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CHEMOTHERAPEUTIC METHODS FOR DENTURE CARE:

Analysis of a novel denture care product

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A Thesis submitted to the University of Glasgow for the Degree of Master of Science (by Research)

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May 2011

Abstract

Oral colonisation by the opportunistic yeast species *Candida albicans* is the major cause of oropharyngeal candidosis (OPC) in a variety of individuals. A sub-set of OPC, denture induced stomatitis (DIS), is predominantly associated with the elderly population is considered to occur due to multiple factors, including improper denture hygiene. Biofilm forming ability of *C. albicans* strains is linked to high carriage and persistence rates following treatment among infected individuals.

These studies aimed to assess various denture decontamination options upon *C. albicans* biofilms grown on both polystyrene and acrylic resin surfaces. 'Gold standard' denture hygiene regimens proved to be partially effective in reducing *C. albicans* numbers therefore combination treatments with two commercial denture cleansers as well as dentifrice were investigated. Sequential treatment with Polident® (pH 8.6) yielded highest efficacy in biofilm inactivity with no re-growth present following incubation in growth medium. Interactions of known morphological modulators upon initial biofilm formation as well as mature biofilm viability were also assessed. Both EDTA; a cation chelator and farnesol; a molecule involved in quorum sensing in *C. albicans* spp. demonstrated anti-biofilm effects. Implications of fungicidal activity presented could impact upon novel anti-candidal formulations.

Finally, host immune responses related to *C. albicans* infection were investigated *in vitro* using OKF6 TERT2 oral epithelial cells. Release of the inflammatory marker; IL-8 demonstrated that the biofilm mode of candidal growth results in elevated levels of host inflammation. Treatment of biofilms with Polident® (pH 7.0) denture cleanser did not impact upon levels of host inflammation suggesting that biofilm components, independent of viability lead to elevated immune responses.

In conclusion, current methods for the treatment of *C. albicans* biofilm forming species are inadequate as the majority of commercially available 'chemical cleaners' merely inactivate the fungal biofilm without dispersing biofilm material. This has implications on subsequent yeast re-growth and prospective infection.

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Acknowledgements

I would like to thank my supervisors Dr Gordon Ramage, Prof Colin Murray and Dr Donald Cameron of the Glasgow University Dental School for their continuous advice and assistance throughout this research project.

I would also like to take the opportunity to thank a few other individuals: Mr Douglas Robertson for his support during my studentship, Steven Milligan, Mr Anto Jose, Dr Elaine McCulloch and Ranjith Rajendran for their particular expertise in the laboratory, Leighann Sherry for help with the final steps of my project, Margaret Mullin for her expert assistance with electron microscopy and Dr David Lappin for his advice on statistics and data analysis.

This research was only made possible thanks to funding from GlaxoSmithKline, attained through Dr Ramage in collaboration with Glasgow University Dental School.

Finally, I would like to thank the remainder of the staff and students at the Glasgow University Dental School who have made my time there very enjoyable.

Declaration of Originality

I am aware of and understand the University's policy on plagiarism and I certify that this thesis is my own work, except where indicated by referencing.

Adriana Zalewska, February 2011

Abbreviations

cDNA:	Complementary deoxyribonucleic acid
CLSI:	Clinical Laboratory Standards Institute
DIS:	Denture induced stomatits
DMEM:	Dulbecco's modified Eagles medium
DMSO:	Dimethyl sulphoxide
EDTA:	Ethylenediaminetetraacetic acid
ELISA:	Enzyme-linked immunosorbent assay
HIV:	Human immunodeficiency virus
IL-8	Interleukin-8
KSFM:	Keratinocyte serum-free medium
MIC:	Minimal inhibitory concentration
NT:	Newton's Type
OKF6-TERT2:	Oral mucosal immortalised keratinocyte cell line
OPC:	Oropharyngeal candidosis
PMIC:	Planktonic minimum inhibition concentration
PMMA:	Polymethylmethacrylate
PBS:	Phosphate buffered saline
rpm:	Revolutions per minute
RPMI:	RPMI 1640 medium
RT-PCR:	Reverse transcriptase polymerase chain reaction
SAB:	Sabouraud dextrose agar
SEM:	Scanning electron microscopy
SMIC:	Sessile minimum inhibition concentration
spp.	Species
w/v:	Weight / volume
XTT:	2,3 bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide
YPD:	Yeast peptone dextrose broth
zym:	Zymosan A from *Saccharomyces cerevisiae* cell wall

Chapter 1: Introduction

1. Introduction

1.1 General introduction

Oropharyngeal candidosis (OPC), commonly referred to as 'oral thrush', is caused by the opportunistic yeast *Candida*. OPC typically presents with white coloured pseudomembraneous lesions covering large areas of the oral mucosa, tongue and palate (Odds, 1988).

Candida spp. are considered to have a commensal relationship with the host. At present, it is estimated that up to 70% of individuals harbour the organism without detrimental effects. It is only when the host becomes immunocompromised that problems arise (Cannon & Chaffin, 1999). Most worryingly, the incidence of *Candida* spp. associated bloodstream infections has increased by more than 75% in the last 30 years and is considered to remain at this elevated level until the introduction of new prevention measures and treatments (Jarvis, 1995; Pfaller & Wenzel, 1992). Although OPC is rarely associated with high instances of mortality, significant morbidity is often experienced. Such morbidity heavily burdens both the individual and the healthcare system (Li *et al.*, 2007b).

Symptoms of OPC include changes in taste and oral pain, which is often referred to as a burning sensation (Ramage *et al.*, 2004). The consequence of such generalised sub-clinical symptoms is that the condition is often under diagnosed and appropriate treatment therefore not administered (Akpan & Morgan, 2002). Even after prescription of anti-fungal medication, colonisation is often re-established. Thereafter, problems can regularly arise if the underlying reason for immunosuppression is not identified or cannot be treated (Lamfon *et al.*, 2004).

One of the commonest types of OPC is the erythematous form. When associated with use of oral prosthetic appliances, this often leads to the development of denture induced stomatitis (DIS). The condition characteristically presents itself as erythema (inflammation) of the oral mucosa in denture wearing individuals (Odds, 1988). The yeast *Candida albicans* is implicated as the main causative organism of

DIS. This is primarily due to the organism's ability to readily adhere to and form resilient biofilms on both soft and hard tissues of the oral cavity (Coco *et al.*, 2008).

1.2. Oropharyngeal candidosis

Clinically, the most prevalent superficial *Candida* infections are those associated with the oral mucosa. OPC can be divided into three main clinical forms, comprising angular cheilitis, pseudomembraneous and erythematous (Akpan & Morgan, 2002). This thesis will specifically concentrate on the erythematous form, of which DIS is a common type.

1.2.1 Erythematous candidosis

Generally, the condition is characterised by erythema of the oral palate and tongue without the typical white plaques evident within the pseudomembraneous form (Palmer *et al.*, 1996). An inflamed mucosa, particularly of the upper palate in a denture wearing individual, is referred to as DIS (Figure 1.1).

DIS is generally considered to be caused by poor denture adaptation to the oral mucosa. Repeated damage to the oral mucosa by an unstable denture leads to the release of an inflammatory secretion from oral epithelial cells that promotes the colonisation and subsequent development of *Candida* species on the oral mucosa (Peltola *et al.*, 1997; Ramage *et al.*, 2004)

Figure 1.1 Inflamed palatal oral mucosa indicative of denture induced stomatitis.

(www.swedishmedical.org/PERT/images/denture.jpg)

1.2.2 Denture induced stomatitis (DIS)

1.2.2.1 Introduction to DIS

There are approximately 15 million denture wearing individuals in the United Kingdom (Coulthwaite & Verran, 2007). Inflammation due to DIS is frequently present in patients who wear either full or partial removable dentures. This indicates that the condition is a widespread problem that is often recurring, and proves to be a considerable healthcare burden (Jose *et al.*, 2010).

1.2.2.2 Historical background

Descriptions of DIS date back to the late 1800's (Black, 1885). Initially the term 'rubber sore mouth' was chosen and subsequently renamed to 'denture stomatitis' in 1965. This highlighted the effect of denture wearing upon oral infection and ensuing inflammation of the oral mucosa (Newton, 1962; Cawson, 1965).

Originally, bacteria were perceived as the principle micro-organisms responsible for the condition. Subsequently, the link between high rates of *Candida* species and DIS was established (van Reenen, 1973). Due to high rates of oral candidal colonisation

and denture wear in the aging population it is possible to comprehend why DIS is the most frequent form of OPC in the elderly population (Ellepola *et al.*, 1999).

1.2.2.3 Predisposing factors for DIS

Predisposing factors for DIS are listed in Table 1.1 (Samaranayake, 1986; Cannon & Chaffin, 1999; Farah *et al.*, 2000; Davies *et al.*, 2006). Poor dietary intake, in particular deficiencies in iron or a high consumption of carbohydrate rich foods, has been established to worsen or prolong OPC (Cawson, 1966; Samaranayake, 1986).

Research has elucidated that the occurrence and severity of DIS is reduced if dentures are routinely removed during the night. This is due to reductions in potential prolonged denture trauma and colonisation of the denture and the soft tissues by microorganisms during this time (Oksala, 1990).

Factors contributing to DIS can continuously change, therefore the condition is considered to be frequently persistent and recurring. Indeed, it is estimated that approximately 11-70% of denture wearers are affected at any one time (Budtz-Jorgensen, 1974; Arendorf & Walker, 1987; Barbeau *et al.*, 2003).

Table 1.1 Factors which predispose an individual to denture induced stomatitis.

Factors predisposing to the development of denture induced stomatitis	
Poor oral hygiene	Xerostomia (dry mouth)
Denture trauma	Immunosuppression
Antibiotic Therapy	Radiation therapy
Smoking	Diet

1.2.2.4 Clinical assessment of DIS

Newton's criteria describe DIS according to severity (Newton, 1962). This ranges from type 0 to type 3, with 3 being the most severe presentation of the condition. Candidal colonisation and ensuing biofilm formation is associated with various types of Newton's grade of erythema (Ramage *et al.*, 2004; Coco *et al.*, 2008). Recent *in vivo* observations demonstrated that all modes; including yeast and hyphal forms of *Candida* biofilm growth are exhibited during DIS infection (Ramage *et al.*, 2004).

Newton's scale of severity was later updated to the simplified version below, and these levels of oral inflammation are represented in Figure 1.2:

0- No inflammation
1- Localised inflammation (NT I)
2- Diffuse inflammation (NT II)
3- Granular inflammation (NT III)

It has become evident that high levels of inflammation are not consistently due to the amount of organisms present. Other factors such as poor oral hygiene appear to play a crucial role (Coco *et al.*, 2008).

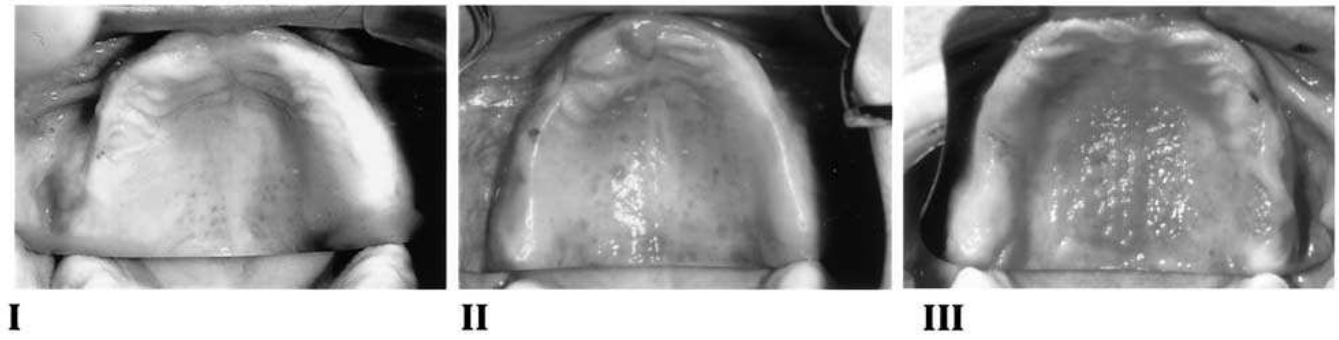


Figure 1.2 Newton's Type scale for the classification of inflammation present in DIS. I) Slight inflammation II) Diffuse inflammation III) Severe inflammation are shown in the figure (Barbeau *et al.*, 2003)

1.2.2.5 Candidal adhesion to dentures

Strong attachment and subsequent biofilm formation of *Candida* spp. on surfaces is fundamental for yeast survival. It is commonly accepted that adherence of yeast to the denture surface is facilitated by the formation of a salivary pellicle on teeth and dentures alike (Edgerton *et al.*, 1993). Candidal attachment has also been reported to occur directly, without initial salivary pellicle formation. So far this process has only been examined *in vitro* as presence of a salivary pellicle cannot be avoided in the oral cavity (Radford *et al.*, 1999).

The main stages involved in adhesion and colonisation include:

- Movement to surface through diffusion or chemotaxis
- Initial adhesion through van der Waal's forces followed by surface free energies between the yeast and surface
- Attachment involving yeast cell wall components such as polysaccharides adhering to various receptors in plaque which is composed mainly of saliva and bacteria
- Colonisation and biofilm formation (Radford *et al.*, 1999)

The initial stage of adherence is thought to occur through surface free energies between the impression surface of the denture and microorganism. The second stage is more precise, involving specific microbial adhesives which bind strongly to receptors on the denture surface (Nevzatoglu *et al.*, 2007). These adhesins are candidal surface proteins that have been found to be associated with germ tube attachment to plastic surfaces (Tronchin *et al.*, 1988). Tronchin and colleagues did not specify the adhesin group involved, but novel research implicates candidal host surface adherence to the agglutinin like sequences (ALS) gene family amongst other proteins (Hoyer, 2001). It is considered that there are 8 cell surface proteins in the family and expression of corresponding genes is linked to greater pathogenicity within *C. albicans* strains (Zhao *et al.*, 2003).

Together, these adhesion mechanisms prevent removal of the organism from both denture surfaces and the oral mucosa by the host's physical innate defence mechanisms, such as saliva. Furthermore, manual removal of *Candida* spp. by brushing the denture surface is made more difficult (Ramage *et al.*, 2004).

Acrylic resin dentures are commonly manufactured from polymethylmethacrylate (PMMA) and it is well documented that surface 'roughness' is an important factor for microbial adhesion. Generally, rougher acrylic surfaces are associated with high *Candida* yields (Nevzatoglu *et al.*, 2007; Yamauchi *et al.*, 1990). This is considered to be related to contact sensing or 'thigmotropism' of *Candida* spp. enabling growth of hyphae along irregularities or cracks in the denture material (Nikawa *et al.*, 1998; Ramage *et al.*, 2004). This subsequently results in maximum protection of the microorganism from physical cleansing and aids retention to the denture surface (Taylor *et al.*, 1998; Watts *et al.*, 1998).

Dentures provide a reservoir for growth of micro-organisms, as well as facilitating candidal contact to adjacent oral mucosa. It is also considered that yeast numbers on denture surfaces are higher than those colonising the oral mucosa (Davenport, 1970). This is due in part to the development of anaerobic and acidic conditions in close proximity to the tissue covered by the denture. Such conditions facilitate growth of micro-organisms (Budtz-Jorgensen, 1974). Furthermore, salivary flow to the palate is frequently impaired resulting in lower clearance of micro-organisms than in dentate individuals (Lamfon *et al.*, 2004). Most importantly, the presence of denture plaque; a diverse biofilm structure, consisting of various oral micro-organisms in addition to *Candida* spp. is considered to be a major contributing factor to candidal retention on dentures (Chandra *et al.*, 2001b).

As previously stated, saliva is an important host innate defence mechanism. Various *in vitro* studies have illustrated that coating of acrylic resin with saliva reduces microbial adherence due to blocking of interactions between yeast and denture surface (Moura *et al.*, 2006; Samaranayake & MacFarlane, 1980). However, a disparity in research findings is apparent as various sources contradict this information and suggest that a saliva coating increases candidal binding to surfaces

(Vasilas *et al.*, 1992). Saliva acts to physically remove organisms and contains active substances such as lysozyme and lactoferrin which inhibit candidal adhesion to and colonisation of oral surfaces (Pereira-Cenci *et al.*, 2008). Interestingly, not all components of saliva are considered to hinder the organism. In particular, binding of *C. albicans* to a specific site in mucin has been described, which facilitates binding to saliva coated surfaces (Edgerton *et al.*, 1993; Hoffman & Haidaris, 1993).

1.3. *Candida* species and disease

1.3.1 Overview of *Candida* species infections

Candida spp. are a common human commensal and generally do not cause harm to the host (Akpan & Morgan, 2002; Ramage *et al.*, 2004). Incidence of infections tends to increase when host defences are compromised. HIV and AIDS patients in addition to the elderly and cancer sufferers commonly experience higher rates of pseudomembraneous and erythematous candidosis compared to healthy individuals (Palmer *et al.*, 1996; Davies *et al.*, 2006).

Candida spp. are also responsible for causing a quarter of nosocomial bloodstream infections (Pfaller *et al.*, 1998). This is thought to be mainly due to formation of biofilms on medical devices, which consequently results in limited treatment options. A high level of biofilm-associated resistance to antifungals represents a significant hurdle for the management of such systemic infections (Kojic & Darouiche, 2004).

Other than bloodstream infections, *Candida* associated problems include intra-abdominal and urinary tract infections (Pappas *et al.*, 2004). Epidemiology studies reveal that mortality rates for patients with invasive candidiasis are as high as 60% (Voss *et al.*, 1997; Wey *et al.*, 1989). It is apparent that there is a continual need for surveillance and research into all forms of *Candida* infections due to extremely high prevalence and associated morbidity and mortality.

1.3.2 *Candida albicans* and the human host

C. albicans is a commensal organism with the ability to become opportunistic when conditions become favourable. The yeast is commonly isolated from 30-40% of healthy individuals and increases to 50-70% for those who wear dentures (Odds, 1988; Akpan & Morgan, 2002). The organism generally resides in the gastrointestinal and genitourinary tracts of healthy individuals. In certain circumstances, such as a shift in normal microflora due to antibiotic use, overgrowth of the microorganism can occur (Akpan & Morgan, 2002; Pappas *et al.*, 2004).

Various *Candida* spp. have been implicated in oropharyngeal candidosis but *C. albicans* is considered to be the most pathogenic (Budtz-Jorgensen, 1974). Studies identify that numerous virulence factors are present which lead to both successful commensal carriage as well as dissemination to pathogenic disease. One such factor is that *C. albicans* has been found to have better adhesive properties than other *Candida* species (Calderone & Braun, 1991). In addition, the pathogen is known to secrete two types of hydrolytic enzymes (proteinases and phopholipases) which are crucial for the breakdown of peptide bonds and phospholipids in the host's tissues (Sugita *et al.*, 2002). This is discussed further in Section 1.4.3.

As well as superficial presentations observed within the oral cavity, *C. albicans* is also an important pathogen contributing towards systemic disease. Major health implications occur when *C. albicans* breaches the epithelial barrier and enters the bloodstream. The commonest types of systemic candidal infection include bloodstream infections, catheter infections, intra-abdominal infections and urinary tract infections (Pappas *et al.*, 2004).

1.3.3 Other common *Candida* species

Twenty *Candida* species are considered to be of medical significance (Akpan & Morgan, 2002). Of these, *C. albicans*, *C. glabrata* and *C. tropicalis* represent more than 80% of clinical isolates (Odds, 1988).

C. glabrata is the second most prevalent commensal *Candida* species found in healthy individuals after *C. albicans* (Coco *et al.*, 2008; Pereira-Cenci *et al.*, 2008). This documented high prevalence may be in part attributed to *C. glabrata's* resistance to azole antifungal medications (Sanglard *et al.*, 1999). In comparison, other *Candida* species are highly susceptible to azoles. This adaptation means that systemic infections due to *C. glabrata* often result in elevated mortality rates as other treatment options need to be sought (Cate *et al.*, 2009; Li *et al.*, 2007b).

Although high numbers of both *C. glabrata* and *C. albicans* occur commensally and in pathogenic infection, significant differences in interaction with the host have been documented. *C. albicans* relies mainly on dimorphic switching between yeast and hyphal forms as well as secretion of adhesins for attachment to host surfaces (Kaur *et al.*, 2005; Whiteway & Oberholzer, 2004). Interestingly, adhesion to host cells is reportedly better developed in *C. albicans* strains while *C. glabrata* strains possess stronger adhesion attributes in relation to denture acrylic (Biasoli *et al.*, 2002; Luo & Samaranayake, 2002).

Release of degradative enzymes such as proteinases and phospholipases is present in both species, although it is documented as being higher in *C. albicans* strains (Chakrabarti *et al.*, 1991; Samaranayake *et al.*, 1994).
Although *C. albicans* has typically been demonstrated as the most frequently isolated *Candida* species, a recent shift has been reported in which non-*albicans* species are being regularly isolated from the oral mucosa of healthy individuals as well as DIS sufferers (Coco *et al.*, 2008). Apart from the species mentioned above, *C. parapsilosis*, *C. krusei* and *C. dubliniensis* are frequent oral inhabitants associated with OPC (Pereira-Cenci et al., 2008; Cate et al., 2009). The recent documented increase in *C. dubliniensis* spp. isolated from OPC patients is due, in part, to the formation of pseudohyphae by this species. Pseudohyphae formation leads to biofilm development on acrylic surfaces within the oral cavity (Ramage *et al.*, 2001b). It is the ability of *Candida* species to form resilient biofilms which facilitates success as an opportunistic pathogen (Chandra *et al.*, 2001a; Mukherjee & Chandra, 2004).

