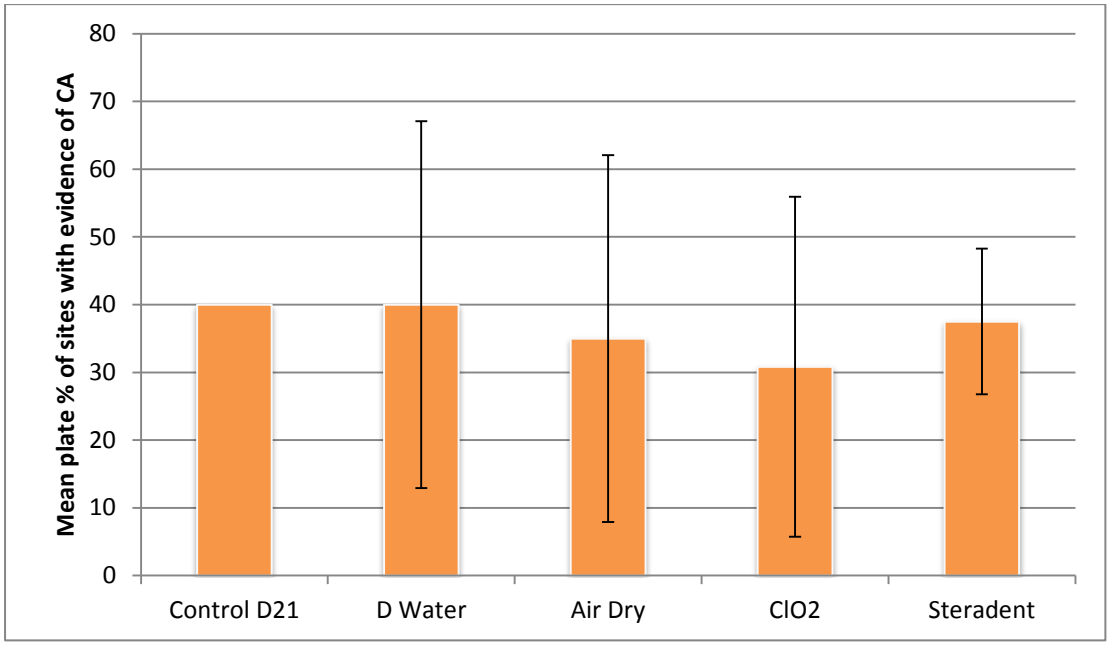

Figure 4.6 Mean percentage of sites with evidence of Candida. Error bars are their 95% confidence intervals.

4.3.1. Viability

The viability of the control (day 21) on surface contamination was determined using the serial dilution technique. The results are illustrated in Figure 4.7. The colony counts were calculated to be 276 000 colony forming units, which is interpreted as a viable count.