1.4. Biofilm formation

1.4.1 Introduction to biofilm formation

A biofilm can be defined as a heterogeneous community of cells, attached to a surface and enclosed in an extra-polymeric matrix (Ramage *et al.*, 2002a). A considerable proportion of infections are a result of biofilm development (Potera, 1999). Apart from formation on dentures and in dental plaque, *Candida* biofilms are known to grow readily on other artificial devices including catheters and prosthetic limbs. This is a serious health care burden as biofilm development on medical implants and appliances leads to a high proportion of nosocomial infections, which are often life threatening (Cateau *et al.*, 2008). Frequently, the only clinical solution is removal of the device and treatment with antibiotics and/ or antifungals to clear the infection (Kojic & Darouiche, 2004).

1.4.2 Morphological stages involved in biofilm progression

C. albicans is considered to be a dimorphic fungus which can undergo morphological changes when triggered by environmental queues such as a temperature of 37ºC and CO_2 levels of 5.5% (Karkowska-Kuleta *et al.*, 2009). Stages of biofilm progression begin with germ tube formation which is the initial stage of hyphal development (Cannon & Chaffin, 1999). This occurs following location of an appropriate surface for colonisation. Pseudohyphae are an intermediate between yeast and hyphal cell whereby cells remain attached to each other but vary in shape (Cate *et al.*, 2009; Odds, 1988). This stage occurs at around 4 hours following attachment to the surface (Ramage *et al.*, 2001c). Hyphae are essential for mature biofilm development as they, alongside the extracellular material play a part in upholding the general structure, drug resistance and adhesive properties of the biofilm (Chandra *et al.*, 2001b). True hyphae are present at around 8 hours following adhesion (Ramage *et al.*, 2001c). Morphological stages of *C. albicans* are represented in Figure 1.3

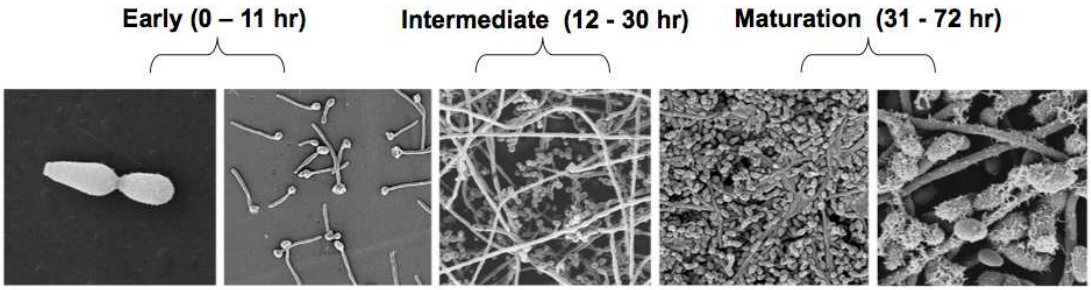


Figure 1.3: Stages of biofilm progression. Early stage occurs between 0 and 11 hours and results in initial hyphae formation. Production of matrix material in intermediate stage (12-30 hours) ensures that the biofilm gains structure and resilience. The final stage of maturation at 31-72 hours results in a resilient three dimensional structure (Ramage *et al.*, 2009).

1. **Early phase:** Adherence of yeast cells to surface. This can occur up to 11 hours after initial colonisation. Initiation of hyphae formation.
2. **Intermediate phase:** Budding yeast cells transform to hyphal cells through phenotypic switching. Matrix material is produced due to differential gene expression and subsequent production of enzymes involved in carbohydrate synthesis. A monolayer structure is achieved 12 to 30 hours after colonisation.
3. **Maturation phase:** Three-dimensional structure is achieved through further growth and production of extra-polymeric matrix which encloses yeast, pseudohyphae and hyphae forms. Microcolonies of yeast form the basal layer with networks of hyphal cells lining the top of the biofilm. Together a resilient biofilm structure formed after 31 to 72 hours (Chandra *et al.*, 2001a; Kruppa, 2009; Lamfon *et al.*, 2004; Ramage *et al.*, 2001c).

1.4.3 Phenotypic switching and gene expression related to pathogenicity

The phenotype of a *Candida* biofilm can alter reversibly in response to environmental changes including alterations in temperature, pH and the presence of serum (Odds, 1988; Cate *et al.*, 2009). Alterations such as a rough or fringed appearance to the phenotype of a colony occur relatively frequently. In fact, such events are described as occurring at a rate of 1 change per 10^1-10^4 colonies (Slutsky

et al., 1985). It is understood that cells which remain as just one of either yeast or hyphal forms have limited virulence traits (Murad *et al.*, 2001).

A widely researched phenotypic switching event present in some *C. albicans* strains is 'white-opaque switching' as it occurs in high frequency and can be observed at the microscopic level (Anderson & Soll, 1987). This type of switching not only assists the fungus in reproduction but also has pathogenicity implications as cells in both white and opaque transitions have been found to have increased virulence in different environments (Kvaal *et al.*, 1999). The overall goal of such phenotypic switching events is to increase chances of pathogenicity when the organism is introduced to a new environment such as a new host.
Phenotypic alterations also prove crucial in the pathogen's ongoing development of resistance to a wide range of antifungals (Mukherjee & Chandra, 2004). In particular, the CDR3 drug resistance gene is expressed during the opaque stage of white-opaque switching of a strain of *C. albicans*. The CDR gene family are associated with efflux pump activity and subsequent resistance to fluconazole (Balan *et al.*, 1997).

Switching events have also been implicated in controlling genes encoding proteolytic enzymes (in particular SAP1 and SAP3) which are vital for virulence attributes characteristic of *C. albicans* (Hube et al., 1994) (Described further in Section 1.4.4). It is the release of enzymes which is responsible for the majority of the tissue damage seen in cases of DIS (Hube, 1996; Rajendran *et al.*, 2010).

1.4.4 Degradative Enzymes

Along with *Candida* species, many other microorganisms are known to secrete enzymes which facilitate disease progression. Collectively, it is proteolytic enzymes which are most commonly associated with *C. albicans* virulence (Naglik *et al.*, 2003).

Four main types of these proteinases are widely known; these are aspartyl, serine and cysteine proteinases and metalloproteinases (Barrett & Rawlings, 1991). The employment of proteolytic enzymes by *Candida* species has been well documented.

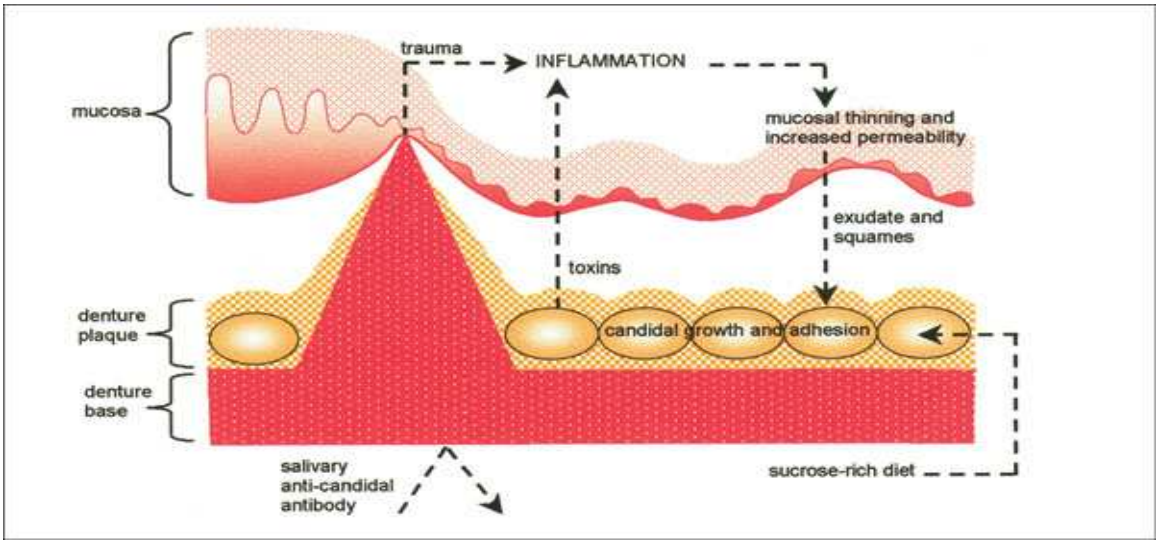
In particular, the secreted aspartyl proteinases (Sap) family which is encoded by 10 Sap genes is an important virulence factor. Sap production is also characteristic of several non *albicans* species including *C. tropicalis* and *C. parapsilosis (*Monod et a*l., 1994)*. It is considered that the secretion of these enzymes is directly linked to the pathogenicity of a species as lesser or non-pathogenic *Candida* species do not express Sap genes (Naglik *et al.*, 2003). In particular, during hyphal stages of *C. albicans* development Saps 4, 5 and 6 are expressed (Hube, 1996).

Along with the production of Sap proteases, *Candida* species also exhibit phospholipase and haemolysin activity. Increased production of these enzymes has been demonstrated in biofilm forming isolates (Rajendran *et al.*, 2010). Phospholipase activity of *C. albicans* isolates is relatively high. 30-100% of strains taken from various infection sites including blood and urine were found to possess phospholipase activity (Price *et al.*, 1982). The role of this enzyme is not yet fully understood (Tsang *et al.*, 2007). The function of haemolysin relates to iron acquisition of the fungus. Iron is a key substrate for the majority of microorganisms and a role for this element has been described in *C. albicans* hyphal penetration when associated with systemic candidiasis (Manns *et al.*, 1994). These hydrolytic enzymes, in combination with cell wall antigens, act as potent mediators of inflammation, which results in the erythema and tissue destruction associated with host-pathogen interactions.

1.4.5 Host pathogen interactions

1.4.5.1 Introduction to host pathogen interactions

Candida species may cause harm to the host if conditions for adherence and subsequently multiplication become favourable (Section 1.2). The role of the oral environment is to protect the oral cavity from adherence of *Candida* spp. to both hard surfaces, such as the teeth and dentures, as well as to the soft epithelium (Decanis *et al.*, 2009). If oral tissues are successfully infected by a micro-organism, inflammation and tissue destruction may ensue as illustrated in Figure 1.4.



(Davenport *et al.*, 2001)

Figure 1.4 Biofilm formation resulting in trauma and consequential inflammation.

Host detection of invading microorganisms occurs primarily through Toll-like receptors which are located on host cells such as epithelial cells (Takeda & Akira, 2004). Activation of Toll-like receptors leads to a plethora of down-stream processes which are required for host defence (Akira & Takeda, 2004)

One of the main host responses consists of leukocyte recruitment which is followed by a release of inflammatory cytokines (Mencacci *et al.*, 2000). It is well established that IL-6, IL-8, and TNF-α are the predominant cytokines released in response to *Candida* infection (Mostefaoui *et al.*, 2004). In particular, an increase in IL-8 release has been documented to occur in response to epithelial cell contact with *C. albicans* hyphal cells (Dongari-Bagtzoglou & Kashleva, 2003).

Other than innate immunity, the adaptive immune response is also thought to be involved in host protection against mucosal *Candida* spp. infections. Sources often demonstrate contradictory information in regard to the anti-candidal role for cell mediated immunity (Fidel *et al.*, 1993), but a particular study reported that Th1 CD4+ T cells have been implicated in a strong cell mediated host defence against candidosis in a mouse model (Cenci *et al.*, 1995).

1.4.5.2. Current in vitro research

The oral epithelium is an effective first line of host defence with both *in vitro* and *in vivo* studies having seldom been able to identify candidal penetration further than the stratum spinosum (Reichart *et al.*, 1995; Sundstrom *et al.*, 2002). Studies indicate that the epithelium can act as a thorough anti-candidal barrier in healthy hosts but immunocompromised individuals, such as those infected with HIV, have impaired oral epithelial function (Steele *et al.*, 2000).

Previously, it has been difficult to assess whether epithelial cells induce fungistatic/cidal anti-candidal effects. However, it has been demonstrated that direct candidal contact with epithelial cells results in arresting growth only temporarily (Nomanbhoy *et al.*, 2002). This therefore means that other host immune responses are necessary to ensure full candidal eradication.

Cell lines are attained, which best indicate host-pathogen interactions *in vivo*. OKF6/TERT human oral keratinocyte cell lines are often used to assess the immune response to candidal infection (Decanis *et al.*, 2009). This cell line has recently been co-cultured with oral keratinocytes to form the 'engineering human oral mucosa'. The aim behind the design of this and other three dimensional model systems is to have a more direct indication of the activities of the human oral mucosa in response to *Candida* spp. infection (Rouabhia & Deslauriers, 2002).

1.4.6 Quorum Sensing

Biofilm formation is partially controlled by quorum sensing, which ensures that a correct cell density and structure can be maintained in a suitable location (Miller & Bassler, 2001). *In vitro* studies have demonstrated that yeast cell concentrations below 1 x 10^6 cells/mL are necessary for successful development of hyphae and therefore a resilient biofilm (Kruppa, 2009). An example of an important quorum sensing molecule is farnesol. It is a natural organic compound which is responsible for the shift from *Candida* yeast to hyphal forms (Oh *et al.*, 2001). Farnesol inhibits a primary step of biofilm development; the attachment of cells to a surface

(Ramage *et al.*, 2002b). Therefore, it has been recognised that microorganisms utilise quorum sensing to 'communicate'. This ensures the success of a three dimensional biofilm which can maintain a variety of microbial oral species (Costerton *et al.*, 1999).

The biofilm structure benefits from a mixed species community that allows the co-habitation of diverse bacteria and fungi. In the oral mucosa, *Candida* spp. most often exists in a mixed fungal-bacterial community which aids in the survival of all species involved (Bamford *et al.*, 2009). Indeed, the adhesion of certain bacteria such as *Escherichia coli* and *Streptococcus* species are improved when in a mixed *Candia* biofilm (Nair & Samaranayake, 1996). The occurrence of a diverse mixed species lifestyle is usually due to environmental stresses such as limited nutrients (Palkova & Vachova, 2006). The complex molecules that are involved in cell density regulation may therefore be used to our advantage to help control OPC as novel therapeutics.

1.5 Treatment of OPC

As previously stated, *Candida* spp. biofilm formation is central to host tissue adherence and the subsequent pathological changes. One of the major difficulties treating *Candida* spp. infections, including OPC, is intrinsic antifungal resistance that is often associated with biofilm forming isolates (White *et al.*, 1998; Chandra *et al.*, 2001b; Ramage *et al.*, 2001c).

1.5.1 Use of antifungals

Following diagnosis, OPC is typically treated by use of topical antifungals applied to the mucosa and/or denture base. Where resistance to topical treatments is observed, systemic antifungals are frequently necessary. Systemic antifungals are also required for individuals receiving chemotherapy (Akpan & Morgan, 2002; Patton *et al.*, 2001). Commonly administered antifungal agents are listed in Table 1.2.

Research has elucidated that survival in a biofilm structure leads to multi-antifungal resistance (Ramage, 2010). In particular, resistance to chlorhexidine, amphotericin B and fluconazole has been documented (Hawser & Douglas, 1995; Chandra *et al.*, 2001b). In addition to emerging resistance to a variety of antimycotics, the efficacy of topical antifungals in the oral cavity is reduced due to a diluting effect of saliva (Martin, 1989). Moreover, a significant factor that results in inefficiency of treatment is that an individual is often unwilling or unable to continue with prolonged antifungal use, for example poor compliance. This often leads to incomplete treatment and implications in the development of antifungal resistance (Ellepola & Samaranayake, 2000). This section will focus on the various options used to treat oral and systemic candidal infections.

Table 1.2 Antifungal drugs currently used against *Candida* infections. Mode of action and target of candidal infection varies between groups

Group	Mode of action	Types	Review
Azole	Inhibition of ergosterol production in candidal cell membrane	fluconazole, voriconazole, clotrimazole, miconazole ketonconazole	(Sheehan *et al.*, 1999) (Kale & Johnson, 2005)
Polyene	Binding to ergosterol	amphotericin b, nystatin, natamycin	(Cornely *et al.*, 2006)
Echinocandin	Inhibition of glucan synthesis in candidal cell wall	caspofungin, micafungin	(Morrison, 2006) (Denning, 2003)
Allylamine	Inhibition of ergosterol synthesis	terbinafine	(Jain & Sehgal, 2000)

Typical treatment of OPC consists of topical nystatin or amphotericin B application once or twice daily, while intravenous fluconazole is administered to immunocompromised patients (Chen & Sorrell, 2007). Amphotericin B is often favoured due its various anti-candidal properties including the prevention of SAP enzyme production/release (Wu *et al.*, 1996), and the ability to target candidal cells in yeast and hyphal forms (Ellepola & Samaranayake, 2000). A drawback of this antimycotic is the extent of side effects experienced, the most adverse being nephrotoxicity (Sabra & Branch, 1990).

Like amphotericin B, nystatin also targets ergosterol in the fungal cell membrane and leads to 'fungal cell leakage' (Ellepola & Samaranayake, 2000). Nystatin is mainly used topically and in tablet form for the treatment of oral candidosis. Unfortunately, the bitter taste associated with the tablets often leads to patient aversion, therefore sweetened lozenges have been manufactured (Greenspan, 1994; Millns & Martin, 1996). Nystatin use has been described in a study for treatment of *Candida* spp. infected dentures. However, it was concluded that soaking of dentures in nystatin solution was ineffective as there was a retention of high numbers of *Candida* spp. (Banting *et al.*, 1995).

Fluconazole is considered to be effective due to relatively low levels of clinical side effects as well as availability of use in both oral and systemic infections. Nevertheless, a disadvantage is emerging resistance to various non albicans species including *C. glabrata* (Pfaller & Diekema, 2004). Voriconazole is often the drug of choice for infections exhibiting fluconazole resistance, but this is primarily for systemic candidiasis (Donnelly & De Pauw, 2004). Clotrimazole and miconazole are used in topical treatments of oral candidosis, and in particular angular cheilitis, due to dual activity against *Candida* and staphylococcal spp. (Sheehan *et al.*, 1999). Studies involving the use of miconazole as a 'denture lacquer' have demonstrated an anti-candidal effect in DIS sufferers (Konsberg & Axell, 1994).

Significantly less research has been conducted in regard to the echinocandins as they are a relatively new class of antimycotics (Denning, 2003). Caspofungin is often the antifungal of choice in fluconazole resistant cases as drug safely of the

echinocandins is considered to be high due to the specificity of interaction with the fungal cell wall (Deresinski & Stevens, 2003). Caspofungin was also found to be more effective than a number of polyenes and azoles in inhibiting biofilm forming *C. albicans* strains *in vitro* (Ramage, 2010).

1.5.2 Alternative antifungal agents

- Chlorhexidine

Various studies have discussed the benefits of chlorhexidine use in both mouth washes and in denture decontamination fluids (McCourtie *et al.*, 1986). In the former, a concentration of 0.2% chlorhexidine gluconate has been reported to exhibit high inhibitory effects on *Candida* spp. as well as other oral microbes (Addy & Hunter, 1987). In contrast, a 2% solution is effective for overnight decontamination of dentures (Ellepola & Samaranayake, 2000). Chlorhexidine is not only toxic to *Candida* spp., but also negatively influences candidal adhesion to surfaces, this property is particularly useful for the treatment of dentures (Lal *et al.*, 1992). This is due to the chlorhexidine property of binding to proteins from the yeast cytoplasm which in turn results in disintegration of the cytoplasm and cell death (Russell, 1986). Overall, chlorhexidine is widely recommended by dentists for the prevention and treatment of oral candidosis (Barasch *et al.*, 2004).

- Sodium Hypochlorite

The practice of soaking dentures in sodium hypochlorite (a key compound in bleach) is recommended by some dentists. Several studies have demonstrated anti-candidal sterilising properties (Rudd *et al.*, 1984). Similar to chlorhexidine, *Candida* spp. anti-adhesive effects have also been demonstrated by soaking infected dentures in sodium hypochlorite (Webb *et al.*, 1995).

1.5.3 Use of denture cleansers

To reduce the incidence of DIS, and therefore subsequent requirement for antifungals, there is a raised importance in the practice of a good oral hygiene regimen including regular decontamination of dentures with over the counter denture cleansers (Akpan & Morgan, 2002). Although regular denture cleanser use results in maintenance of manageable *Candida* spp. levels, biofilms are not effectively disrupted. This can ultimately lead to candidal overgrowth if normal cleansing mechanisms are disrupted (Jose *et al.*, 2010).

Brushing dentures with regular toothbrushes has been suggested as a means of physically removing biofilms. However, it has been demonstrated that brushing does not adequately remove plaque from denture surfaces compared to soaking in denture cleanser (Dills *et al.*, 1988). Research has also revealed that regular use of a toothbrush upon denture surface leads to surface damage leading to irregularities (Ramage *et al.*, 2004; Charman *et al.*, 2009). As mentioned previously, imperfections in the denture surface allow increased attachment of *Candida* spp. (Charman et al., 2009).

Another method of denture cleansing utilised by certain individuals consists of rinsing dentures in water alone. Unsurprisingly this technique does not yield satisfactory effects in terms of removal and inhibition of oral microorganisms (Dills *et al.*, 1988; Srinivasan & Gulabani, 2010).

Denture cleansers are categorised as either alkaline hydrochlorites, alkaline peroxides, neutral peroxides with enzymes or enzymes (Budtz-Jorgensen, 1979). Some common use, over the counter denture cleansers are listed in Table 1.3.

Denture cleansers vary in composition and recommended time for soaking dentures. Denture cleanser solutions usually consist of a tablet dissolved in enough water to cover the entire denture surface.

Table 1.3 Over the counter denture cleansers.

DENTURE CLEANSER	CATEGORY	pH	MANUFACTURER
Polident	Neutral/alkaline peroxide	7	GlaxoSmithKline
Efferdent	Alkaline peroxide	7.5	Pfizer
Steradent	Alkaline peroxide		Reckitt Benckiser
Fittydent	Alkaline peroxide		Fittydent International GmbH

There have been many studies concentrating on the efficacy of denture cleansers in comparison to one another, and to other cleansing methods commonly used. The following highlights the most interesting studies to date.

A trend throughout the literature brings to light the effectiveness of alkaline peroxides in comparison to other categories of denture cleansers (Minagi *et al.*, 1987; Dills *et al.*, 1988; Gornitsky *et al.*, 2002). These include Polident and Efferdent (Table 1.3).

A recent study investigated the reduction in colony forming units of *Candida* spp. and *Streptococcus mutans* following treatment of dentures with overnight denture cleansers. The outcome of the research suggested that Polident was more effective at reducing both *Candida* spp. and *S. mutans* numbers compared to Efferdent denture cleanser (Gornitsky *et al.*, 2002), which is in agreement with a more recent study (Uludamar *et al.*, 2010). Another study demonstrated similar effects by both denture cleansers on *Candida* spp. biofilms grown on denture liners. Although a decrease in colony forming units was observed, viability of organisms was not sufficiently reduced which presents implications for future re-growth (Vieira *et al.*, 2010).

Hypochlorite based denture cleansers, in particular sodium hypochlorite, are also considered to be 'efficient', following studies performed using acrylic resins colonised with *C. albicans in vitro* (Montagner et al., 2009). Drawbacks of regular hyphochlorite use for the decontamination of surfaces often outweigh the benefits. Reported undesired effects include corrosive properties (Requa-Clark, 1983) as well as an after-taste present following return of dentures to patients (de Souza *et al.*, 2009).

Generally, establishment of a regular and thorough denture cleanser regimen is considered to be satisfactory for the maintenance of low levels of *Candida* spp. thereby avoiding of the development of DIS (Gornitsky *et al.*, 2002; Srinivasan & Gulabani, 2010). Indeed, a recent survey demonstrated that soaking dentures in denture cleanser was the most commonly advised method of denture decontamination, being recommended by 72% of dentists (Dikbas *et al.*, 2006).

1.6 Summary

The oral cavity provides an environment within which complex multi-species interactions can vary significantly between individuals. *Candida* species, in particular *C. albicans*, can be isolated from a large proportion of healthy and immunocompromised patients. Oropharyngeal candidosis is a common problem for individuals with impaired immunity as well as the elderly. Denture induced stomatitis, a recognised form of oral candidosis, is a significant health concern for denture wearing individuals in which inflammation of the mucosa can result in discomfort amongst other symptoms.

It is necessary to emphasise that correct diagnosis and treatment of DIS and OPC, in general, is crucial for reducing the incidence of the condition. Due to the limitations of the various treatment options outlined, the aim of this project was to concentrate on the current treatments and novel anti-candidal compounds available and how these impact on both *Candida* spp. infection and the human host.

1.7 Hypotheses

Current methods of denture care are largely ineffective at permanently inhibiting *Candida albicans* upon acrylic resin denture surfaces. This therefore leads to rapid colonisation and re-establishment of infection within the oral cavity. It was the hypotheses of this study that improved denture cleanser formulations were more effective than current oral hygiene measures used for denture decontamination, and that these may have further health benefits by reducing subsequent inflammation.

1.8 Aims of study

1. To evaluate the effects of treatment with a commercial denture cleanser upon a model system of *Candida albicans* biofilms.
2. To assess the host immune response to treated and untreated *C. albicans* biofilms.
3. To consider the impact of novel compounds for the treatment of *C. albicans* biofilms.

Chapter 2: Materials and Methods

2. Materials and Methods

C. albicans strains related to denture induced stomatitis are regularly isolated from the mucosal surface of the upper palate as well as from the denture surfaces. The following experiments were designed to investigate the role of *C. albicans* strains in relation to the pathogenesis and treatment of oral candidosis, with particular emphasis on denture induced stomatitis.

2.1 Clinical isolates

Clinical *C. albicans* isolates used in these investigations were obtained from a previous study on denture induced stomatitis within Glasgow Dental School and Hospital (Coco, 2008). All clinical isolates used in this current study were obtained from Microbead tubes (Prolab Diagnostics) stored at -80ºC. Isolates were originally obtained from denture sonicate samples and were cross referenced to clinically defined levels of inflammation, as classified by the Newton Type (NT) scale (Newton, 1962). Briefly, patients were classified as having healthy pink tissue (0), slight inflammation (NT1), reddening and pin-point localised inflammation (NT2), or diffuse chronic inflammation (NT3). The strains used for these studies are listed in Table 2.1. Strains were grouped according to which Newton's grade of inflammation was represented. Three type strains were also assessed for biofilm forming ability. Strain ATCC90028 was used as a positive control in experimentation.

One *C. albicans* strain isolated from each of the three Newton Types and one from a healthy individual were selected for use in the outlined studies following experimentation in relation to good biofilm forming ability.

Table 2.1 Newton's Type strains used for assessment of *C. albicans* biofilm formation.

Control	Baseline	NT 1	NT2	NT3
3153A	BC016	BC019	BC011	BC005
ATCC 90028	BC052	BC030	BC015	BC070
SC5314	BC053	BC031	BC055	BC071
	BC054	BC032	BC015	BC072
	BC088	BC033	BC168	BC138

2.2 Microbiological analysis of *Candida albicans* species

2.2.1 Culture and identification

Isolates were sub-cultured onto Sabouraud's dextrose agar plates (SAB [Life Technologies, UK]). These were incubated for 24 h at 37ºC. The identity of *Candida* species was validated by use of the API ID 32C (bioMérieux UK Ltd, Basingstoke) yeast identification system, a standard biochemical system for the identification of yeasts. A suspension of each isolate was prepared to a McFarland standard of 2 in sterile saline, which was then dispensed into each cupule consisting of various dehydrated carbohydrate substrates. The strip of cupules was then sealed to prevent evaporation and incubated at 30ºC for 48 h. Based on the microorganisms ability to ferment or utilise different substrates a 10-digit numerical profile was generated that could be inputted in to the Apiweb™ identification software (bioMérieux UK Ltd, Basingstoke), to inform of the likely species identity (https://apiweb.biomerieux.com/). Following confirmation of the isolates identity, strains were assessed for their ability to form biofilms.

2.2.2 Biofilm formation

A single colony of each isolate was taken from the appropriate SAB plates and inoculated into 10 mL of yeast peptone dextrose (YPD [Sigma-Aldrich, UK]) within a 50 mL centrifuge tube (BD Falcon™). The isolates were grown in an orbital incubator at 30ºC overnight at 200 rpm. Cells were then washed by centrifugation at 2000 rpm for 5 min, the resulting pellet re-suspended in sterile PBS, and cells counted in a Naeuber haemocytometer. These were then standardized to 1 x 10^6 cells/mL in RPMI (Roswell Park Memorial Institute) 1640 growth media (Sigma-Aldrich, UK). Biofilms were subsequently grown by dispensing 500 µL and 200 µL of the standardized solution to 24 and 96 well flat-bottomed microtitre plates, respectively (Corning Life Science, UK). Biofilms were then incubated overnight at 37ºC. Following biofilm formation, culture media was removed, biofilms washed with sterile PBS to remove non-adherent cells, and the biomass quantified using a crystal violet assay. Biofilms were grown for periods of 4 h (early) and 24 h (mature).

2.2.2.1 Quantification of biofilm formation

Biofilm biomass (Section 3.1.3) was assessed using a modified version of a protocol first developed by Christensen *et al*, and later modified by Kolter and O'Toole (Christensen *et al.*, 1985; O'Toole & Kolter, 1998). For each biofilm formed under specified conditions, spent culture media was removed and the biofilm washed three times with PBS. Suitable negative control wells containing no fungi were included within each microtitre plate. Biofilms were air-dried and 100 µL of 0.5% w/v crystal violet solution (Fisher Scientific) added for 5 min to permit sufficient staining. The solution was removed by carefully rinsing the biofilms under running water, until all unbound stain was removed. Biofilms were de-stained by adding 100 µL of 95% (v/v) ethanol into each well. The ethanol was gently pipetted to completely solubilise the crystal violet, and the ethanol transferred to a clean 96 well microtitre plate and absorbance read at 570 nm in a plate reader (FLUOstar Omega). A minimum of eight replicate wells were used for each test condition and

results are given as an average of the absorbance and adjusted to account for any absorbance due to CV using the negative control well readings.

2.3 Antifungal susceptibility testing

2.3.1 Denture hygiene and 'gold standard' denture care products

Polident® denture cleanser and a novel formulation of this denture cleanser; Polident® partials (courtesy of GlaxoSmithKline, Parsippany, NJ, USA) were used in this study alongside Colgate® cavity protection toothpaste (Colgate® Palmolive, USA). 'Gold standard' denture care products; Milton® sterilising tablets and Corsodyl were also assessed for biofilm inhibition properties (Section 3.1.4). Concentrations used are listed in Table 2.2 alongside novel antifungal compounds tested (Section 2.6). Antifungals were prepared according to manufacturer's instructions or prior optimisation studies.

Table 2.2 Quantity/concentrations of antifungals used in studies.

Compound	Active Ingredients	Manufacturer	Instructions
Polident® (pH 7.0)	Sodium bicarborbonate, citric acid, sodium carbonate, potassium caroate, sodium perborate, TAED, sodium benzoate, PEG-180, sodium lauryl sulfoacetate	GlaxoSmithKline, Parsippany, NJ, USA	Dissolve 1 tablet in 100 mL of distilled water and use at 40°C
Improved Polident® formulation (pH 8.6)		GlaxoSmithKline, Parsippany, NJ, USA	Dissolve 1 tablet in 100 mL of distilled water and use at 40°C
Colgate® cavity protection toothpaste	Sodium monofluorophosphate (0.15% w/v fluoride ion)	Colgate Palmolive, Guilford, Surrey, UK	Prepare a 10:90% (w/v) slurry in sterile distilled water at room temperature
Milton® sterilising tablets	Sodium dichloroisocyanurate (19.5% m/m)	Cueta Healthcare Ltd, Bournemouth, Dorset, UK	Dissolve 1 tablet in 5 L of distilled water
Corsodyl	0.2% w/v chlorhexidine digluconate, 7% v/v ethanol	GlaxoSmithKline, Brentford, Middlesex, UK	Dilute to working concentration with RPMI
Farnesol	N/A	Sigma, Gillingham, Dorset, UK	Working solutions of 600, 300 and 150 μM in RPMI
EDTA	N/A	Sigma, Gillingham, Dorset, UK	Working solutions of 500, 250 and 125 mM in RPMI

2.3.2 *Candida albicans* biofilm inhibition by treatment with Milton® sterilising tablets and Corsodyl

Early and mature biofilms of 4 NT strains were grown in triplicate in a 96 well microtitre plate as previously described. Excess media was removed and 100 µL neat solutions of Milton® sterilising tablets and 0.2% Corsodyl (Table 2.2) were used to treat biofilms for 3 min and 24 h. Solutions were subsequently removed, biofilms washed briefly with sterile PBS and an XTT assay performed to assess biofilm viability following treatment (as described in Section 2.3.4)

2.3.3 CLSI broth microdilution susceptibility testing

Antifungal testing (Section 3.3.2) to determine the minimal inhibitory concentrations (MIC) of defined compounds in planktonic *C. albicans* cells was performed using the Clinical Laboratory Standards Institute M27-A3 broth microdilution method for yeasts (CLSI, 2008). All preparations for microtitre plates were performed in a Microflow laminar flow biological safety cabinet (Bioquell UK Limited, Andover, Hants, UK). 100 µL aliquots of RPMI were dispensed into columns 2 to 10 in a 96-well microtitre plates (round-bottomed wells). 200 µL of each compound was dispensed into column 1 and then serially double diluted to give a final concentration range of 50% to 0.098% for Polident® and Polident® partials in columns 1 to 10 after the addition of yeast suspensions. Column 11 served as a positive control (no compound), and column 12 served as a negative control (media only). Because of the opacity of Colgate® dentrifice, then MIC testing could not be performed based on the visual reading of the test.

Each clinical strain was propagated on SAB agar overnight at 30^{o}C. A loopful of culture was removed and a yeast suspension prepared in sterile phosphate buffered saline (PBS: 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4 [Oxoid, Cambridge, UK]). This suspension was further diluted 1:10 in PBS to enable enumeration of cells/ml in a Neubauer haemocytometer (Fisher Scientific, UK). Each strain was subsequently adjusted to a density of 10^4 cells/mL in RPMI-1640. 100 µL of cellular suspension was added to microtitre wells containing

defined compounds, including the antifungal-free positive control. The microtitre plates were then incubated at 37°C for 24 to 48 h. The MIC was determined by directly visualising yeast growth within the microtitre plate and selecting the lowest concentration of each derivative that inhibited growth of the test isolate, compared to positive control.

2.3.4 Sessile susceptibility testing

Antifungal susceptibility testing of sessile cells was performed as previously reported (Ramage *et al.*, 2001a). Isolates were propagated from a loopful of cells from SAB agar plates and inoculated into 10 mL of yeast peptone dextrose broth (YPD [Oxoid, UK]). These were incubated at 30°C overnight in an orbital shaker at 400 rpm (Vortemp1550, Labnet International Inc, Woodbridge, NJ, USA). All strains grew in the budding–yeast phase. Cells were centrifuged at 3000 rpm for 10 min, and the pellet resuspended in 5 mL PBS. This was diluted 1:100 in PBS and enumerated as described above. All isolates were adjusted to 1×10^6 cells/ml in RPMI 1640. Biofilms were formed on commercially available pre-sterilised, polystyrene, flat-bottomed, 96-well microtitre plates (Corning, Sigma-Aldrich, UK) by pipetting standardised cell suspensions (200 μl of the 1×10^6 cells/mL) into each well of the microtitre plate (final inoculum of 2×10^5 cells/well) and incubating for 24 h at 37°C. Following biofilm growth, media was removed and antifungal components serially double-diluted to give a final concentration range of 50%-0.098% for Polident®, Polident® partials and Colgate® within the microtitre plate. These were incubated at 37°C for 24 h. Antifungal-free wells and biofilm-free wells served as positive and negative controls, respectively. Sessile minimal fungicidal concentrations ($SMFC_{50}$ and $SMFC_{80}$) were determined as the lowest concentration of antifungal giving a 50% or 80% reduction in biofilm metabolic activity relative to the antifungal-free control, using the XTT reduction assay, as described below (Pierce *et al.*, 2008; Ramage *et al.*, 2001a; Ramage & Lopez-Ribot, 2005). Testing of these isolates was performed in duplicate.

2.3.5 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT)-reduction assay

A semi-quantitative measure of biofilm formation was calculated using an XTT-reduction assay, adapted from previous studies (Pierce *et al.*, 2008). Briefly, XTT (Sigma-Aldrich) was prepared in a saturated solution at 0.5 g/L in PBS. The solution was filter sterilised through 22 µm-pore size filter, aliquoted, and stored at -80^{o}C. Prior to each assay, an aliquot of stock XTT was thawed, and menadione (Sigma-Aldrich, 10 mM prepared in acetone) added to a final concentration of 10 µM. A 100 µL aliquot of XTT/menadione solution was then added to each pre-washed biofilm, and to negative control wells containing no *C. albicans* to measure background XTT-reduction levels. The plates were then incubated in the dark for 3 h at 37^{o}C and colour change measured using a microtitre plate reader (FluoStar Optima, BMG Labtech, Basingstoke, UK) at 490 nm. A minimum of eight replicate wells were used for each test condition and absorbance results averaged and adjusted to correct for any absorbance of XTT/menadione using the negative control well readings. The colorimetric change in the XTT-reduction assay directly correlates with the metabolic activity of the biofilm as previously described (Ramage *et al.*, 2001a).

2.3.6 Time kill kinetics

C. albicans killing efficacy of the two Polident® denture cleansers and Colgate® toothpaste (described fully in Section 2.4) was assessed over a set time period (Section 3.3.5). Initially overnight biofilms of the 4 NT strains were grown in 96 well microtitre plates as described previously. Excess media was discarded and 100 µL of each of the above solutions (Table 2.1) were allowed to interact with biofilms for time points of 30 s, 1 min, 3 min, 5 min and 10 min. Solutions were subsequently removed and biofilms washed briefly with sterile PBS. An XTT assay was conducted to assess biofilm viability following treatment.

2.4 Treatment of *Candida albicans* biofilm with Polident® denture cleansers and Colgate® toothpaste

Results are described in Section 3.3. Two commercially available denture cleansers with different optimal pH's of 7.0 and 8.6 (Polident®, GlaxoSmithKline, USA) and a commercially available toothpaste (Colgate Cavity Protection Toothpaste, Colgate® Palmolive, USA) were used throughout the study. Each *C. albicans* isolate were inoculated onto 1 cm^2 acrylic resin sections within a 24 well tissue culture plate (Costar®, Corning Inc, MA, USA) at an optimised concentration of 1 x 10^6 cells/mL in RPMI for 4 h at 37ºC, as previously described (Ramage *et al.*, 2001b), prior to a series of treatment regimens. These sections were initially treated after 4 h with the denture cleansers and sequentially thereafter at 24, 48 and 72 h. Each 1 cm^2 section was treated by immersing in denture cleanser solution for 3 min using sterile forceps, which were removed and gently washed in sterile PBS to remove excess denture cleanser solution. Following treatment, sections were transferred to bijou tubes (Sterilin Ltd, UK) containing 1 mL of sterile PBS, and sonicated at 35 kHz for 5 min (Ultrasonic bath [Fisherbrand, Fisher Scientific, UK]). Total viable counts were then quantified using the Miles and Misra plate counting technique onto SAB agar plates. In parallel, total viable counts were quantified on sections with no treatment at each time point (control) and on sections treated and re-incubated in RPMI at 37ºC overnight prior to the next treatment phase to assess regrowth. All experiments were performed on all strains at least two separate occasions and in triplicate.

Intermittent studies were also performed to assess how brushing with toothpaste impacted denture cleansing. These experiments were identical to those described above, but at 24 h and 48 h the sections were brushed with a 10% w/v slurry of toothpaste (25ºC) in ddH_2O for approximately 2 s using a soft bristle toothbrush (Oral-B ®, Proctor and Gamble, USA). The 2 s time was calculated based on the proportional surface area of an upper denture being brushed for an average of 2 min. All sections were processed as described above, including appropriate controls.

2.4.1 Visualisation of treated biofilms by scanning electron microscopy (SEM)

An early *C. albicans* (NT3) biofilm was grown on denture material sections (GlaxoSmithKline, USA). Biofilms either remained untreated or were treated with Polident®, Polident® followed by brushing with toothbrush, Polident® partials or Colgate® followed by brushing, as described above. Preparation for SEM was conducted as previously outlined (Erlandsen *et al.*, 2004). Following treatment, sections were transferred to 24 well plates and covered with 500 µL of fixative (2% v/v para-formaldehyde, 2% v/v gluteraldehyde, 0.15M sodium cacodylate and 0.15% w/v Alcian Blue). Sections were fixed for 2 h, fixative removed and sections rinsed in 0.15M sodium cacodylate ready for processing. Sections were then rinsed a further three times for 5 min with sodium cacodylate. Following this, sections were incubated in 1% osmium tetroxide for 1 h and washed three times for 10 min in distilled water. Sections were then incubated in uranyl aceytate aqueous solution in the dark for 1 h following by a series of dehydration steps in alcohol: 30%, 50%, 70%, 90% and absolute alcohol for 10 min/alcohol, excluding absolute alcohol in which sections remained for 2 x 10 min. Sections were dried twice for 5 min in hexamethyldisilazane (HMDS) in a dessicator before mounting on aluminium stubs with double sided copper tape. Samples were covered by painting with silver paint, using fine brush to increase conductivity. Finally, sections were covered by gold sputter coating and viewed using a JEOL JSM 6400 scanning electron microscope.

2.5 Manufacture of denture material sections

Bespoke denture material sections were manufactured for use in cell culture studies. There was a requirement for thin (< 0.5 mm) sections for use in the epithelial cell/biofilm model outlined in Figure 2.3. All other studies employed the 1cm^2 denture material sections provided by GlaxoSmithKline.

The mould for the formation of denture material sections was formed using a 1 mm thick PETG polyethyleneterephthalate – glycol modified/ Ethylene 1, 4-cycloexylene

terepthalate copolymer disc. The disc dimension was cut to 5 cm^2 and scored into 10 mm x 10 mm squares approximately 0.5 mm deep (5 x 5 or 25 sections).

2.5.1 Formation of dental stone

The mould was made using a two-part denture flask. Dental stone (Super yellow, John Winter & Co Ltd, UK) was mixed with water to a ratio of 100 g powder to 20mL water and vacuum mixed to avoid air entrapment in the mixture. The stone mix was placed into the shallow half of the denture flask and the PETG disk placed onto this having previously been covered with a wetted film of the dental stone mix as demonstrated in Figure 2.1. The mix was allowed to set. A separating medium (50% Sodium Silicate solution) was added to the set dental stone surface and rinsed off. The mould was completed by filling the second part of the flask with a second dental stone mixture spread on top of the PETG sheet, followed by the flask being positioned in place with the remainder of the dental stone mix poured to fill the mould through the top of the flask. During this process the denture flask was placed on a vibrating platform to ensure any air bubbles were removed from the surface of the PETG sheet. The dental stone was allowed to set for 1 h.

Figure 2.1 PETG sheet placed in dental stone mix to create mould.

2.5.2 Preparation of denture acrylics

The PMMA acrylic was mixed according to manufacturer's instructions. 24 g of power was added to 10 mL of denture base liquid (Chaperlin & Jacobs Ltd, UK) and thoroughly mixed together for 30 s then left for approximately 10 min until a snap-dough texture was achieved. To facilitate opening, the denture flask containing the mould was immersed in boiling water for 10 min. Following opening of the flask; the PETG disc was removed and the mould cleaned with water and detergent to remove any possible greasy residue which would prevent the mould being coated with a sealant. A mould seal isolating liquid (Metrodent, UK) was painted on the surface of the mould halves and allowed to dry. The acrylic dough was placed on one half of the mould and pressed by hand. A cellophane sheet was placed on top of the dough and the flask lid (second half of the mould) secured on top.

The flask mould containing the acrylic was then compressed in a hydraulic press at 100 bar pressure which allowed the excess acrylic to escape from the sides. The flask was opened and the cellophane sheet removed before a final closure of the flask as observed in Figure 2.2. The flask was placed into a spring compress to maintain a continual pressure during the processing procedure.