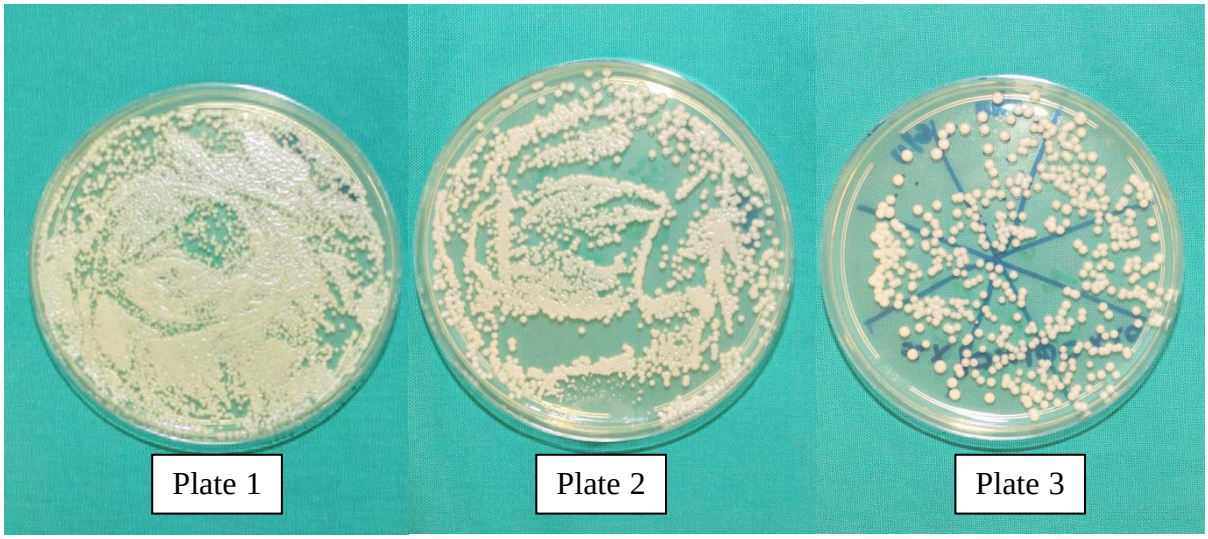

Figure 4.7 Viability of surface contamination on day 21 (control)

CHAPTER 5
DISCUSSION

C. albicans is a normal commensal of the oral cavity in healthy individuals. It is the main aetiological factor associated with denture stomatitis affecting many denture wearers (Buergers et al., 2008; Skupien et al., 2013). Treatment consists of antifungal agents as well as oral and denture hygiene, often using commercially available disinfectants. However, some studies have shown that despite a treatment regimen, symptoms of denture stomatitis returned once the regimen was completed, and this was assumed to be due to the observed penetration of the denture acrylic surface by the *Candida* (Glass et al., 2004). However, the depth of penetration was not known, nor whether that would influence disinfection.

A biofilm, as seen in Figures 4.3 to 4.5 was evident on the surface after exposure of acrylic plates to *C. albicans*. This finding corroborates other studies where a biofilm was present within hours after exposure (Ramage et al.,2004; Ramage et al.,2005; Jackson et al., 2014; Radford et al., 1999). *C. albicans* attaches to surfaces by van der Waals forces. Initial adherence of *C. albicans* to mucosal cells is thermodynamically favoured. After adhesion, attachment occurs through specific cell-surface components (adhesins and mannoproteins) (Radford et al., 1999). This is followed by colonisation of *C. albicans* through its growth as well as ability to change morphologically, where yeast cells are found intertwined with filaments and hyphae. In addition, other micro-organisms would co-aggregate with *C. albicans*, and contribute to the complexity and protection of the biofilm (Radford et al., 1999; Yang, 2003; Williams and Lewis, 2011).

Unpolished acrylic plates were used, as it has been shown that the larger surface area as well as the roughness promotes the initial adherence of plaque and creates a more sheltered environment that allowed for an increase in plaque growth, retention as well as decreased dislodgement of *C. albicans* (Verran and Maryan, 1997; Radford et al., 1999; Dua and Kashinath, 2008; Gendreau and Loewy, 2011; Skupien et al., 2013).

This study has shown that the denture base resin has a labyrinth of pores and crevices, as seen in Figure 4.3. The denture base used in this study is a polymethyl methacrylate, which is a heat cured acrylic resin, and the most common material used in the construction of dentures. The intaglio surface of the denture base is not polished and remains rough and this, together with the physical properties of the denture base, promotes colonisation. This was evident in a study by Richmond et al (2004) who found that acrylic resin exhibited water sorption, which helps with surface colonisation as well as penetration into the acrylic resin. Other materials used as denture bases have also shown colonisation of *C. albicans* (Radford et al., 1999; Skupien et al., 2013).

Attempts have been made to produce a denture base resin that is resistant to *Candida* adhesion and biofilm formation. One study examined the introduction of a silver nanoparticle that exhibits antimicrobial activities and found that with increasing nanosilver solution concentrations, there was a decrease in the activity and mass of the biofilm (Li et al., 2014). Other studies examined the addition of a surface charge and the application of a self-bonding polymer or a coating, where modifications made were effective methods in reducing adhesion of *C. albicans* to PMMA surfaces (Dhir et al., 2007; Park et al., 2008; Yodmongkol et al., 2014; Tsutsumi et al., 2016). However, to date, there are no materials commercially available that have been shown to inhibit the biofilm.