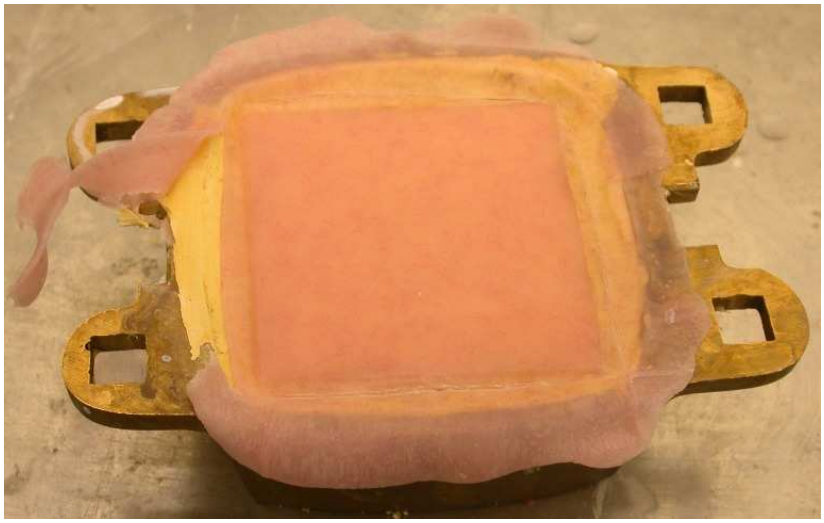


Figure 2.2 Packing of PMMA acrylic.

The compress was placed in a dry heat bath for 9 h in accordance with the manufacturer's instructions (initially the temperature is taken up to 70ºC over a seven hour period). The temperature was then increased to 100ºC and this temperature is maintained for 3 h to ensure removal of any residual monomer.
Following polymerisation and cooling of the flask to room temperature, the flask was removed from the compress and opened. The processed acrylic disc was cut into 1 cm sections and filed to ensure a smooth finish.

2.6 Novel antimicrobial compounds

Results described in Section 3.4. Due to ineffective denture care products; non-oral hygiene compounds were tested for fungal inhibition. These included EDTA; a cation chelator which has previously been documented as having an effect on *C. albicans* morphology from yeast to hyphal structure (Ramage *et al.*, 2007) and farnesol; a quorum sensing molecule associated with the yeast morphotype of *C. albicans* (Ramage *et al.*, 2002b).

2.6.1 *Candida albicans* biofilm treatment with Farnesol and EDTA

Mature *C. albicans* biofilms of 4 NT strains were grown in triplicate in 96 well microtitre plates as previously described. Excess media was removed and 100 µL of each solution at decreasing doubling concentrations (Farnesol at 600-150 µM and EDTA at 500-125 mM). Polident® denture cleanser at 100-25% was also used as a direct comparison to treat biofilms. Following a 4 h treatment, solutions were removed and biofilms washed with repeatedly with sterile PBS. An XTT assay was conducted to assess strain viability following treatment.

2.6.2 Inhibition of *Candida albicans* by pre-coating of substrates with antimicrobials

96 well microtitre plates and PMMA denture material sections were coated with 100 µL of farnesol, EDTA and Polident®. Antimicrobials were allowed to evaporate from surfaces over a 24 h period. Subsequently, mature biofilms of NT strains were grown in triplicate in 96 well microtitre plates as well as on PMMA denture material sections coated with antimicrobials as previously described. A positive control consisted of mature NT III biofilms grown without prior pre-coating. To assess biofilm viability following treatment of surface compared to positive control; an XTT assay was conducted in 96 well microtitre plates and a colony forming unit count was conducted following ultra sonication of denture material sections containing biofilms as described in section 2.3.7.

Biofilm formation following coating of 96 well microtitre plates with antifungal substrates was also assessed through utilisation of the crystal violet staining method described in section 2.2.2.1. Briefly, mature biofilms were air dried and stained with 0.5% w/v crystal violet solution, rinsed under running water and de-stained with 95% ethanol. Absorbance was read at 570 nm.

2.7 Cell Culture

Described in Section 3.5. Epithelial cells (OKF6-/TERT2) were grown as adherent monolayers in 5% CO_2, 37^{o}C. All culture media and supplements were obtained from Invitrogen, UK.

2.7.1 OKF6/TERT2 immortalised oral epithelial cell line

OKF6/TERT2 cells were provided by Rheinwald laboratory (Brigham and Woman's Hospital, Boston). This keratinocyte cell line was immortalised through forced expression of telomerase. OKF6/TERT2 cells have been shown to resemble primary oral keratinocytes in cytokine induction and toxicity studies (Dongari-Bagtzoglou & Kashleva, 2003). Cells were cultured in keratinocyte serum free medium (K-SFM, [10725 Invitrogen, UK]), supplemented with 10,000 units penicillin and 10 mg streptomycin per mL in 0.9% NaCl, 25 μg/ mL bovine pituitary extract (BPE), 0.2 ng/ mL epidermal growth factor (EGF) and 0.2 mM $CaCl_2$ (0.4 mM total Ca^{++}). BPE and EGF were filter-sterilised through 0.2 μm filter pore. Cells were seeded at 1 x 10^5/mL in a 75 cm^2 flask (Corning Life Sciences, NY). Cells were allowed to reach 90-100% confluence before passage. This was achieved by removing K-SFM and washing cells with 10 mL of PBS (D8662, Sigma-Aldrich, UK). 5mL 0.05% trypsin + EDTA solution (13924, Sigma-Aldrich, UK) was added to detach cells from flask. Trypsin was allowed to act for ~ 5 min and cells were observed under light microscopy to ensure full detachment. The trypsin was neutralised by addition of 15 mL of Dulbecco's Modified Eagle Medium [(DMEM, D8662 Sigma-Aldrich, UK)] and centrifuged for 5 min at 1000 rpm. The resulting cell pellet was resuspended in 10 mL Hank's Balanced Salt Solution (H9269, Sigma-Aldrich, UK) and centrifuged as above. The resulting pellet was resuspended in K-SFM and live cells counted by

staining with trypan blue solution (0.4%) (Sigma-Aldrich, UK). To assess cytokine responses cells were seeded in a total of 1.5 mL at a density of 1 x 10^5 cells of defined keratinocyte free medium (DK-SFM, 10785 [Invitrogen, UK]) in a 24 well culture plate. This media contained 0.2 mM $CaCl_2$ (0.4 mM total Ca^{++}) and DKSFM growth supplement (10784, Invitrogen, UK).

2.7.1.1 Stimulation of OKF6/TERT2 cells

To assess the response of OKF6/TERT2 cells to various stimulants, IL-8 protein release was quantified using ELISA technology. Studies were designed to assess the effect of both planktonic and sessile *C. albicans* on IL-8 release, and how antifungal treatment impacted upon this. *C. albicans* strain BC071 (NT3) was used for all these experiments. OKF6/TERT2 cells were grown in 24 well plates at 1 x 10^5 cells/mL until 90-100% confluent. These were then stimulated with planktonic *C. albicans* at concentrations of 1 x 10^5 and 1 x 10^6 cells/mL. Treatment of planktonic *C. albicans* cells consisted of cells being initially centrifuged at 3000 rpm for 10 min, and the pellet resuspended in 1 mL of neat Polident ® for 3 min. Cells were washed with sterile PBS and centrifuged to remove excess denture cleanser. This process was repeated in triplicate. Resulting cells were used for OKF6/TERT cell stimulations. Early (4 h) and mature (24 h) biofilms were grown on manufactured denture material sections, as described above (see section 2.3). Biofilms were treated with Polident ® by immersion of denture material sections containing early and mature biofilms in denture cleanser for 3 min. Sections were then repeatedly dipped in sterile PBS to remove excess denture cleanser. Sections containing treated and untreated biofilms were attached to hanging inserts with sterile Vaseline® as shown in Figure 2.3. Inserts were then placed into wells containing epithelial cells. This model allowed a 0.5 mm gap between the biofilm and the cells and avoided direct media starvation of the epithelial cells.

Zymosan A (zym) from *S. cerevisiae* cell wall was used as a positive control for IL-8 release. OKF6/TERT2 cells were stimulated for 4 and 24 h in 5% CO_2 at 37^{o}C. Cell supernatants were then removed and used to perform IL-8 ELISAs while OKF6/TERT cells were used for gene expression analysis.

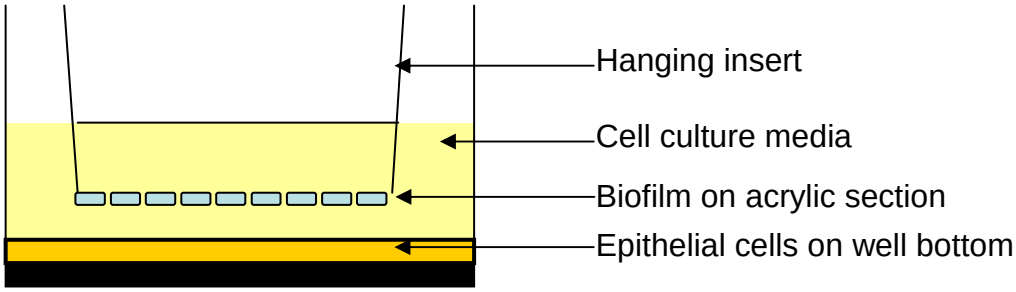


Figure 2.3 *In vitro* model of *Candida* biofilm interaction with epithelial cell layer.

2.7.1.2 Analysis of gene expression

OKF6/TERT2 gene expression was measured through quantitative reverse transcription polymerase chain reaction (QRT-PCR). This method allowed rapid and specific identification of up/down regulation of IL-8 gene expression in response to the previously stated stimulants.

2.7.1.2.1 RNA extraction, quantification and DNA synthesis

Following the removal of cell supernatants, RNA was extracted from OKF6/TERT2 cells through the use of the RNeasy mini kit (Qiagen, UK), according to manufacturer's instructions. The RNA obtained was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Scientific). 1.5 µL of RNA from each sample was loaded onto NanoDrop pedestal. The resultant RNA was of high quality, approximating 2.0 (260/280). cDNA synthesis was achieved using SuperScript® II Reverse Transcriptase (Invitrogen, UK).

2.7.1.2.2 Real time quantitative PCR

Expression levels of IL-8 were measured in relation to the house keeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The thermal profile was optimised for primers used (Tables 2.3 and 2.4). Each well contained 12.5 µL of

SYBR green master mix (Invitrogen, UK), 0.5 µL of forward and 0.5 µL of reverse primers, 2 µL of cDNA and 9.5 µL of DNase/RNase free water (Qiagen, UK) to make up a total volume of 25 µL.

The mean efficiencies were determined in the sample triplicates and used to adjust the cycle threshold (Ct) values. Ct values were used for the quantitative comparison of the amplification rates. After baseline subtraction, the mean Ct values of the triplicates were determined and transformed into relative quantities.

Table 2.3 Primers used in the study. A) Primers were chosen in line with previous literature. B) Primer conditions; including temperature and number of cycles.

A)

Target	**Primer**	**Reference**
IL-8 Forward	TTAGCACTCCTTGGCAAAAC	(Li *et al.*, 2007a)
IL-8 Reverse	CAGAGACAGCAGAGCACACAA	
GAPDH Inner 1	CAAGGCTGAGAACGGGAAG	(McKimmie *et al.*, 2008)
GAPDH Inner 2	GGTGGTGAAGACGCCAGT	

B)

Function	**Temperature (°C)**	**Time**	**Cycles**
Denaturation	55	2 min	1
Denaturation	95	10 min	40
Annealing	95	30 s	
Extension	59	1 min	
Extension	72	1 min	1

2.7.1.3 IL-8 Cytokine Assay

ELISA kits (BioSource, Invitrogen, UK) were used in accordance with the manufacturer's instructions. Buffers used are listed in Table 2.5. All assays were optimised and validated prior to use. An Immulon 4 HBX flat-bottom 96-well microtitre plate (Fisher Scientific, UK) was coated with coating buffer, sealed and incubated overnight at 4ºC. Between steps, the plate was washed 5 times with wash buffer to remove reagents and unbound antibody. 100 µL of standards ranging from 2000-31.25 pg/mL and samples of cell supernatants were added to wells in duplicate or triplicate. The final colour change from clear to bright blue was measured after the addition of TMB substrate at an absorbance of 450 nm (FLUOstar Omega, BMG LabTech). The results were calculated using a 4-parameter curve fit.

Table 2.4: Buffers used for IL-8 ELISA.

Coating Buffer	0.1 M $NaHCO_3$ (pH 8.2)
Assay Buffer	5 g BSA and 1 mL Tween 20/1L PBS (pH 7.4)
Wash Buffer	500 µL Tween 20/1L PBS (pH 7.4)

Chapter 3: Results

3. Results

3.1 Phenotypic characterisation of NT strains

3.1.1 Introduction

An important characteristic of the opportunistic pathogen *C. albicans* is its ability to exist in both yeast and hyphae forms. These are both important in the development and maintenance of biofilm consortia (Bachmann *et al.*, 2003). Biofilm formation of *C. albicans* strains is associated with the ability to successfully colonise surfaces and resist removal through chemical and physical cleansing (Ramage *et al.*, 2004). Other pathogenic traits of the species include the production of degradative enzymes, which are associated with virulence. A correlation between the production of these enzymes and biofilm formation by *C. albicans* strains has been recently demonstrated (Rajendran *et al.*, 2010).

The aim of this component of the study was to characterise the biofilm properties of *C. albicans* clinical strains isolated from denture stomatitis patients. This included biofilm formation and their sensitivity to commonly used antimicrobial agents used to control oral candidosis and disinfect dentures. Four strains representing each of Newton's Type's of inflammation of the palate (BC052 [0], BC030 [I], BC015 [II] and BC071 [III]) were used for these studies, including an American Type Culture Collection strain (ATCC 90028) as a positive biofilm control.

3.1.2 Planktonic growth

Growth rates of planktonic cells were assessed in order to demonstrate that potential differences in biofilm formation or sensitivity were not related to restricted growth kinetics, and could therefore be compared equally. All strains reached exponential and lag phase at a similar rate (Figure 3.1). Rates of exponential growth were as follows: 0.33 O.D/h (ATCC 90028), 0.36 O.D/h (BC052), 0.41 O.D/h (BC030) and 0.48 O.D/h (BC015 and BC071). NT I strain BC030 was shown to reach exponential phase at the quickest rate after approximately 5 h.

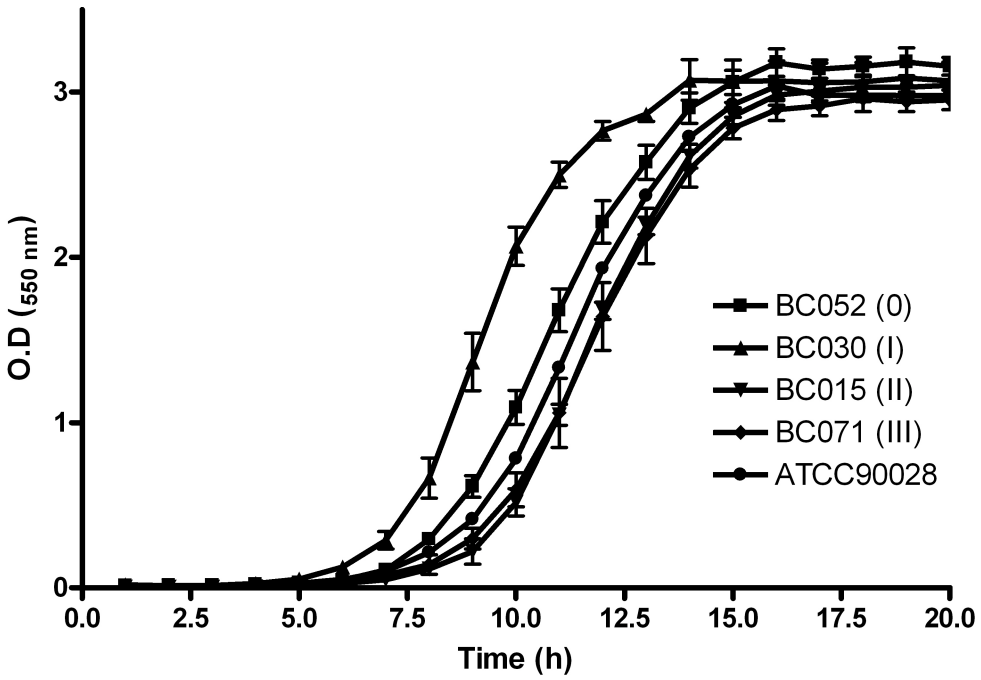


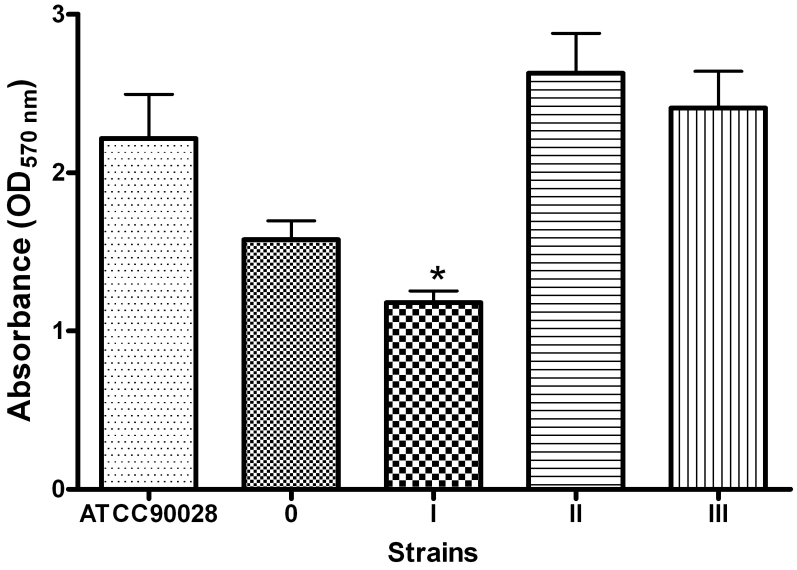
Figure 3.1: Planktonic *Candida albicans* NT strains grow at comparable rates. NT strains BC052 (0), BC030 (I), BC015 (II) and BC071 (III) and type strain ATCC90028 were grown in planktonic form for 20 h in a round bottomed 96 well microtitre plate in RPMI from an initial concentration of 1 x 10^4 cells/mL.

3.1.3 Biofilm formation

3.1.3.1 Assessment of biofilm biomass

Biofilm formation was shown to vary between strains as illustrated in Figure 3.2. NT strains 0, II and III formed stable biofilms covering the well (Figure 3.2B). Statistically poorer biofilm formation was observed with NT strain I compared to ATCC90028 ($p < 0.05$).

A



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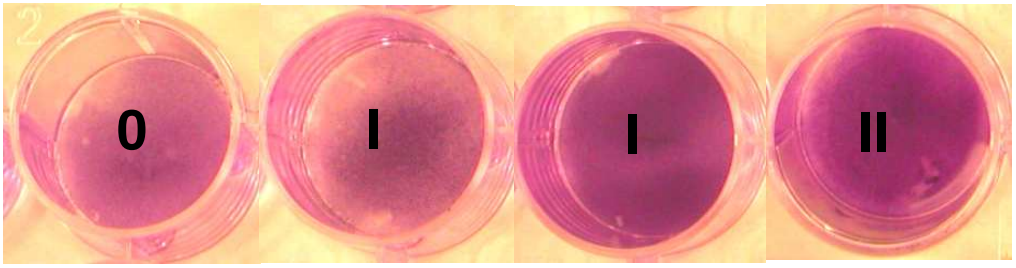


Figure 3.2: The biofilm forming ability of *Candida albicans* NT biofilms is variable A) Absorbance readings of biofilm biomass. B) Visual representation of NT strain biofilms. Biofilms were formed in microtitre plates by standardisation of planktonic cells to a concentration of 1 x 10^6 cells/mL in RPMI and subsequent incubation at 37°C overnight. Biofilms were dried, stained with 0.5% w/v crystal violet, washed and de-stained with 100% ethanol. Absorbance was measured at 570 nm.

3.1.3.2 Assessment of Candida albicans metabolism

Assessment of biomass does not provide an indication of *C. albicans* viability. Therefore, biofilm viability studies (Section 2.3.5 2) were performed to assess whether there was a variation in metabolic activity in NT biofilms. Metabolic activity of mature biofilms formed by different NT strains was assessed using the XTT assay (Figure 3.3). A significantly lower ($p = < 0.01$) *C. albicans* metabolic activity was observed for NT strains 1 and 3 compared to control strain ATCC90028. Variation in biofilm metabolism was observed independent of biofilm biomass. This suggests that metabolic assays do not necessarily reflect good biofilm formation.

Following the variation of *C. albicans* metabolism observed in Figure 3.3 a study was designed to determine the sensitivity of the XTT assay to *C. albicans* viability (Figure 3.4). Depending on sensitivity levels, this assay would then be further utilised for investigations relating to treatment of *C. albicans* cells with antimicrobial compounds. Metabolic activity of NT *C. albicans* planktonic cells ranging from 1×10^1 – 1×10^8 cells/mL was quantified. Linear regression analysis indicated that there was a positive correlation between cell concentrations and absorbance ($R^2 = 0.8133$). The assay did not detect cell concentrations $<1 \times 10^5$ cells/mL. These results present implications for future viability assays as *C. albicans* cell numbers of $< 1 \times 10^5$ cells/mL cannot be detected.