The pores and crevices present in the acrylic plate support the attachment and colonisation of *Candida* through micro colony formation and biofilm attachment, which further increases retention in the denture base (Wendt and Glass, 1987; Glass et al., 2004; Glass et al., 2010). Wendt and Glass (1987) studied the effect of time on the increased presence of *C. albicans* by means of colony forming units on the surface as well as in the depth of the acrylic resin. After exposure of acrylic strips for a period of time (up to a week), the results showed that surface contamination occurred as early as 30 minutes, with penetration into the acrylic strip occurring as early as 4 hours, and the numbers of *C. albicans* found within the strip increased over time. This led to the conclusion that a denture base may, after one day of contact with contaminated mucosa, be considered contaminated within the depth of the denture. As such, treatment would be by means of either effective sterilisation or the fabrication of a new denture. These findings were further supported by Glass et al (2004), Glass et al (2010) and Radford et al (1999). In the present study, the first observation was made only after 7 days exposure of denture base to *C. albicans*. Nevertheless, these results suggest that regular cleaning and the use of disinfectants is important.

Constant exposure of *C. albicans* to the denture base allowed for its penetration after 7, 10, 14 and 21 days. These results are comparable to similar findings obtained by Wendt and Glass (1987), Glass et al (2004) and Glass et al (2010). Wendt and Glass (1987), found increasing colony forming units over time.

A significant increase in depth of penetration was noted from day 0 to day 7 and 14 ($p<0.0001$). However, this increase plateaued between14 and 21days ($p=0.99$). The average depth per plate after 7 days was 33 µm, and at 14 and 21 days it was 97 µm. It is likely that as

the depth of penetration increases over time, less nutrients are available, thus contributing to the plateau effect. These results also suggest that the surface disinfection is not sufficient and the penetration of disinfectants is necessary to eradicate the deep seated organisms.

The SEM micrographs showed that the penetration was not only by the hyphal form of *Candida* but it was also with oval form of *Candida* cells in clumps. This was more evident after 21 days, where there was an increase in *C. albicans* forming microcolonies within the biofilm and denture base. These microcolonies provide a niche environment for increased Candidal growth and survival in the denture base. The presence of hyphae highlights the increase in biofilm mass as well as the increase in resistance to removal and the greater pathogenicity to adhere to surfaces and allow for further dissemination (Trochin et al., 1991; Jackson et al., 2014). Pathak et al (2012) described biofilm formation as three overlapping phases, the early phase (0-11 hours) which is characterised by adherence and microcolony formation; an intermediate phase (12 – 30 hours) where the biofilm community is composed of yeast cells, germ tubes and young hyphae; and the maturation phase (38 – 72 hours) where the biofilm thickens and matures with a dense network of yeast cells, hyphae, pseudohyphae and nutrients (carbohydrates and proteins). This suggests that dentures of patients with stomatitis can be heavily contaminated with mature biofilms which are difficult to eradicate.

This study has shown that *C. albicans* did not evenly penetrate the acrylic in spite of it being submerged in the culture media. This relates to the characteristic of the resin which shows uneven porosity, and explains the variance in the results over the different fracture sites observed, which required that many sites had to be examined. It was found that some areas had no penetration, but in others, the maximum depth on day 7 was 225 µm, on day 10 it was 300 µm, and 588 µm on day 14 and 631 µm on day 21 (Table 4.3). However, submersion n

inoculum does not reproduce the situation in the oral cavity, where denture surfaces are in immediate contact with mucosal surfaces and may in fact be more contaminated and possibly to a greater depth.

It therefore would be useful to have a denture cleansing regimen that would penetrate the denture surface to at least 1 mm in order to remove and kill any biofilm that has penetrated the denture surface. Many different cleansing regimens have been advocated in the literature, categorised as either mechanical or chemical or both, with mechanical methods including the use of toothbrushes, denture brushes, nail brushes, agitators, sonic vibrators and ultrasonic cleansers, to name a few. Chemical methods include the use of commercial denture cleansers (e.g. Steradent®, Polident®), household solutions (e.g. diluted sodium hypochlorite, vinegar), and cold sterilants. Microwave radiation has also been recommended, though the danger of warping the denture base makes this not universally acceptable. Most studies, however, have concentrated on the surface contamination and have not related the efficacy of surface biofilm removal to the possible penetration of the biofilm into the porous resin.