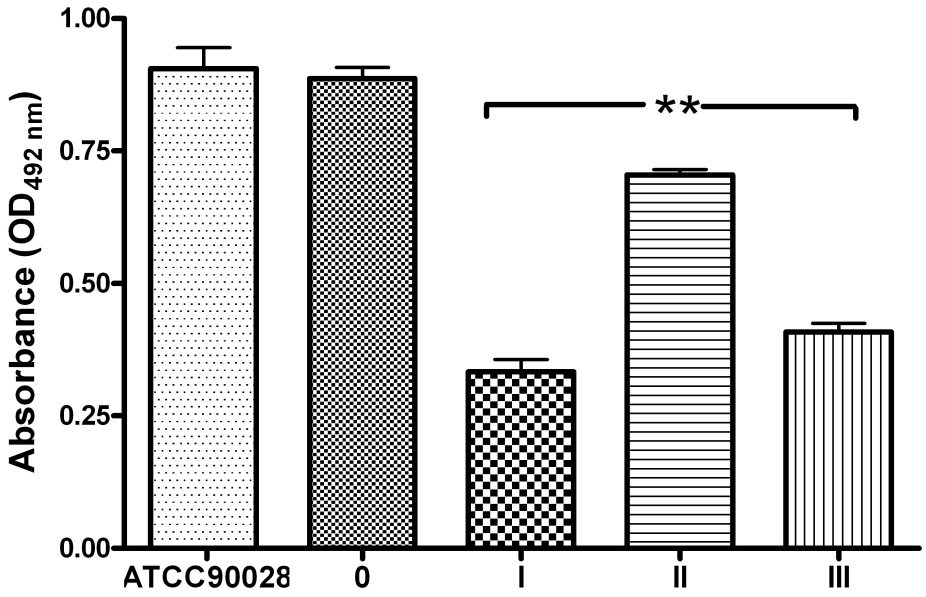


Figure 3.3: The metabolic activity of *Candida albicans* NT biofilms is variable. Biofilms were formed in microtitre plates by standardisation of planktonic cells to a concentration of 1 x 10^6 cells/mL in RPMI and subsequent incubation at 37 ºC overnight. The XTT assay was performed and absorbance measured at 492 nm.

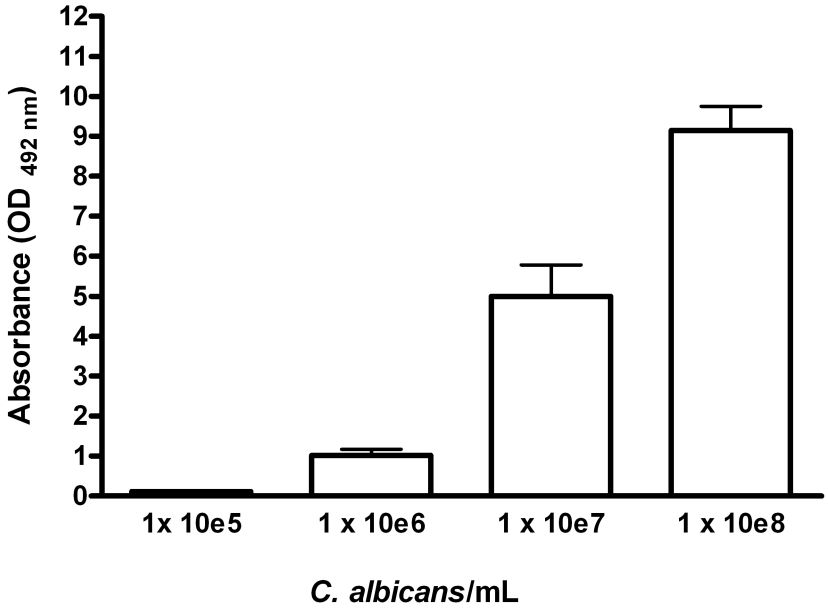


Figure 3.4: The XTT assay has a dynamic but limited sensitivity to *Candida albicans* metabolism. *C. albicans* planktonic cells were serial diluted in PBS to give a concentration range of 1 x 10^1 – 1 x 10^8 cells/mL. Cells were centrifuged and pellets re-suspended in XTT. Following a 3 h incubation at 37ºC, absorbance was measured at 492 nm.

3.1.4 Rapid *in vitro* assessment of 'gold standard' denture care products against *Candida albicans* biofilms

3.1.4.1 Introduction

Lasting treatment of denture stomatitis often requires a number of factors, including establishment of an appropriate oral hygiene regimen as well as daily treatment of dentures to reduce the possibility of re-establishment of infection. 'Gold standard' treatments recommended by dental practitioners for the decontamination of dentures include soaking dentures in solutions containing chlorhexidine (Pusateri *et al.*, 2009b; Redding *et al.*, 2009) or Milton® sanitizer (Clarkson *et al.*, 2003).

3.1.4.2 Biofilm treatment with Milton® sterilising tablets and Corsodyl

These studies aimed to assess biofilm inhibition through the use of products recommended by dental professionals for the treatment of oral candidosis. Milton sterilising tablets (sodium dichloroisocyanurate [19.5% m/m]) and Corsodyl (chlorhexidine [0.2%]) were used to treat early and mature NT biofilms.

Results indicate that both Milton® and Corsodyl completely eradicate early and mature biofilms after a 24 h treatment. However, a 3 min treatment with both products resulted in >90% inhibition of early biofilms (4 h). A disparity in treatment efficacy between the two products was observed following 3 min treatment of mature biofilms. Corsodyl treatment was significantly ($p = 0.0113$) more effective than treatment with Milton® sterilising tablets at reducing *C. albicans* viability; 24 h biofilms were inhibited by >40% and >80% by Milton® and Corsodyl, respectively, compared to untreated control.

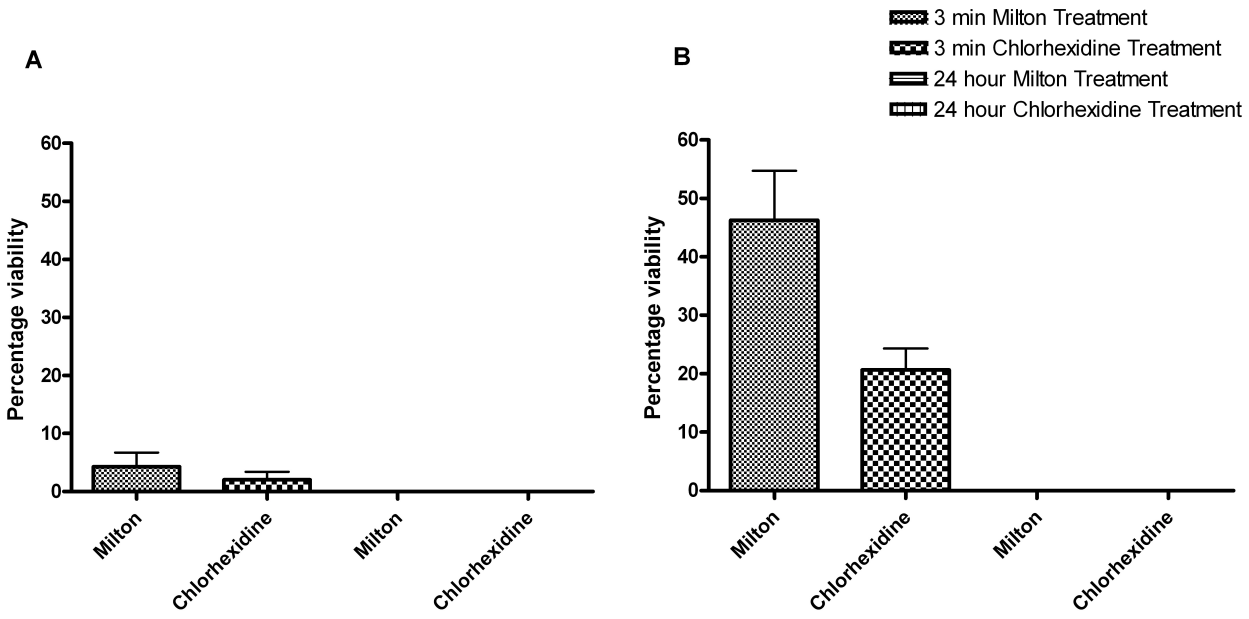


Figure 3.5: Reduction in *Candida albicans* viability after treatment of NT biofilms with Milton® Sterilising Tablets and Corsodyl. Biofilms of NT strains were grown for (A) 4 and (B) 24 h in 96 well microtitre plates before treatment with Milton® sterilising tablets (sodium dichloroisocyanurate [19.5% m/m]) and Corsodyl (chlorhexidine 0.2%) for 3 min and 24 h.

3.2 *Candida albicans* biofilm formation on denture acrylic

3.2.1 Introduction

Long standing evidence indicates that *C. albicans* adheres to and forms biofilms on a variety of surfaces. These include various medical devices as well as oral prosthesis (Kojic & Darouiche, 2004; Nevzatoglu *et al.*, 2007). Candidal colonisation of dentures leads to the onset of denture induced stomatitis which ranges in severity of symptoms (Coco *et al.*, 2008; Ramage *et al.*, 2004).

Since the study of biofilm formation on whole dentures is impractical and inherently problematic, it was decided that biofilm investigations were conducted on acrylic denture material sections and in 96 well microtitre plates when high throughput analysis was required.

3.2.2 Optimisation of *Candida albicans* cell concentration for biofilm growth on denture material

C. albicans cell concentrations of 1 x 10^6 cells/mL are used throughout the literature as the standard for biofilm formation (Ramage *et al.*, 2001a). However, this is optimised for the 96 well format and may not be applicable to denture material studies. Therefore, biofilm formation of *C. albicans* on 1 cm^2 denture material sections was initially assessed using both 1 x 10^6 and 1 x 10^7 cells/mL, as illustrated in Figure 3.6. Due to experimental constraints during optimisation NT III strain BC071 was used as it was previously demonstrated that this isolate formed stable biofilms (Figure 3.2). 24 h biofilms were grown from both concentrations on pre-sterilised denture material sections. Although there was a significant difference between biofilm growth of the two concentrations after 24 h (***p= 0.0022), inoculation of sections with 1 x 10^7 cells/mL of *C. albicans* resulted in an 80% decrease in cell concentration from initial inocula while inoculation with 1 x 10^6 cells/mL resulted in an overall 16% increase in final cell concentration. Following this result concentrations of 1 x 10^6 cells/mL were used for the remainder of studies.

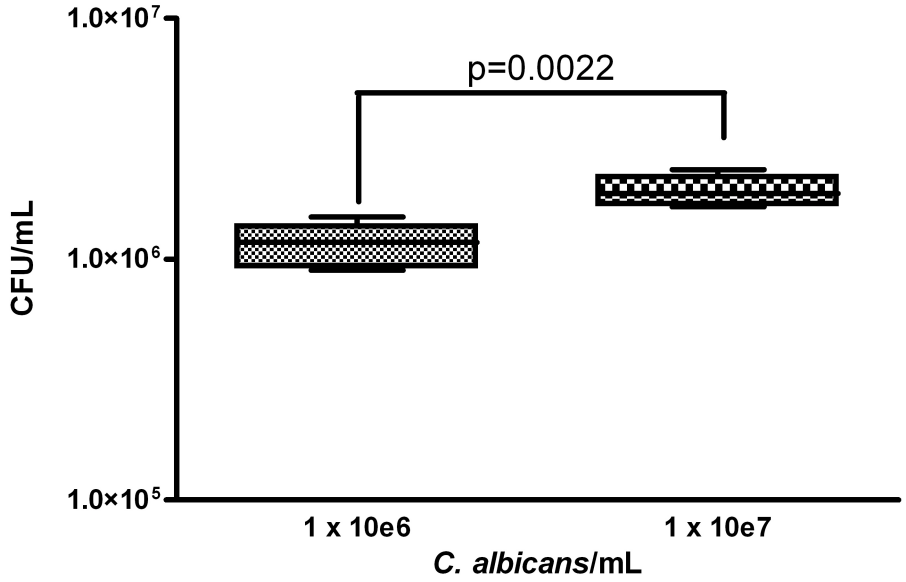
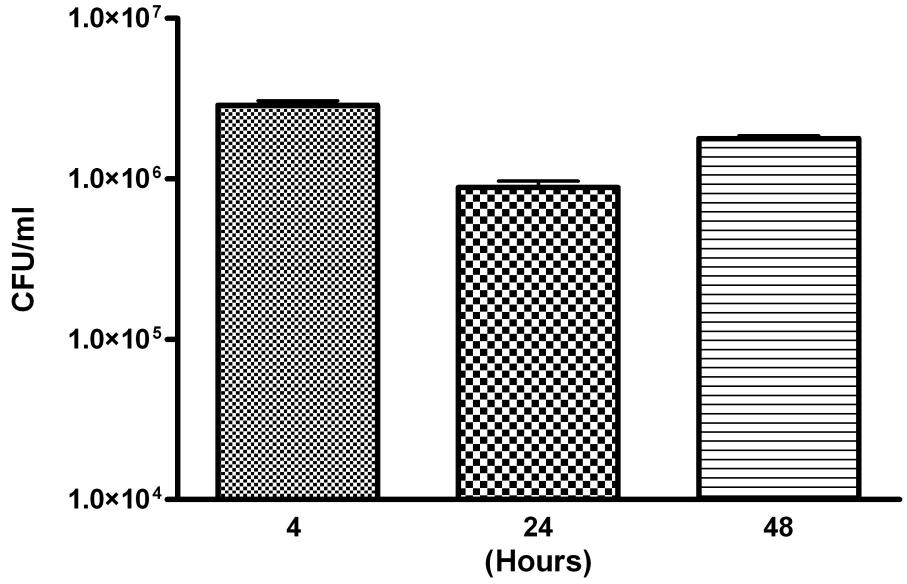


Figure 3.6: Optimisation of *Candida albicans* cell concentration for biofilm formation on denture material. Strain BC071 (III) at concentrations of 1 x 10^6 and 1 x 10^7 cells/ mL was used to form biofilms on 1 cm^2 denture material sections in RPMI at 37^{o}C. After 24 h, viable *C. albicans* cells were assessed through sonication of denture material sections and conducting colony forming unit counts. A significant difference (***p=0.0022) between biofilm growth of the two concentrations was observed. Ultimately, an initial inoculum of 1 x 10^6 cells/mL resulted in a 16% increase in cell concentration after 24 h compared to an 80% decrease in cell concentration following an initial inoculum of 1 x 10^7 cells/mL.

3.2.3 Time kinetics of biofilm formation

Growth of NT III strain biofilms on acrylic sections was investigated over a defined time period (4, 24, 48 h). The Miles and Misra technique for conducting colony counts was utilised and *C. albicans* cell concentrations/mL were calculated as demonstrated in Figure 3.7. Biofilm viability remained moderately constant. Mean CFU's/mL were as follows: 2.88 $x10^6$, 8.83 x 10^5 and 1.78 x 10^6 cells/mL for each time point, respectively (Figure 3.7a). Scanning electron microscopy images taken at the above time points indicate an increase in biofilm biomass with time, showing a progression from early hyphae formation to mature biofilm, but this did not significantly impact the colony counts (Figure 3.7b)

A



B

i) ii) iii)

Figure 3.7: Sustained biofilm growth for 48 hours on denture material sections. A) Colony forming units of *C. albicans* from sonicated sections. B) SEM images of biofilms on sections after i) 4 ii) 24 and iii) 48 h. Biofilms of strain BC071 (III) were grown on denture material sections for a total of 48 h in RPMI at 37°C. Biofilm containing sections were sonicated after 4, 24 and 48 h and colony forming units counted. Levels of *C. albicans* viability on sections remained stable independent of time. In contrast SEM images indicate hyphal progression after 4 h which can be visualised as a denser biofilm with many yeast and hyphal cells after 24 and 48 h.

3.3 Effect of denture cleansers on *Candida albicans* biofilms

3.3.1 Introduction

Conventional denture cleansing techniques comprise of soaking dentures in an over-the-counter denture cleanser solution or brushing with dentifrice. Recent evidence has shown that this second method should not be minimised due to physical damage to dentures caused by brushing, which subsequently facilitates attachment of *Candida* species (Charman *et al.*, 2009). In contrast, denture cleansers are highly effective in inhibiting *C. albicans* biofilms, although retention of biofilm biomass on the denture surface leads to potential regrowth (Jose *et al.*, 2010). These studies were designed to assess two commercial denture cleansers in comparison to brushing with commercially available dentifrice to determine their impact on biofilms and candidal retention, and to assess intermittent use of a denture cleanser with brushing with dentifrice.

3.3.2 Planktonic and sessile minimum inhibition concentration (MIC)

MICs of the two denture cleansers and dentifrice were established for both planktonic and sessile *C. albicans* NT strains as well as type strain ATCC 90028 as described in Table 3.1. The MIC for planktonic cells did not differ when incubated with either of the Polident® denture cleansers. In comparison, the MIC for sessile cells was decreased by one dilution from 50% to 25% by treatment with novel formulation of the Polident® (pH 8.6) denture cleanser. Both Polident® denture cleansers were more effective than Colgate® dentifrice against planktonic cells. All strains showed equivalent sensitivity under each experimental parameter.

Table 3.1 Decrease in planktonic and sessile MIC with denture cleansers in comparison to dentifrice. Planktonic and sessile MICs were conducted for 4 NT strains and type strain ATCC 90028 treated with two denture cleansers and dentifrice using CLSI and biofilm sensitivity testing (Ramage *et al.*, 2001a). The planktonic MIC with both denture cleansers remained the same while the sessile MIC was reduced from 50% to 25% following treatment with Polident® (pH 8.6). No strain differences were observed.

Strain	POLIDENT® (pH 7.0)		POLIDENT® (pH 8.6)		COLGATE®	
	PMIC	SMIC	PMIC	SMIC	PMIC	SMIC
ATCC90028 (C)	6.25%	50%	6.25%	25%	25%	50%
BC052 (NT0)	6.25%	50%	6.25%	25%	25%	50%
BC030 (NT1)	6.25%	50%	6.25%	25%	25%	50%
BC015 (NTII)	6.25%	50%	6.25%	25%	25%	50%
BC071 (NTIII)	6.25%	50%	6.25%	25%	25%	50%

3.3.3 Preliminary assessment of denture cleanser (pH 7.0) and dentifrice treatment on *Candida albicans* biofilms decontamination

Early biofilms (4 h) of all NT strains were formed on pre-sterilised denture material sections (1 cm^2). These were treated with Polident® (pH 7.0) alone, Polident® (pH 7.0) followed by brushing with a soft bristle toothbrush, or with dentifrice followed by brushing. Treatment after 4 h biofilm formation and *C. albicans* capacity to regrow after 24 h incubation in RPMI was investigated by sonicating denture material discs, performing serial dilutions and subsequently conducting colony forming unit counts, as illustrated in Figure 3.8a. Results indicate that early biofilms were completely inhibited with both Polident® (pH 7.0) treatments and significantly reduced (**p= < 0.01) by treatment with dentifrice and brushing. *C. albicans* colony counts increased in all treatment groups on sections re-incubated in RPMI after 24 h. SEM images of *C. albicans* biofilms on PMMA sections following outlined treatments (Figure 3.8b) indicated evidence of varying degrees of residual biofilm structure after all treatment options.

No significant difference was observed in the Polident® (pH 7.0) treatment groups ± brushing, so all further studies were conducted by treating with Polident® (pH 7.0) alone to reduce experimental error.

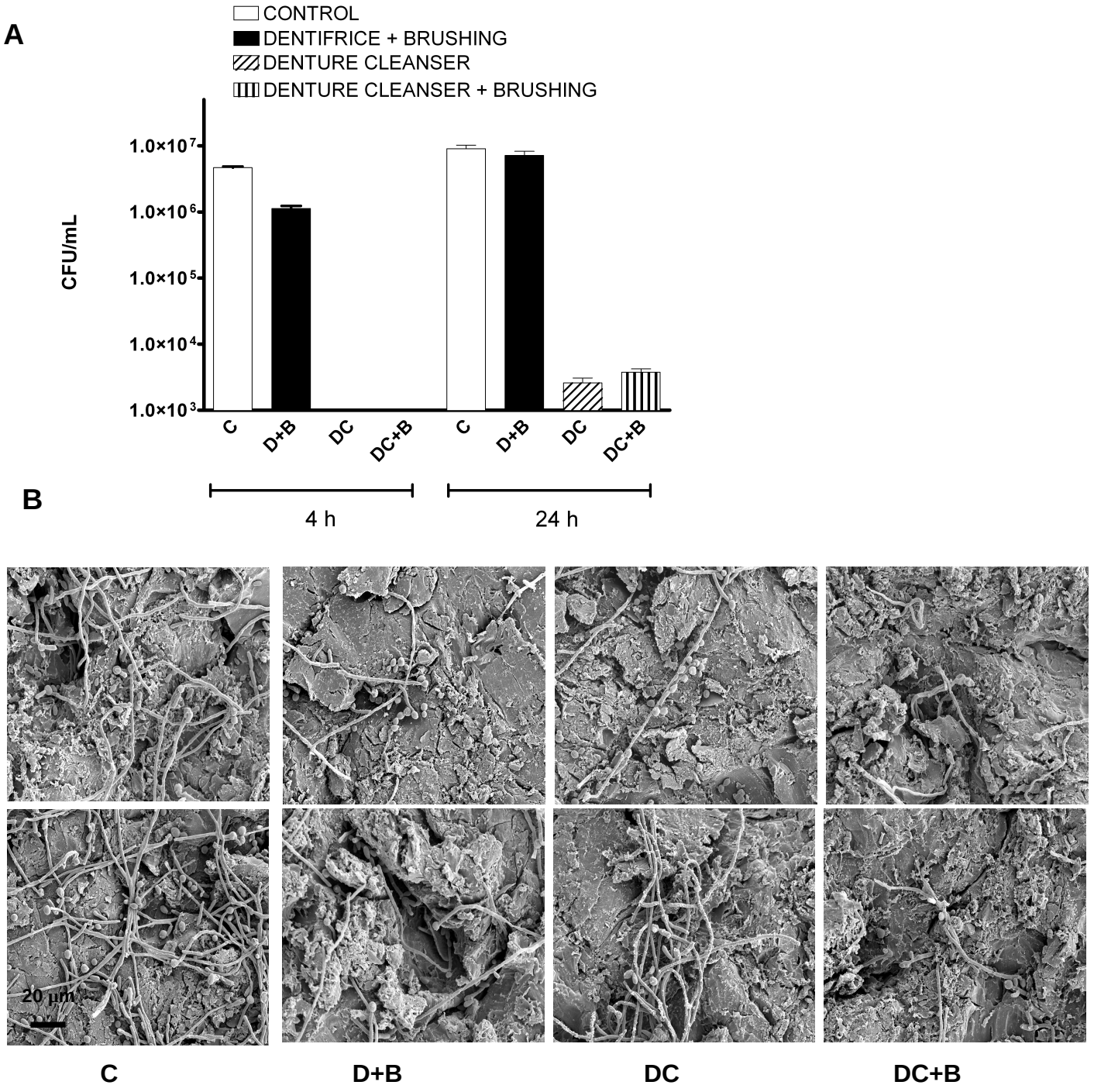


Figure 3.8: Complete inhibition of early *C. albicans* biofilms by treatment with denture cleanser (pH7). A) 4 h NT biofilms of all strains were grown on acrylic resin denture material (1 cm^2) sections and treated with Polident® (pH 7.0) ± brushing with a soft bristle toothbrush, and brushing with dentifrice. Regrowth of *C. albicans* after 24 h was assessed using the Miles and Misra colony counting technique. B) SEM images showing yeast and hyphal *C. albicans* forms.

3.3.4 Daily intermittent treatment of biofilms

The aim of this study was to investigate the effect of daily treatment of biofilms grown on denture material sections. Intermittent treatments with Polident® (pH 7.0) and Polident® (pH 8.6) and dentifrice/brushing were compared to daily sequential treatments with either denture cleanser alone.

3.3.4.1 Sequential denture cleansing with Polident® (pH 8.6) inhibits biofilm growth

Treatment of immature 4 h biofilms with Polident® (pH 7.0) initially inhibited *C. albicans*, but after three sequential treatments (24, 48 and 72 h) biofilm growth was observed, with approximately 8 x 10^5 CFU/mL detected at 72 h (Table 3.2). Comparison of treated and untreated controls demonstrated a significant reduction of cells at each time point ($p<0.001$). It was also shown that after each treatment *C. albicans* was able to regrow to cellular levels equivalent to 89%, 457% and 714% of the 4 h untreated biofilms, indicating that the denture cleanser was unable to completely inhibit and kill mature biofilm growth (Figure 3.9). Treatment of immature 4 h biofilms with Polident® (pH 8.6) was shown to inhibit the biofilm throughout the study, with no growth detected for any of the treated acrylic section (Table 3.2). No regrowth following treatment was detected at each treatment phase (Figure 3.10).

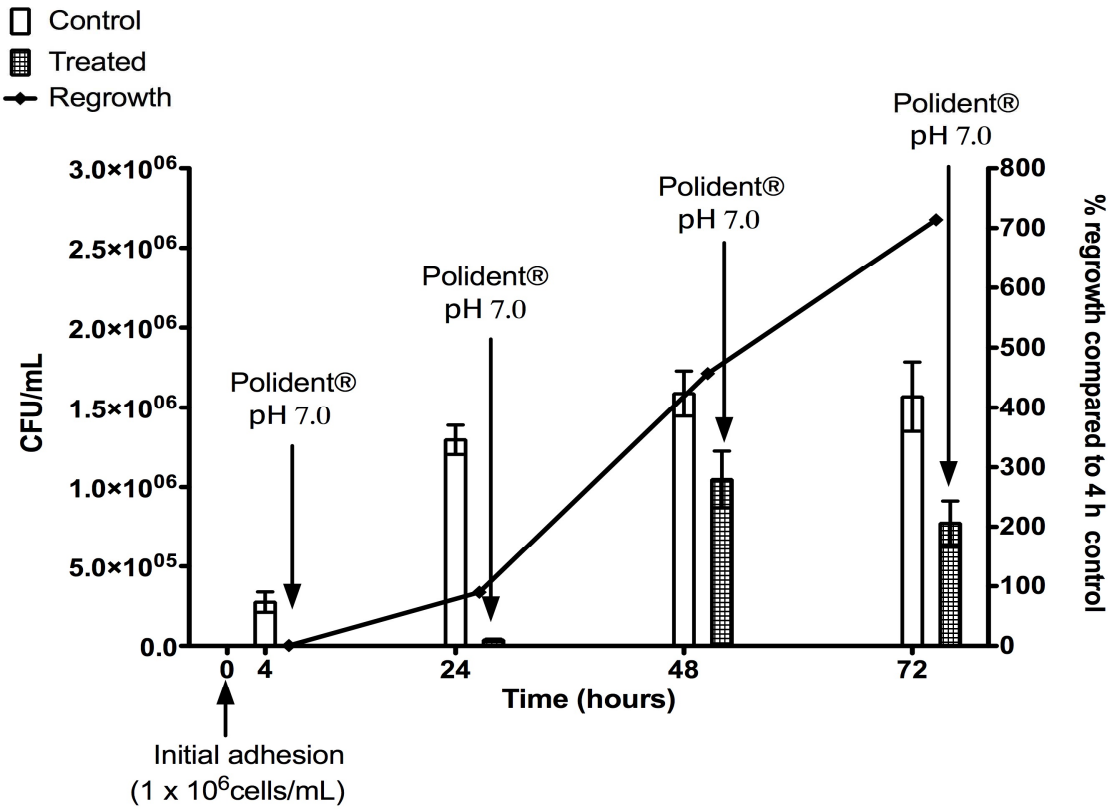


Figure 3.9: Daily sequential treatment with Polident® (pH 7.0) does not inhibit biofilm regrowth. An inoculum of 1 x 10^6 cells/mL from each NT isolate were added to a 1 cm^2 of denture acrylic section and incubated for 4 h to form an early biofilm, then treated directly with Polident® (pH 7.0) and sequentially thereafter at 24, 48 and 72 h and the total viable cells enumerated. In parallel, acrylic sections that were treated at each time point were reinoculated into RPMI and the levels of regrowth enumerated. Untreated biofilms were also enumerated throughout the experiment.

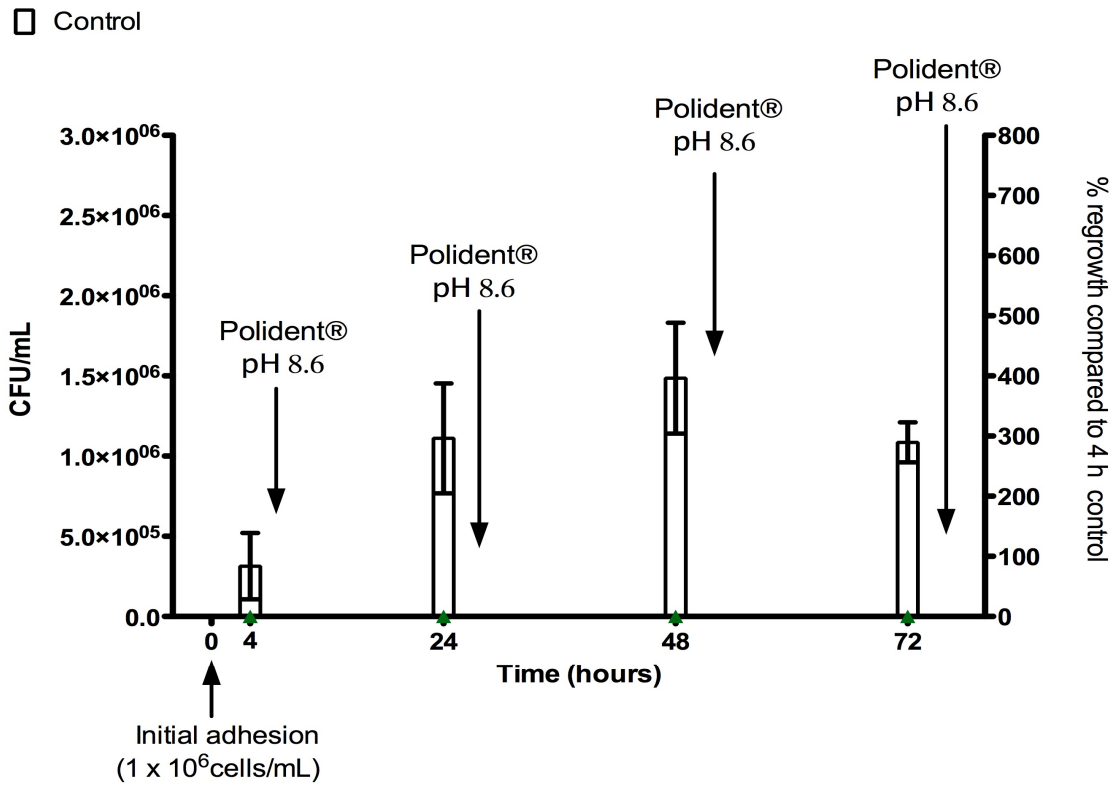


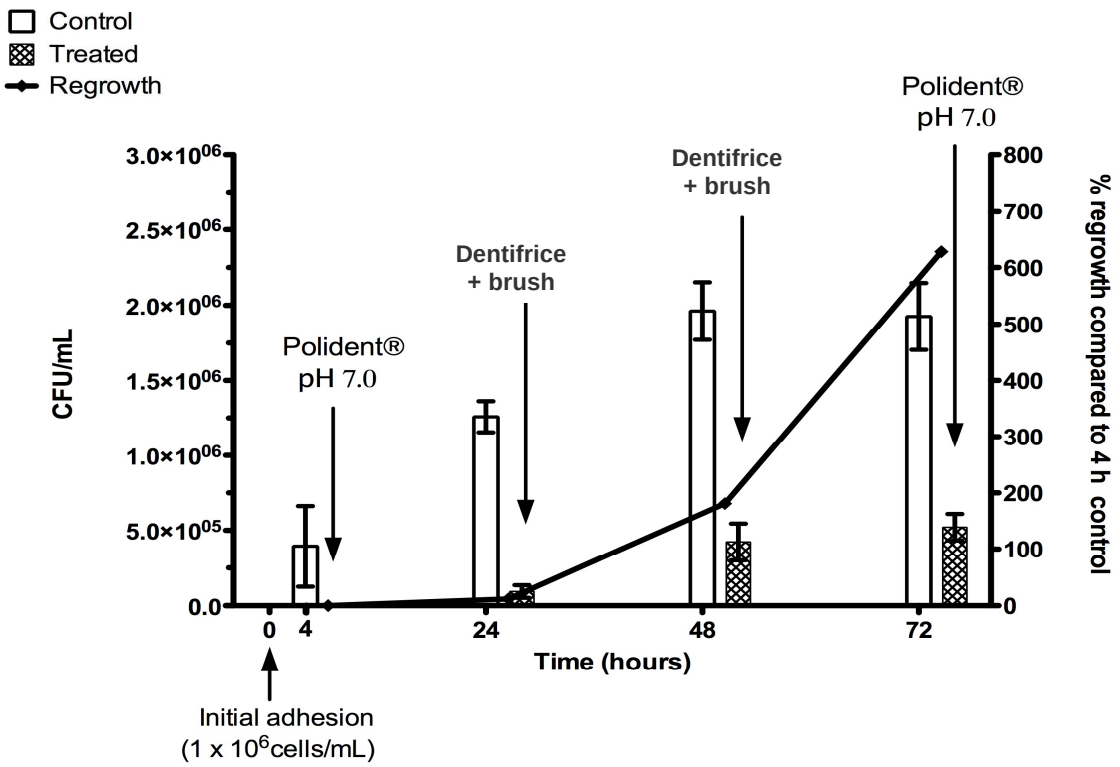
Figure 3.10: Daily sequential treatment with Polident® (pH 8.6) inhibits biofilm regrowth. An inoculum of 1 x 10^6 cells/mL from each NT isolate were added to a 1 cm^2 of denture acrylic section and incubated for 4 h to form an early biofilm, then treated directly with Polident® (pH 8.6) and sequentially thereafter at 24, 48 and 72 h and the total viable cells enumerated. In parallel, acrylic sections that were treated at each time point were reinoculated into RPMI and the levels of regrowth enumerated. Untreated biofilms were also enumerated throughout the experiment.

3.3.4.2 Intermittent denture cleansing with Polident® (pH 7.0 and 8.6) and Colgate® dentifrice and brushing does not inhibit biofilm growth

Treatment of immature 4 h biofilms with Polident® (pH 7.0) was shown to completely inhibit *C. albicans* biofilms and prevent regrowth. However, it was shown subsequently that a residual population of cells persisted on the acrylic following brushing with dentifrice, with the 24 and 48 h treated sections supporting the growth of *C. albicans*, which resulted in a 12% and 181% regrowth, respectively, compared to the untreated 4 h control (Table 3.2). A final treatment with Polident® (pH 7.0) was unable to inhibit biofilm growth, but a 26% reduction in biofilm was observed nonetheless. However, the mature biofilm was able to flourish following treatment, increasing the cellular viability by 628% to that of the initial biofilm (Figure 3.11).

Treatment of immature 4 h biofilms with Polident® (pH 8.6) was also shown to completely inhibit *C. albicans* biofilms after 4 h, but following sequential brushing with Colgate® dentifrice residual levels were detected, which increased to 0.6% and 50% of their time matched controls at 24 and 48 h, respectively (Table 3.2). Regrowth of the biofilms was also observed at 24 h (8%) and 48 h (343%). A final Polident® (pH 8.6) treatment reduced the biofilm viability by 90%, but regrowth was also observed from the residual cells to 434% of the 4 h untreated biofilm (Figure 3.12).

SEM imaging confirmed that biofilm structure remains following cleansing with both Polident® formulations (Figure 3.13).



3.1

1: Daily intermittent treatment with Polident® (pH 7.0) does not inhibit biofilm regrowth. An inoculum of 1 x 10^6 cells/mL from each NT isolate were added to a 1 cm^2 of denture acrylic section and incubated for 4 h to form an early biofilm, then treated directly with Polident® (pH 7.0) and then with Colgate® dentifrice and brushing at 24 and 48 h, followed by a final treatment with Polident® (pH 7.0) after 72 h, and the total viable cells enumerated. In parallel, acrylic sections that were treated at each time point were reinoculated into RPMI and the levels of regrowth enumerated. Untreated biofilms were also enumerated throughout the experiment.

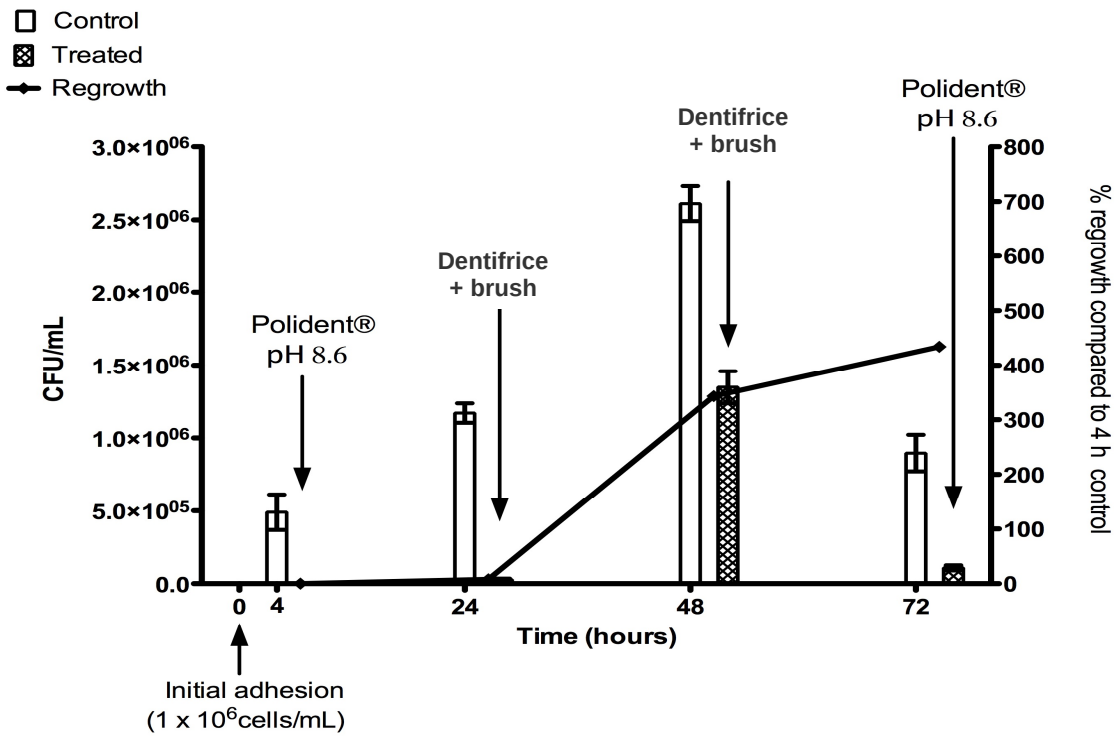


Figure 3.12: Daily intermittent treatment with Polident® (pH 8.6) does not inhibit biofilm regrowth. An inoculum of 1 x 10^6 cells/mL from each NT isolate were added to a 1 cm^2 of denture acrylic section and incubated for 4 h to form an early biofilm, then treated directly with Polident® (pH 8.6) and then with Colgate® dentifrice and brushing at 24 and 48 h, followed by a final treatment with Polident® (pH 8.6) after 72 h, and the total viable cells enumerated. In parallel, acrylic sections that were treated at each time point were reinoculated into RPMI and the levels of regrowth enumerated. Untreated biofilms were also enumerated throughout the experiment.

Table 3.2 Enumeration of *Candida albicans* cells following sequential and intermittent treatment on acrylic sections at 4, 24, 48 and 72 h.

	Polident® (pH 7.0)		Polident® (pH 8.6)	
	Sequential	**Intermittent**	**Sequential**	**Intermittent**
4	ND	ND	ND	ND
24*	$3.64 \times 10^4 \pm 7.02 \times 10^3$	$9.32 \times 10^4 \pm 4.17 \times 10^4$	ND	$2.76 \times 10^4 \pm 6.73 \times 10^3$
48*	$1.05 \times 10^6 \pm 1.78 \times 10^5$	$4.23 \times 10^5 \pm 1.22 \times 10^5$	ND	$1.35 \times 10^6 \pm 1.11 \times 10^5$
72	$7.73 \times 10^5 \pm 1.39 \times 10^5$	$5.21 \times 10^5 \pm 8.84 \times 10^4$	ND	$1.08 \times 10^5 \pm 1.77 \times 10^4$

*For intermittent treatment dentifrice and brushing performed at these time points

[ND] No colonies detected for these treatments

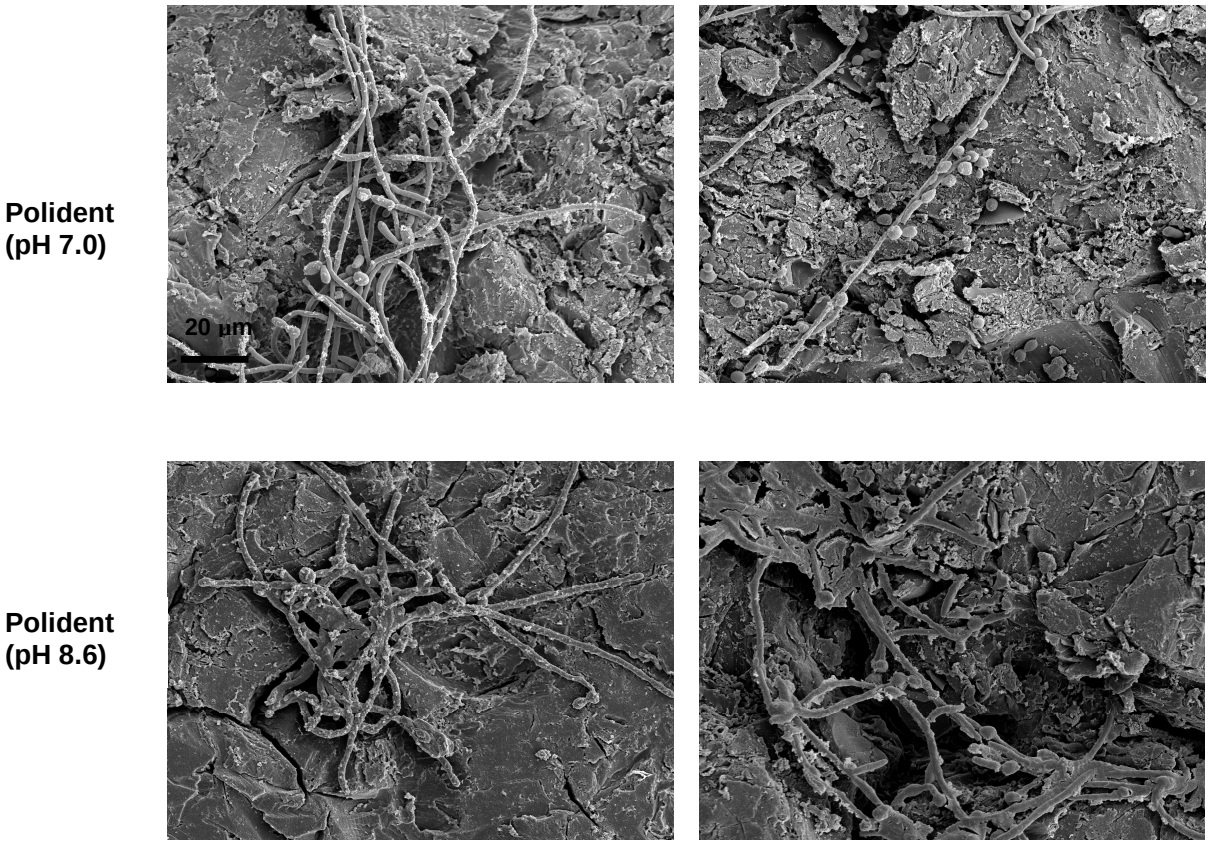


Figure 3.13: Residual fungal biofilm remains following treatment with denture cleanser. NT strain BC071 was grown on denture material for 4 h, treated with Polident denture cleansers (pH 7.0 and pH 8.6) and allowed to re-grow overnight (24 h).