In this study a popular and readily available commercial cleanser, Steradent®, was used because it has been shown to be effective at removing surface biofilm in some studies (Gedik and Ozkan, 2009; Sampaio-Maia et al., 2012; Meric et al., 2014), although mechanical disruption has been advocated to remove residual biofilm (Jose et al., 2010). Chlorine dioxide has been shown to kill *C. albicans* and its biofilm (Patel et al., 2012; Herczegh et al., 2013), and on irreversible hydrocolloid impression material it was shown to remove all contaminants after 90 seconds immersion (Rweyendela et al., 2009). Gel form chlorine dioxide has also been used in the treatment of denture stomatitis (Uludamar et al., 2011). Air drying of contaminated dentures was shown to be more effective than leaving

dentures overnight in water (Stafford et al., 1986) but few studies have compared this with other methods.

The present study has shown that none of the regimens tested were effective in removing the organisms from within the acrylic resin. It was not known, though, whether these would be viable organisms. However, when the control plate which had been exposed to inoculum for 21 days was subjected to the same regimens, organisms remained viable. It cannot be concluded that these were only surface or only penetrated organisms or both, but it would be logical to infer that none of the methods tested are likely to be successful at disinfecting the denture resins. It is also quite possible that the surface tension of the solutions used was such as to prevent the penetration into the micropores of the resin. Clearly further studies need to be carried out to determine a successful cleansing regimen.

CHAPTER 6
CONCLUSIONS AND RECOMMENDATIONS

6.1. Summary and Conclusions

C. albicans contaminated dentures are implicated in the development of denture stomatitis and therefore effective cleaning and disinfection of acrylic dentures remains an important concern. Under the conditions of this study, it was found that *C. albicans* could penetrate acrylic plates to 631 µm. The penetration was exposure time dependent. Significant penetration was found within the first two weeks, thereafter, the depth of penetration plateaued, which could be due to a decrease in nutrients present as the depth of penetration increased. The first null hypothesis was therefore rejected.

None of the cleaning and disinfecting techniques used achieved a complete eradication of *C. albicans*. The second null hypothesis was therefore accepted. These results suggest that either the recommended concentrations of the disinfectants or the contact time requires modification, or that other regimens must be sought.

6.2. Recommendations

In the absence of a proven denture cleansing procedure that would eradicate biofilm from both the surface of a denture base resin, as well as in any areas where the biofilm may have penetrated, it would seem sensible to recommend that the intaglio surfaces of dentures of patients exhibiting denture stomatitis of whatever type, should be removed to a depth of at least 1 mm and the denture relined or, preferably, replaced.

Further research is required to determine a denture cleansing regimen that would be more effective than those currently available. In particular the viability of *Candida* cells within the denture acrylic base must be assessed.

Research should also continue into the incorporation of antimicrobial compounds such as surface charges and silver nanoparticles into the denture base as they have shown some promising results. This aspect can be further studied with regards to the biofilm formation and penetration of *C. albicans*.

Penetration of other oral bacteria with *C. albicans* into the denture base and the role of other oral bacterial species in the pathogenesis of stomatitis and the interaction with *Candida* should also be studied.

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APPENDIX A

ETHICS WAIVER CERTIFICATE

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: P V Tobias Health Sciences Building, 2nd floor Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265

Ref: W-CJ-141205-1 05/12/2014

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **Dr Y Osman Latib (student no 828054).**

Project title: **Penetration of *Candida albicans* into denture base resin.**

Reason: This is an *in vitro* laboratory study using a denture base resin and laboratory cultured *Candida albicans*. There are no human participants

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON - JONES
HREC (MEDICAL)
2014 -12- 05
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu.

APPENDIX B

FACULTY PROTOCOL APPROVAL

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Private Bag 3 Wits, 2050
Fax: 027117172119
Tel: 02711 7172076

Reference: Ms Thokozile Nhlapo
E-mail: thokozile.nhlapo@wits.ac.za

17 March 2015
Person No: 828054
PAG

Y Osman Latib
Postnet Suite 242
Private Bag x 2600
Houghton
2041
South Africa

Dear Osman Latib

Master of Science in Dentistry: Approval of Title

We have pleasure in advising that your proposal entitled *The penetration of candida albicans in denture base resin* has been approved. Please note that any amendments to this title have to be endorsed by the Faculty's higher degrees committee and formally approved.