3.3.5 Killing efficacy of denture cleansers and dentifrice

This study was designed to investigate killing activity of denture care products. Denture cleansers are quicker and more effective at inhibiting biofilm metabolism than dentifrice. The metabolic reduction of all four isolates was evaluated using an XTT reduction assay. It was shown that dentifrice slurry was minimally inhibitory, reducing the metabolism by only 53% after 10 min exposure. In contrast, Polident® (pH 7.0) was able to reduce the metabolism by 40% after 30 s, 75% by 3 min and 81% by 10 min. Polident® (pH 8.6) was highly active, inhibiting the metabolism by 96.5% after only 30 s, which reached 97% after 10 min.

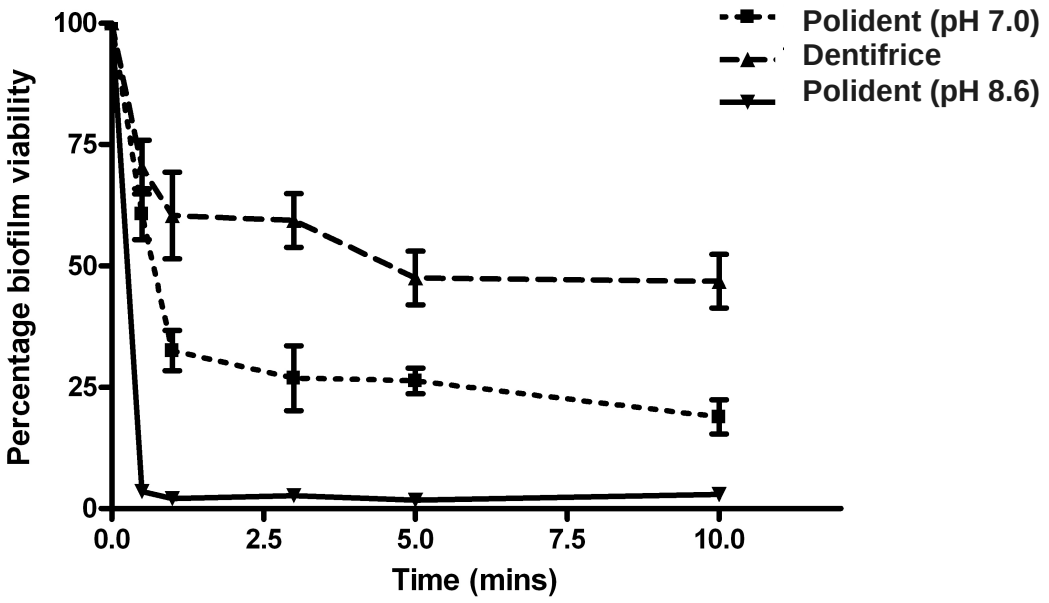


Figure 3.14: Time-kill analysis of biofilms treated with denture care products demonstrated that Polident® (pH 8.6) rapidly kills *Candida albicans*. Biofilms were grown in 96 well microtitre plates for 24 h before treatment with denture care products at time points of 0.5, 1, 3, 5 and 10 min. Metabolic activity of the treated biofilm was assessed by an XTT assay and the proportional decrease in viability compared to the untreated biofilms.

3.4 Use of alternative antimicrobials for the treatment of *Candida albicans* biofilms

3.4.1 Introduction

In recent years there has been a wide interest in novel antimicrobial compounds used for the treatment of a variety of infections (Liu *et al.*, 2010; Simmons *et al.*, 2010). Current studies have been conducted using ethylenediaminetetraacetic acid (EDTA) a divalent ion chelator (Ramage *et al.*, 2007) and farnesol; a quorum sensing molecule released by *C. albicans* which affects phenotypic switching (Decanis *et al.*, 2009; Ramage *et al.*, 2002b). Depending on the levels of fungal inhibition exhibited by these antimicrobials, there is possible scope for incorporation of these compounds into current products, for example denture cleansers to improve the action of these formulations.

3.4.2 Optimisation of antimicrobial concentrations for efficient treatment of *Candida albicans* biofilms

The aim of this study was to assess optimal concentrations of antimicrobials for the treatment of *C. albicans* biofilms. Biofilms were found to be inhibited by all treatments (Figure 3.15). Treatment with Polident® (pH 7.0) at all concentrations reduced biofilm viability by 100%. Treatments with farnesol and EDTA inhibited biofilms by >25% at lowest concentrations used. Increasing concentrations of EDTA did not alter effectiveness of treatment while treatment with farnesol at 600 μM reduced biofilms by >75%.

3.4.3 Pre-coating of 96 well microtitre plates with antimicrobial compounds

Pre-coating with all antimicrobials reduced biofilm formation. Coating with EDTA at the highest concentration negatively impacted biofilm formation by ~75%. Pre-treatment with farnesol and Polident® (pH 7.0) reduced biofilm formation by a mean of 25%.

3.4.4 Pre-coating of denture material sections with antimicrobial compounds

To assess whether there was a similarity between biofilm inhibition by pre-coating of substrates with antimicrobials; denture material sections were coated with above compounds prior to biofilm formation. Antimicrobials were allowed to evaporate prior to biofilm formation of NT III strain BC071 (Figure 3.16).

In contrast to section 3.5.3, statistically significant (**$p < 0.01$) levels of biofilm inhibition were observed after pre-treatment with both EDTA and denture cleanser. Pre-coating of sections with farnesol did not affect biofilm formation.

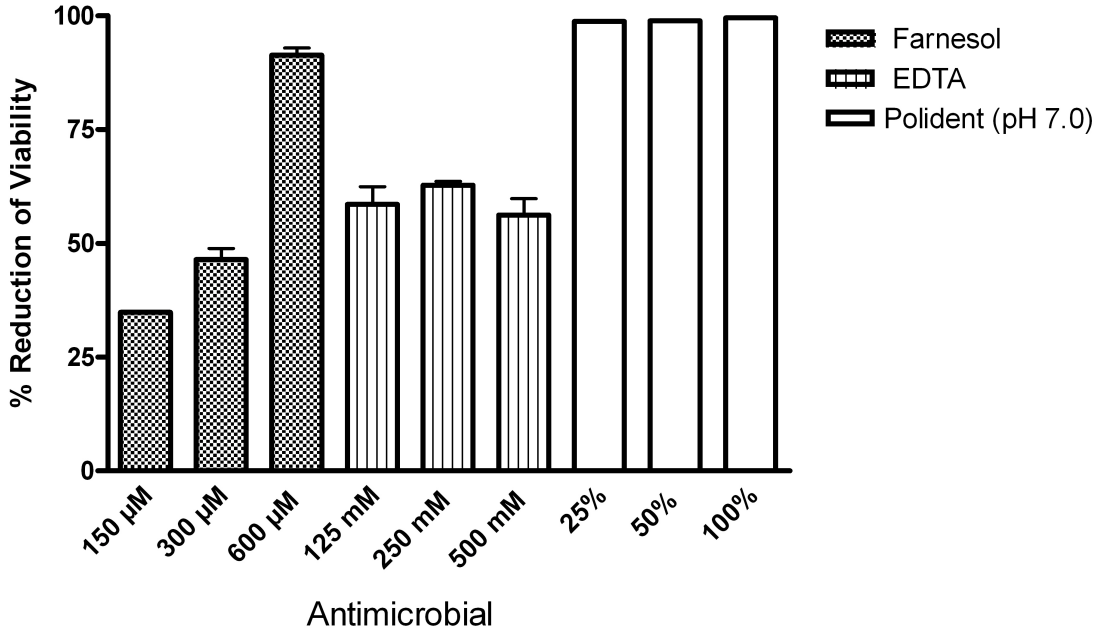


Figure 3.15: Anti-biofilm action of antimicrobial compounds is less effective than treatment with denture cleanser. Biofilms of NT III strain (BC071) were grown for 24 h in 96 well plates. Treatment with farnesol, EDTA and Polident® (pH 7.0) was conducted for 4 h at decreasing concentrations. A reduction in biofilm viability was observed with increasing concentrations of farnesol.

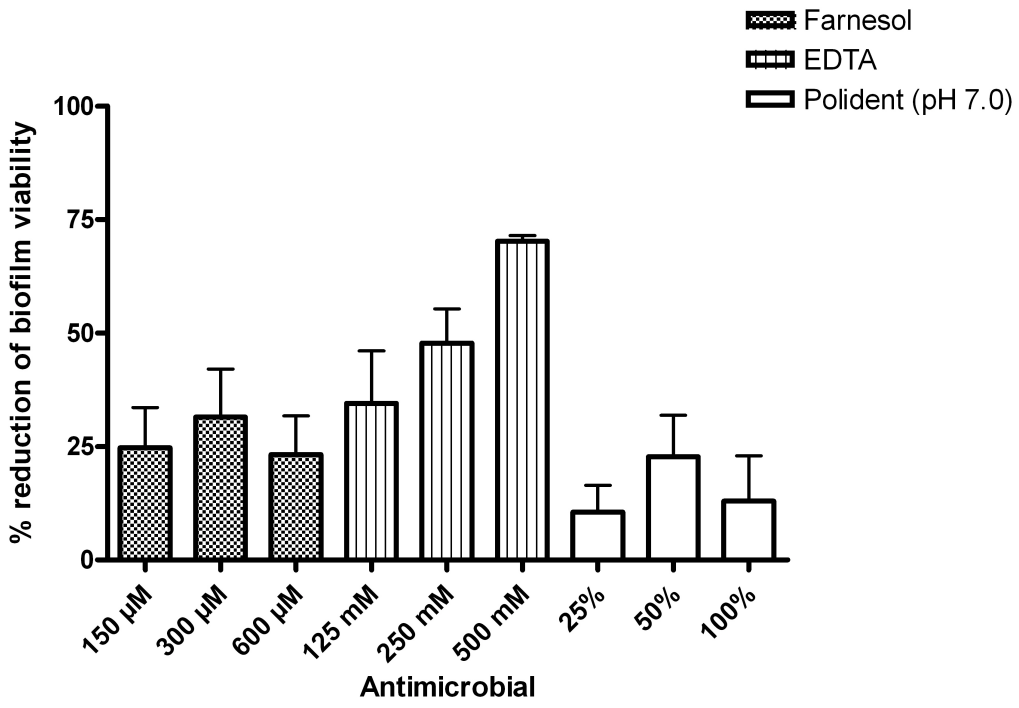


Figure 3.16: Coating of 96 well microtitre plates with antimicrobials reduces subsequent biofilm formation. Decreasing concentrations of A) farnesol, B) EDTA and C) Polident® (pH 7.0) were aliqouted into 96 well microtitre plates and allowed to evaporate. 24 h biofilms of NT III strain BC071 were subsequently formed.

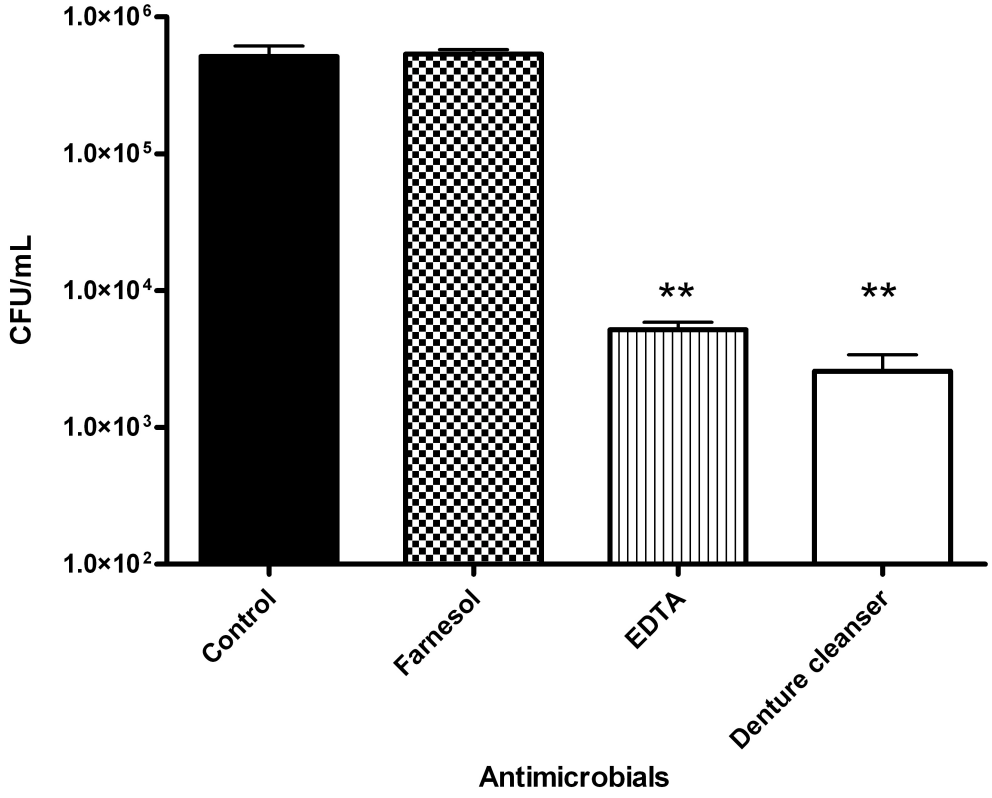


Figure 3.17: Coating of denture material with antimicrobials leads to significant reduction in biofilm formation. Pre-sterilised denture material sections were coated with farnesol at a concentration of 600 μM, EDTA at 500 mM and neat Polident® (pH 7.0). Excess antimicrobial was allowed to evaporate over 24 h. 24 h biofilms of NT III strain BC071 were grown on coated sections.

3.5 Innate immune response to *Candida albicans* biofilms and denture cleansers

3.5.1 Introduction

Successful colonisation of the oral epithelium by *Candida* species is associated with the recruitment of a variety of host responses. In particular the recruitment of mediators subsequently results in the release of pro-inflammatory cytokines including IL-6, IL-8 and TNF-α, which trigger phagocyte deployment to the site of infection (Mostefaoui *et al.*, 2004). Oral epithelial cell lines are commonly used as models for candidal infection *in vivo* (Dongari-Bagtzoglou & Kashleva, 2003).

3.5.2 Toxicity of antimicrobials to oral epithelial cells

The aim of this study was to investigate the impact on viability of OKF6/TERT oral epithelial cells following treatment with EDTA, farnesol and Polident® (pH 7.0). Epithelial cell stimulation with neat Polident® (pH 7.0) resulted in complete cell death after 1 min, while stimulation with EDTA at a concentration of 500 mM did not adversely affect cell viability. Stimulation with farnesol decreased cell viability in a time dependant manner, reducing to ~70% and ~40% after 5 and 10 min, respectively (Figure 3.18).

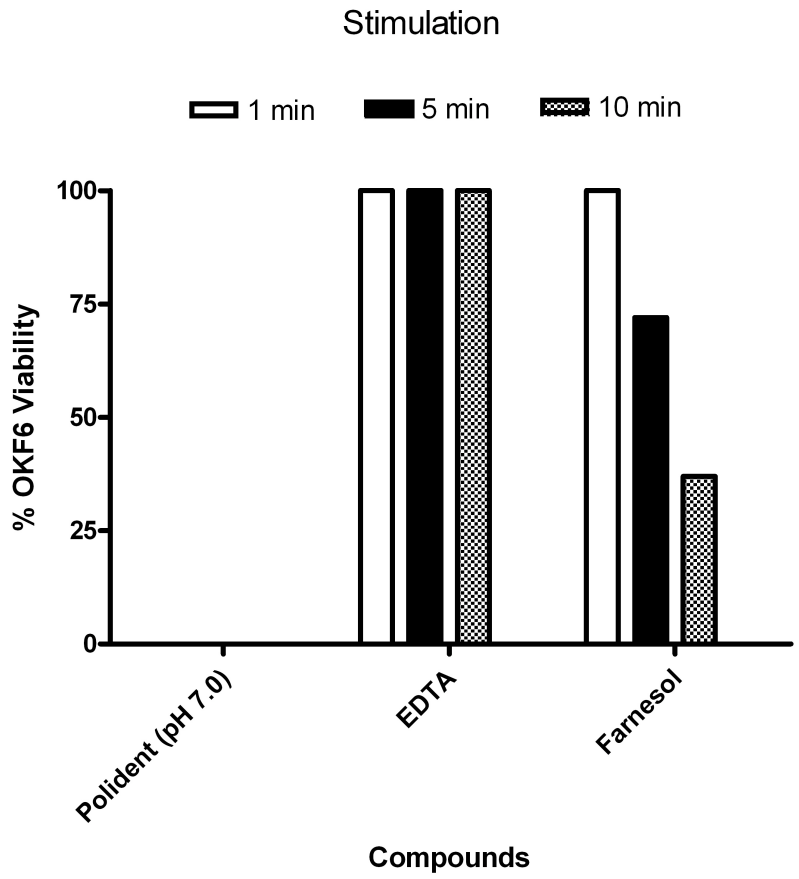


Figure 3.18: Stimulation of oral epithelial cells with antimicrobial compounds affects viability. OKF6/TERT oral epithelial cells were cultured in 24 well tissue culture plates until 90-100% confluent. Cells were stimulated with Polident® (pH 7.0), EDTA and farnesol for 1, 5 and 10 min.

3.5.3 Gene expression of IL-8 in relation to *Candida albicans* planktonic and sessile cells

Real-time PCR was conducted to assess the levels of IL-8 gene expression from OKF6/TERT oral epithelial cells in relation to *C. albicans* grown in planktonic and biofilm forms. A cell culture model system was designed which facilitated direct stimulation of OKF6 epithelial cells with early/mature biofilms grown on denture material sections (Section 2.6.1.1). Gene regulation was standardised to housekeeping gene GAPDH and zym was used as a positive inflammatory agonist (Figure 3.19). Fold change was determined in relation to media control.

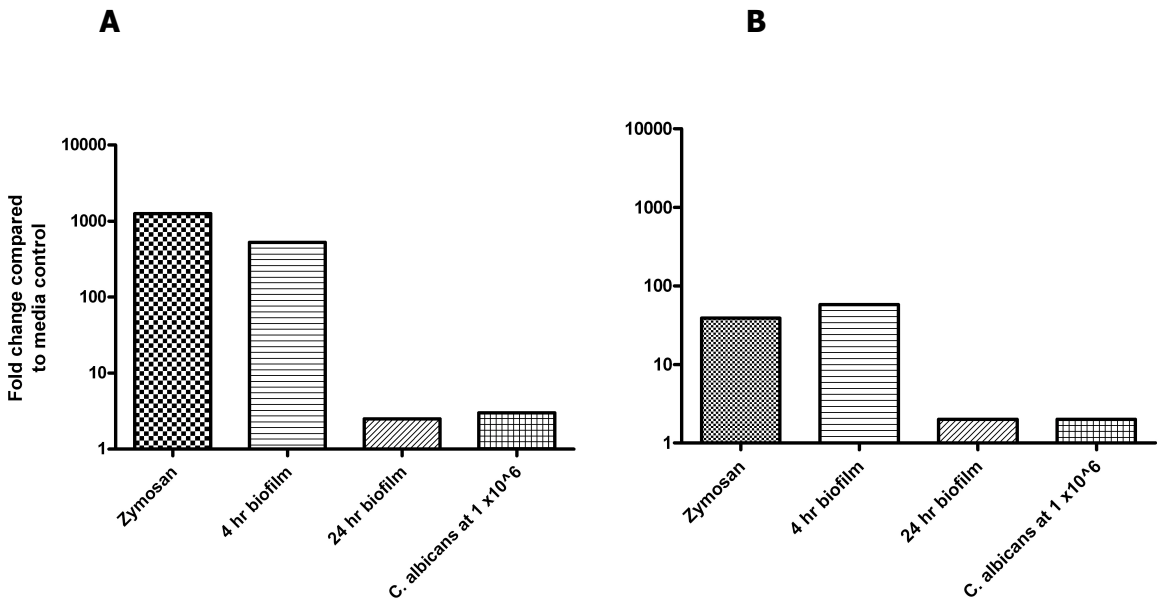


Figure 3.19: IL-8 expression is upregulated in response to early *Candida albicans* biofilms. A) Gene expression following a 4 h epithelial cell stimulation and B) 24 h stimulation. IL-8 gene regulation in OKF6/TERT cells stimulated with planktonic *C. albicans* cells, and early and mature biofilms, that were assessed through real-time PCR. Fold change was calculated in relation to media control using GAPDH as a housekeeping gene.

3.5.4 OKF6/TERT stimulation with denture material particles

The aim of this study was to assess whether manufactured denture material elicited an inflammatory response when used to stimulate OKF6/TERT oral epithelial cells without the presence of yeast. Following a 4 and 24 h stimulation with both concentrations there was no significant IL-8 release associated with the denture material. Stimulation with zym resulted in a significant IL-8 response as illustrated in Figure 3.20.

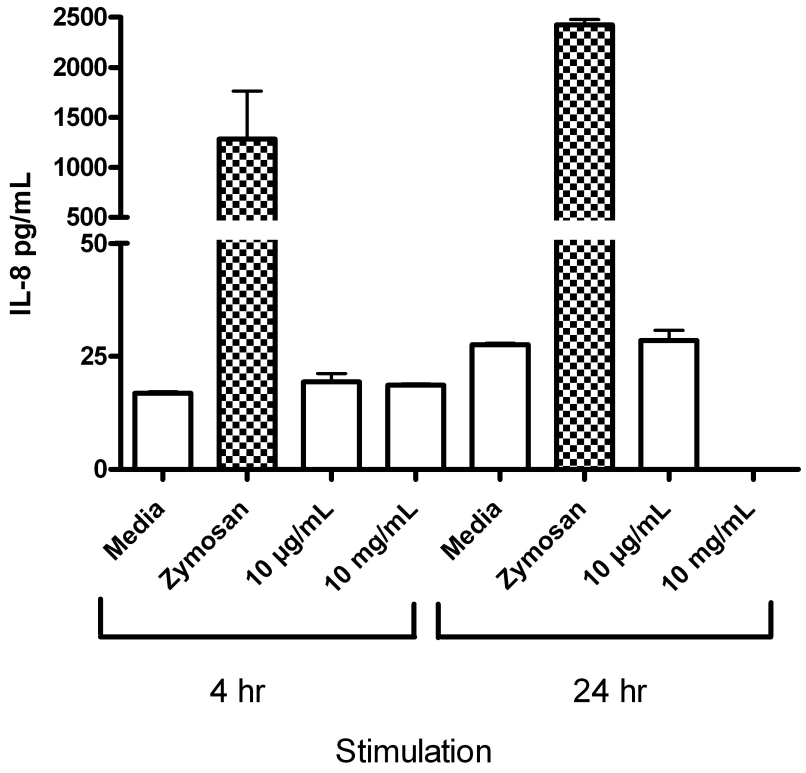


Figure 3.20: OKF6/TERT stimulation with denture material does not result in a significant IL-8 response. OKF6/TERT oral epithelial cells were cultured until confluent and stimulated with denture material particles at concentrations of 10 µg/mL and 10 mg/mL in cell culture media.