Yours sincerely

Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences

APPENDIX C

DEPTH OF PENETRATION STUDIES

Table C -1 Depth of penetration of *C. albicans* on day 7 at each of the 10 interval positions.

Day	Plate		Interval 1	Interval 2	Interval 3	Interval 4	Interval 5	Interval 6	Interval 7	Interval 8	Interval 9	Interval 10
7	1	FS1	107.50	0.00	35.00	0.00	0.00	0.00	0.00	82.50	0.00	0.00
7	1	FS2	0.00	0.00	0.00	127.50	0.00	28.75	32.50	0.00	30.00	0.00
7	1	FS3	0.00	0.00	0.00	0.00	142.50	0.00	0.00	132.50	0.00	127.50
7	1	FS4	0.00	0.00	108.75	0.00	70.00	80.00	0.00	27.50	90.00	0.00
7	2	FS1	0.00	0.00	0.00	120.00	135.00	0.00	98.75	165.00	0.00	0.00
7	2	FS2	0.00	96.25	0.00	0.00	30.00	140.00	0.00	0.00	31.25	117.50
7	2	FS3	0.00	0.00	0.00	42.50	128.75	0.00	71.25	0.00	70.00	0.00
7	2	FS4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	77.50
7	3	FS1	0.00	0.00	65.00	0.00	0.00	0.00	225.00	0.00	0.00	165.00
7	3	FS2	0.00	0.00	92.50	0.00	0.00	142.50	0.00	85.00	0.00	26.25
7	3	FS3	56.25	26.25	52.50	42.50	0.00	60.00	0.00	0.00	0.00	105.00
7	3	FS4	0.00	30.00	30.00	120.00	0.00	0.00	0.00	0.00	80.00	0.00

Table C -2 Depth of penetration of *C. albicans* on day 14 at each of the 10 interval positions.

Day	Plate		Interval 1	Interval 2	Interval 3	Interval 4	Interval 5	Interval 6	Interval 7	Interval 8	Interval 9	Interval 10
14	1	FS1	0.00	376.50	400.00	136.25	0.00	0.00	0.00	27.50	0.00	25.00
14	1	FS2	0.00	0.00	105.00	197.50	278.75	251.25	170.00	0.00	330.00	127.50
14	1	FS3	197.65	89.40	0.00	517.65	0.00	0.00	0.00	0.00	0.00	277.65
14	1	FS4	0.00	0.00	75.29	395.29	56.47	414.12	0.00	338.82	0.00	0.00
14	2	FS1	136.47	230.59	0.00	0.00	0.00	272.94	122.35	508.24	0.00	588.24
14	2	FS2	329.41	0.00	0.00	212.50	162.50	0.00	0.00	0.00	131.76	0.00
14	2	FS3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	118.75	25.00
14	2	FS4	0.00	94.11	0.00	282.35	0.00	0.00	0.00	0.00	338.82	0.00
14	3	FS1	75.00	115.00	0.00	46.25	25.00	0.00	0.00	65.00	240.00	300.00
14	3	FS2	75.29	65.88	0.00	141.78	0.00	70.59	0.00	0.00	282.35	0.00
14	3	FS3	0.00	0.00	376.47	0.00	110.00	0.00	0.00	75.29	508.24	0.00
14	3	FS4	244.71	0.00	0.00	207.06	106.25	66.25	0.00	0.00	95.00	0.00

Table C -3 Depth of penetration of *C. albicans* on day 21 at each of the 10 interval positions.

Day	Plate		Interval 1	Interval 2	Interval 3	Interval 4	Interval 5	Interval 6	Interval 7	Interval 8	Interval 9	Interval 10
21	1	FS1	0.00	0.00	18.82	0.00	37.25	0.00	508.24	0.00	28.24	70.59
21	1	FS2	348.24	442.00	0.00	197.65	0.00	0.00	150.59	0.00	0.00	0.00
21	1	FS3	0.00	470.00	0.00	0.00	0.00	0.00	75.24	0.00	508.24	0.00
21	1	FS4	0.00	56.47	174.12	51.76	0.00	131.76	0.00	0.00	0.00	61.18
21	2	FS1	0.00	0.00	0.00	339.82	0.00	0.00	0.00	0.00	0.00	202.35
21	2	FS2	80.00	0.00	428.24	0.00	0.00	197.65	0.00	0.00	0.00	574.12
21	2	FS3	527.06	0.00	0.00	235.29	0.00	56.47	385.88	0.00	0.00	0.00
21	2	FS4	0.00	28.24	0.00	0.00	437.65	0.00	252.12	0.00	0.00	0.00
21	3	FS1	296.47	0.00	588.24	0.00	0.00	272.94	32.94	0.00	0.00	330.00
21	3	FS2	0.00	0.00	89.41	0.00	630.59	80.00	0.00	0.00	136.47	0.00
21	3	FS3	508.24	0.00	65.88	235.29	0.00	0.00	0.00	432.94	0.00	0.00
21	3	FS4	338.82	42.35	0.00	244.71	0.00	0.00	0.00	18.82	225.88	0.00

Table C -4 Depth of penetration of *C. albicans* on day 10 at each of the 10 interval positions.

Day	Plate		Interval 1	Interval 2	Interval 3	Interval 4	Interval 5	Interval 6	Interval 7	Interval 8	Interval 9	Interval 10
10	1	FS1	25	142,5	0	72	0	148	0	0	0	23,75
10	1	FS2	12,5	0	0	0	0	137,5	262,5	0	0	152,5
10	1	FS3	0	0	0	0	0	75	60	50	0	22,5
10	1	FS4	0	0	0	0	15	200	0	0	81,5	68,5
10	2	FS1	0	26,25	0	187,5	0	0	0	0	20	235
10	2	FS2	225	0	0	217,5	0	0	122,5	0	0	237,5
10	2	FS3	0	155	0	0	0	0	0	192,5	22,5	142,5
10	2	FS4	122,5	0	0	12,5	0	0	45	36,25	0	0
10	3	FS1	36,25	0	22,5	0	0	0	0	0	20	0
10	3	FS2	212,5	0	0	27,5	0	0	0	0	0	0
10	3	FS3	0	0	105	0	66,25	293,75	0	0	0	0
10	3	FS4	128	175	52,5	162,5	0	0	56,25	0	0	0
10	4	FS1	300,00	0	0	10	180	0	0	212,5	0	0
10	4	FS2	215	152,5	0	145	10	0	0	0	0	91,25
10	4	FS3	0	0	0	35	0	0	0	173,75	0	0
10	4	FS4	187,5	20	0	0	0	56,25	0	37,5	56,25	50
10	5	FS1	0	67,5	0	135	0	0	0	25	0	0
10	5	FS2	237,5	0	287,5	0	0	0	142,5	42,5	0	97,5
10	5	FS3	272,5	40	0	27,5	172,5	0	0	0	0	0
10	5	FS4	0	0	0	0	0	281,25	25	30	0	147,7

APPENDIX D

DISINFECTION STUDIES

Table D - 1 Presence (=Y) of *C. albicans* on day 21 (control)

Control	Plate		Interval 1	Interval 2	Interval 3	Interval 4	Interval 5	Interval 6	Interval 7	Interval 8	Interval 9	Interval 10
	1	FS1	N	N	Y	N	Y	N	Y	N	Y	Y
	1	FS2	Y	Y	N	Y	N	N	Y	N	N	N
	1	FS3	N	Y	N	N	N	N	Y	N	N	N
	1	FS4	N	Y	Y	Y	N	Y	N	N	N	Y

Table D - 2 Presence (=Y) of *C. albicans* on day 21 after exposure to air drying for 8 hours