3.5.5 Comparison in immune response to treated and untreated biofilms used to stimulate oral epithelial cells

OKF6/TERT epithelial cells grown in a confluent layer were used to assess the inflammatory response to planktonic and sessile *C. albicans* cells (Section 3.2.1). DMSO, amphotericin B and zym were used as appropriate controls. Planktonic and sessile cells were treated with denture cleanser for 3 min to investigate whether treatment of *C. albicans* cells impacts on IL-8 release. Following a 4 h stimulation there was a significant (***p= < 0.001) IL-8 response observed only after zym stimulation. A 24 h epithelial cell stimulation resulted in elevated IL-8 release in response to both zym as well as early and mature biofilms. Treatment of biofilms and planktonic cells did not significantly alter the IL-8 response.

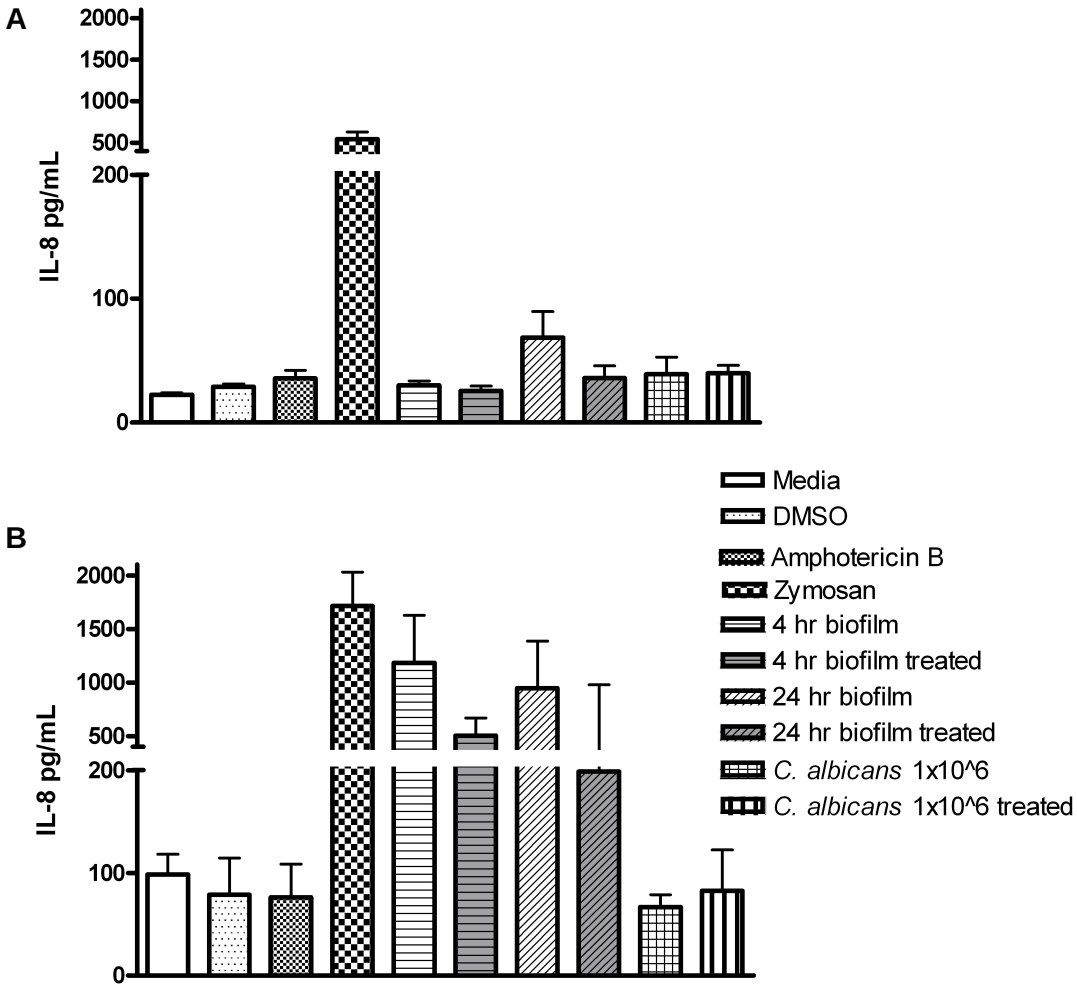


Figure 3.21: Treatment of planktonic and sessile *Candida albicans* cells does not impact IL-8 release. OKF6/TERT oral epithelial cells were stimulated with planktonic and sessile *C. albicans* cells of NT III strain BC071 for A) 4 and B) 24 h. DMSO, Amphotericin B and zym were used as negative and a positive control respectively.

Chapter 4: Discussion

4. Discussion

Candida albicans is the major yeast species isolated from OPC (including denture induced stomatitis) cases and the success of this micro-organism is partly due to the ability to form biofilms on a variety of surfaces (Chandra *et al.*, 2001a; Ramage *et al.*, 2004; Redding *et al.*, 2009). Whilst denture induced stomatitis is not life threatening *per se*, it affects a large proportion of the 15 million denture wearers in the UK and is associated with undesirable symptoms (Coulthwaite & Verran, 2007). In addition to those clinically diagnosed with the disease, an equal number of denture wearers are asymptomatic. Clinical management is required in all cases when possible. This is particularly important given the possibility of *C. albicans* to prosper within the oral cavity, which has been implicated to be associated with oral malignancies (Cannon & Chaffin, 1999; Rautemaa *et al.*, 2007). Therefore, in addition to daily oral hygiene practices, denture decontamination is pivotal to reducing and eliminating *Candida* spp. retained upon the denture as adherent biofilm communities.

This series of investigations has highlighted potential inadequacies of 'gold standard' oral hygiene techniques against *C. albicans* biofilms, and demonstrated that sequential treatment with Polident® (pH 8.6) is superior to intermittent treatment regimens with either denture cleanser in combination with dentifrice and brushing. These investigations have additionally demonstrated the potential utility of morphological modulators; EDTA and farnesol to augment or potentiate the activity of these denture cleansers. Nevertheless, based on the overall data *C. albicans* has the ability to persist upon denture acrylic, which following treatment retains the capacity to induce inflammatory mediators. The role of biofilms and denture disinfection will now be discussed.

Biofilm formation is an adapted lifestyle that enables *C. albicans* to thrive within the oral cavity on biological and innate substrates. Indeed, *C. albicans* biofilm formation is capable of providing defence from the immune system and

contributes towards antifungal resistance (Ramage *et al.*, 2009). In this study, a range of clinical *C. albicans* isolates from denture stomatitis patients (Coco *et al.*, 2008) were tested for their ability to form biofilms in order to identify strains for subsequent analysis. Strains representing different stages of inflammation were selected, including suitable controls. It was established that these strains formed visible biofilms to different extents on polystyrene substrates, irrespective of individual growth rates. Interestingly, those isolates from Newton's type inflammation II and III appeared to form biofilms with greater biomass than other strains. However, metabolic assessment of these strains revealed a contrasting pattern. One possible explanation is that the amount of biomass present was not indicative of cell viability and a proportion of the cell biomass might actually have been inactive. It is probable matrix material accounted for the majority of biomass present (Mukherjee & Chandra, 2004).

Studies have listed disadvantages in using the XTT assay to assess cell viability (Kuhn *et al.*, 2003). Consequently, an experiment was designed that tested the sensitivity of the XTT assay. The results demonstrated that the XTT assay is only sensitive to a *C. albicans* cell concentration of 1×10^5 cells/mL. This implies that viable cells at a concentration $< 1 \times 10^5$ cells/mL would not be detected and other assays measuring cell viability should be employed in future studies. The alamar blue indicator assay is commonly used as an alternative to the XTT assay due to a reported higher sensitivity to a variety of compounds (Uzunoglu *et al.*, 2010). Other options for the assessment of *Candida* spp. cell vaibility include colony plate counting as well as use of various vital stains (Jin et al., 2005).

Proteolytic and haemolytic activity was also variable amongst strains (data not shown). Overall, this demonstrated that strain variation is important with respect to inducing overt disease in OPC patients. However, *C. albicans* innate ability to form biofilms remains problematic for treatment options.

Previous studies have established that *C. albicans* biofilms form readily on plastic surfaces (Tronchin *et al.*, 1988; Ramage *et al.*, 2001c). It was therefore also important to establish whether biofilm formation occurs similarly on acrylic resin denture materials sections used in this study. Results indicated that biofilms of an average cell density of 1 x 10^6 cells/mL were maintained on PMMA denture material sections over a period of 48 h. Resilient biofilm structure was confirmed by SEM imaging, which illustrated both yeast and hyphal *C. albicans* forms indicative of a biofilm organisation. Surface topography is an important factor for biofilm formation. Studies conducted by Verran and colleagues (1997) indicated that *C. albicans* cell numbers were significantly higher on rough versus smooth acrylic and silicone surfaces following a 1 h incubation (Verran & Maryan, 1997). In contrast, PMMA surface roughness did not upon impact biofilm formation in a recent investigation. Marked deviations in biofilm formation were considered to be due to inter-strain variation only (da Silva *et al.*, 2010).

The field of denture decontamination is still undergoing much research due to the general lack of efficiency of many commercially available products. DIS patients often treat their dentures with a dentifrice rather than denture cleanser (Barreiro *et al.*, 2009). Previous research has indicated that brushing dentures with a dentifrice is an ineffective cleansing mechanism as damage to denture surface occurs over time, which then facilitates further attachment of microbial species (Charman *et al.*, 2009). The results of these investigations confirmed that brushing dentures with dentifrice is a significantly less effective inhibition method of both early and mature *C. albicans* biofilms compared to treatment with tested denture cleansers.

It has been revealed that denture wearers often lack a consistent denture cleansing routine, which can impact greatly on their overall denture hygiene. In order to investigate this further, a study was devised a study to compare daily treatment of *C. albicans* biofilms with Polident® (pH 7.0) denture cleanser to daily intermittent treatment with Polident® (pH 7.0) and brushing with Colgate® dentifrice. Results indicated that daily treatment with either

Polident® (pH 7.0) alone or intermittent treatment with Polident® (pH 7.0) and brushing with Colgate® dentifrice were ineffective at reducing *C. albicans* numbers once a mature biofilm had formed. This finding is in agreement with research outlining the difficulties associated with decontamination of biofilm covered surfaces, due to previously outlined biofilm characteristics as well as individual patient problems involved in routine cleansing (Kulak-Ozkan *et al.*, 2002). Resistance to a variety of antifungals occurs due to protective matrix formation associated with *C. albicans* biofilm formation. Various explanations for the action of matrix material have been suggested (Al-Fattani & Douglas, 2006). One possible theory involves the glucan synthase gene; FKS1, which has been implicated as being crucial for resistance to a variety of antifungal agents (Nett *et al.*, 2010). In addition, *C. albicans* cells remaining on decontaminated surfaces may exhibit the high-persister cell phenotype, which has been isolated from individuals suffering from long-term candidal carriage (Lafleur *et al.*, 2010).

The subsequent stage of this study involved repeating the above experiments with a novel formulation of the denture cleanser; Polident® (pH 8.6) which yielded a lower sessile MIC value compared to both Polident® (pH 7.0) and Colgate® dentifrice. The active ingredients of Polident® (pH 8.6) and the original Polident® (pH 7.0) are similar. However, Polident (pH 8.6) benefits from increased levels of oxidising agents present in the formulation. Oxidising agents are crucial for effective denture cleanser formulation due to their microbial cleansing effects (Pavarina *et al.*, 2003).

In contrast to my previous findings, daily treatment of *C. albicans* infected denture material sections with Polident® (pH 8.6) resulted in complete fungal inhibition throughout a 72 h time period. Together, this series of experiments indicated that daily treatment with Polident® (pH 7.0) as well as a combination of Polident® (pH 7.0) and Colgate® dentifrice was not adequate in maintaining low *C. albicans* levels. Dissimilarly, combination cleaning with alkaline peroxide solution and brushing with a dentifrice yielded the highest

decontamination effect on *C. albicans* mature biofilms compared to chemical or mechanical treatment alone (Paranhos *et al.*, 2009).

Since it has been revealed that candidal progression into a biofilm structure is a key virulence trait leading to pathogenesis, factors involved in the 'switch' between planktonic and sessile forms are a crucial target for future drug development. Farnesol is a known candidal quorum sensing molecule responsible for inhibiting the modification from yeast to hyphae (Hornby *et al.*, 2001). In agreement with previous research, treatment of biofilms with varying concentrations of farnesol resulted in impaired biofilm formation (Ramage *et al.*, 2002b). The same effect was observed following treatment with EDTA, which has been found to impact candidal filamentation through a mechanism of cation chelation (Ramage *et al.*, 2007). EDTA demonstrated lower anti-candidal activity; this is in contrast to previous findings which indicated that EDTA had greater anti-candidal activity than a variety of antifungal drugs as well as various denture hygiene products (Sen *et al.*, 2000). A possible reason for this discrepancy could be the variation in *C. albicans* strains tested as well as the use of a variety of antifungals, which do not match those used in this study.

A study was also designed which examined the anti-biofilm properties of farnesol and EDTA through the coating of plastic substrates with these compounds prior to biofilm growth. Coating of both 96 well microtitre plates and PMMA denture material sections with set concentrations of Farnesol, EDTA and Polident (pH 7.0) resulted in a marked decrease in biofilm formation. Other studies conducted using PMMA as a growth substrate for *C. albicans* biofilm formation indicate that use of EDTA is effective during decontamination of these surfaces without, importantly, affecting the surface roughness (Devine *et al.*, 2007).

Antimicrobial effects of farnesol are not fungal specific and have also been documented in regard to various bacteria including Staphylococci (Jabra-Rizk *et al.*, 2006a) and Streptococci species (Koo *et al.*, 2002). Similarly, EDTA has been recognised as reducing the infectivity of herpes simplex virus (Devine *et*

al., 2007). These species are known to be commonly isolated from the oral cavity. This suggests that there are wider beneficial aspects of using morphological modulators in relation to treatment of oral microorganisms. Future manipulations of morphological modulators such as EDTA and farnesol include the potential of combination with current denture hygiene products to aid efficiency against a variety of microorganisms. It has recently been demonstrated that treatment of a range of microorganisms with farnesol increases sensitivity to both antibiotics and antifungals, which suggests implications for adjuvant properties of the molecule (Brehm-Stecher & Johnson, 2003; Jabra-Rizk *et al.*, 2006b). Likewise, EDTA has been used since the 1960's due to the synergistic effects observed when used in combination with antimicrobials as well as antibiotics (Brown & Richards, 1965; Weiser *et al.*, 1969). In particular, EDTA has been utilised for catheter lock technology as recent studies have indicated that combinations of EDTA and amphotericin B lipid complexes result in significantly lowered Candida spp densities on silicone surfaces (Raad *et al.*, 2008).

Whilst EDTA and farnesol have proven antimicrobial effect, it is also important to consider host interactions. This aspect of the study investigated the innate immune response to *Candida* spp. infection and specifically the impact of biofilm treatment upon host inflammation.

Initially, it was important to assess whether EDTA or farnesol were toxic to OKF6/TERT oral epithelial cells as future combinations into antimicrobial products could require antimicrobial to host cellular contact for mode of action. Following epithelial cell stimulation with compounds, an XTT assay was conducted that evaluated cell viability post exposure. EDTA was found not to impact upon cell viability. In contrast, cell contact with farnesol resulted in decreased cell viability with time. Other studies assessing the toxicity of EDTA describe this compound as being toxic *in vivo*, but only at higher concentrations than used in these studies (Lanigan & Yamarik, 2002). Previous studies relating to farnesol toxicity demonstrated that cells derived from normal tissue exhibit lower levels of toxicity to farnesol while a farnesol

concentration of 20 μM had a toxic effect on HeLa cells (Yazlovitskaya & Melnykovych, 1995).

Polident® (pH 7.0) was also used to stimulate an oral epithelial cell line and resulted in complete cell death following a 1 min exposure. Limited intra-oral exposure to human cells is advised in relation to this product as it is currently formulated for application out with the host.

Due to the retention of biofilm biomass following a variety of decontamination procedures (Ramage *et al.*, 2002c; Pusateri *et al.*, 2009a; Jose *et al.*, 2010) it was essential to assess whether host inflammation occurred independent of treatment. This can be achieved through monitoring of various inflammatory markers. In particular, high IL-8 production has been documented in response to epithelial and endothelial stimulation with *C. albicans* (Orozco *et al.*, 2000).

Therefore, IL-8 release by OKF6/TERT cells was quantitatively measured in this study as this pro-inflammatory cytokine is recognised as being released at high levels in response to *C. albicans* stimulation (Dongari-Bagtzoglou & Kashleva, 2003). IL-8 gene expression was initially assessed through real-time PCR. Stimulation with biofilms resulted in upregulation of IL-8 gene expression compared to OKF6/TERT cell stimulation with media alone. Eberhard and colleagues established that significant IL-8 gene upregulation occurs during oral epithelial cell stimulation with *S. mutans* biofilms (Eberhard *et al.*, 2009). Interestingly, early biofilms induced highest IL-8 gene upregulation at both time points while cell stimulation with mature biofilms resulted in upregulation at a similar level to planktonic *C. albicans* cells. A possible explanation for this is that extrapolymeric material formed during mature biofilm progression may result in a 'shielding effect' of the microorganism from cytokines released in response to infection. This phenomenon has been reported in various microorganisms including *Staphylococcus epidermidis* (Richards et al., 1994) and *Pseudomonas aeruginosa* (Bjarnsholt et al., 2010).

Gene expression results were in agreement with IL-8 ELISAs conducted; cell stimulation with early biofilms resulted in the highest IL-8 release. This was only observed after 24 h epithelial cell stimulation due to general low IL-8 release observed following 4 h stimulation. Treatment of both planktonic *C. albicans* as well as biofilms with Polident® (pH 7.0) did not impact upon the inflammatory response. This suggests that the biofilm and associated material are responsible for the immune response discussed independent of whether the biofilm is viable during epithelial cell stimulation.

Another element of this study assessed whether sterile denture material positively influenced and therefore subsequently amplified the inflammatory response during *Candida* spp. infection. Varying concentrations of ground PMMA were used in contact with OKF6/TERT oral epithelial cells. Denture material did not significantly induce IL-8 stimulation by OKF6/TERT oral epithelial cells. This suggests that inflammation detected in DIS occurs only in response to *Candida* spp. as well as other oral microorganisms. Contradicting evidence has been elucidated which suggests that leaching of substances, in particular methyl methacrylate and formaldehyde from acrylic denture base materials, occurs readily during patient denture wear. Both substances are associated with allergy and inflammation observed in a number of individuals. Pre-soaking of dentures in water is therefore recommended prior to denture wear (Tsuchiya *et al.*, 1994).

Future work in relation to chemotherapeutic approaches for the treatment of *C. albicans* biofilms should concentrate on reducing the working concentration of Polident® (pH8.6) used for the treatment of biofilms on denture acrylic. MIC investigations found that this denture cleanser functions at a quarter of its neat concentration. Reductions in working concentration may allow for combinations of other active ingredients to achieve a denture cleanser which not only permanently inhibits yeast viability but also physically removes the biofilm burden from denture surfaces.

Raab and colleagues identified that ultrasonication is an effective method of denture cleansing (Raab *et al.*, 1991). The study indicated that a 90 s treatment resulted in significant removal of denture plaque. In fact, this use of mechanical technology is often readily available for patient use in nursing homes or hospital environments (Roessler, 2003). Currently, ultrasonic baths are not purchased on a commercial scale for patient home use but are certainly a good future option for the provision of thoroughly decontaminated dentures.

4.1 Conclusions

C. albicans strains isolated from varying degrees of DIS inflammation cases represented contrasting metabolic and biofilm forming traits. Strains forming stable biofilms were maintained on PMMA denture material sections at an average density of 1 x 10^6 cells/mL throughout this study. Results indicated that brushing of denture material sections containing pre-formed early biofilms with dentifrice and soft bristle toothbrush was a less effective method of biofilm inhibition compared to treatment with both Polident (pH 7.0 and pH 8.6). Only Polident (pH 8.6) was effective at fully inhibiting mature biofilms. Treatment of biofilms with morphological modulators farnesol and EDTA resulted in marked impairment in *C. albicans* biofilm formation.

The release of the inflammatory chemokine IL-8 from OKF6/TERT oral epithelial cells in response to biofilm stimulation did not significantly change following treatment of biofilms with Polident (pH 7.0) suggesting that biofilm viability does not influence the host inflammatory response.

4.2 Future work

Possible future suggestions to counteract ineffective treatment associated with mature biofilm formation could entail twice daily denture decontamination with denture cleanser or overnight removal of dentures following treatment with denture cleanser, which would hinder regrowth of yeast due to elimination of the growth environment. Neither of these methods is particularly convenient therefore, patients should be advised to treat dentures with Polident® (pH 8.6) due to complete candidal inhibition documented as well as > 95% direct killing efficacy established following a 30 s treatment of mature biofilms.

As these investigations are limited to the use of one yeast species it would be vital to repeat the various outlined studies with a number of micro-organisms, both fungal and microbial, which are native to the oral cavity.

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