Air Dry	Plate		Interval 1	Interval 2	Interval 3	Interval 4	Interval 5	Interval 6	Interval 7	Interval 8	Interval 9	Interval 10
	1	FS1	Y	Y	N	N	N	N	Y	Y	N	N
	1	FS2	Y	Y	N	N	Y	N	N	Y	Y	N
	1	FS3	Y	N	N	Y	Y	N	N	Y	N	Y
	1	FS4	N	N	Y	N	N	N	Y	N	N	Y
	2	FS1	Y	N	N	N	Y	N	N	Y	N	N
	2	FS2	N	N	N	N	N	Y	N	N	Y	N
	2	FS3	Y	N	N	N	N	N	N	Y	N	N
	2	FS4	N	N	N	Y	N	N	N	Y	N	N
	3	FS1	Y	N	N	N	N	N	Y	N	N	Y
	3	FS2	N	Y	N	N	Y	Y	N	Y	N	N
	3	FS3	N	N	Y	Y	Y	N	Y	N	N	Y
	3	FS4	Y	Y	N	N	Y	N	N	N	Y	N

Table D - 3 Presence (=Y) of *C. albicans* on day 21 after exposure to distilled water for 8 hours

Distilled Water	Plate		Interval 1	Interval 2	Interval 3	Interval 4	Interval 5	Interval 6	Interval 7	Interval 8	Interval 9	Interval 10
	1	FS1	Y	N	Y	Y	N	N	N	N	Y	N
	1	FS2	N	N	Y	N	Y	N	N	Y	N	N
	1	FS3	Y	N	N	Y	N	Y	Y	N	N	Y
	1	FS4	N	Y	N	N	Y	Y	Y	Y	N	Y
	2	FS1	N	N	Y	N	N	N	N	Y	N	Y
	2	FS2	Y	N	N	Y	N	N	Y	N	N	N
	2	FS3	N	Y	N	N	N	N	N	N	N	N
	2	FS4	Y	N	Y	N	N	N	Y	Y	N	N
	3	FS1	N	N	N	Y	N	N	Y	Y	N	Y
	3	FS2	Y	Y	Y	N	Y	Y	N	N	Y	N
	3	FS3	N	N	Y	Y	N	Y	N	N	Y	N
	3	FS4	Y	N	Y	N	N	Y	N	N	Y	Y

Table D – 4 Presence (=Y) of *C. albicans* on day 21 after exposure to chlorine dioxide for 8 hours

ClO2	Plate		Interval 1	Interval 2	Interval 3	Interval 4	Interval 5	Interval 6	Interval 7	Interval 8	Interval 9	Interval 10
	1	FS1	Y	Y	N	N	N	Y	N	Y	Y	Y
	1	FS2	Y	N	N	N	Y	N	Y	Y	N	N
	1	FS3	N	N	Y	Y	N	N	N	N	Y	N
	1	FS4	Y	N	N	Y	N	N	N	N	N	Y
	2	FS1	N	N	N	N	N	N	N	N	N	N
	2	FS2	N	Y	N	N	N	N	Y	N	Y	N
	2	FS3	Y	N	N	N	N	N	N	Y	N	N
	2	FS4	N	N	Y	N	N	Y	Y	N	N	N
	3	FS1	Y	N	N	N	N	N	Y	N	Y	N
	3	FS2	N	N	Y	N	N	Y	Y	N	Y	N
	3	FS3	N	Y	Y	N	Y	N	N	Y	N	Y
	3	FS4	N	N	N	N	N	Y	N	N	N	N

Table D – 5 Presence (=Y) of *C. albicans* on day 21 after exposure to steradent® for 20 minutes

Steradent®	Plate		Interval 1	Interval 2	Interval 3	Interval 4	Interval 5	Interval 6	Interval 7	Interval 8	Interval 9	Interval 10
	1	FS1	N	N	N	Y	N	N	Y	N	N	Y
	1	FS2	Y	Y	N	N	Y	N	N	Y	N	N
	1	FS3	Y	N	Y	Y	Y	N	N	Y	Y	N
	1	FS4	Y	N	N	Y	N	Y	N	N	N	Y
	2	FS1	N	N	N	Y	Y	N	N	N	N	Y
	2	FS2	Y	N	N	N	Y	N	Y	N	N	N
	2	FS3	N	N	N	N	Y	Y	Y	N	Y	N
	2	FS4	N	Y	N	N	N	N	Y	Y	N	Y
	3	FS1	Y	N	N	Y	N	N	Y	N	Y	Y
	3	FS2	Y	N	N	N	Y	N	N	N	Y	N
	3	FS3	N	N	Y	Y	N	N	Y	N	N	N
	3	FS4	N	Y	N	N	N	Y	N	Y	N	N