

Overcoming the antifungal drug resistance of biofilms on dental acrylic

Kathryn Newsham-West
BDS (Otago)

A thesis submitted in partial fulfilment for
the degree of Doctor of Clinical
Dentistry in Prosthodontics

At the University of Otago, Dunedin,
New Zealand

August 2013

Abstract

Introduction

RC21v3 is an octapeptide derivative that inhibits the drug efflux pump that confers azole resistance on the yeast *Candida albicans* -an opportunistic pathogen that can cause denture stomatitis. *C. albicans* often forms biofilms on oral surfaces including denture prostheses, and biofilm cells are more drug resistant than planktonic cells growing as a suspension in liquid medium.

Aim

The objective of this research was to determine whether RC21v3 acts synergistically with fluconazole (FLC) to inhibit the growth of *C. albicans* cells in planktonic cultures and during growth as a biofilm.

Methods

Minimum growth inhibition concentration (MIC) assays were undertaken to measure the inhibitory effect of FLC and RC21v3 on *C. albicans* cells growing as planktonic cultures. Checkerboard MIC assays were used to measure the effect of combinations of FLC with RC21v3 on both planktonic and biofilm growth. The inhibition of *C. albicans* biofilm growth on denture acrylic was measured using crystal violet staining of biofilm cells. The effect of FLC and/or RC21v3 on biofilm structure was investigated using confocal microscopy.

Results

Checkerboard MIC assays showed that RC21v3 acted synergistically with FLC to inhibit the planktonic growth of three *C. albicans* strains: ATCC 10261 and MML610 which are FLC-susceptible, and MML611 which is FLC-resistant. In addition, the combination of RC21v3 with FLC in growth assays resulted in FLC becoming fungicidal to all three strains.

A novel dental acrylic biofilm model was developed. As it demonstrated reproducible growth of *C. albicans*, it could be used to measure biofilm growth inhibition. FLC

inhibited the growth of *C. albicans* biofilms and checkerboard assays showed that RC21v3 acted synergistically to reduce the growth of ATCC10261 biofilms.

Conclusion

This is the first demonstration that RC21v3 can chemosensitize *C. albicans* biofilms to fluconazole, and so may be of use in treatment of patients with FLC-resistant biofilms on denture surfaces.

Acknowledgements

First, I wish to thank my supervisors: Professor Richard Cannon, and Dr Kyoko Niimi whose ideas, knowledge, patience and guidance, made this work possible; and Professor Karl Lyons for his support, guidance and encouragement throughout the course of my Clinical Doctorate.

I am grateful to the members of the Molecular Oral Microbiology laboratory for valuable suggestions and guidance, particularly Dr Nick Heng, Dr Mikhail Keniya and Dr Geoffrey Tompkins and to Mrs Jenine Uprichard for laboratory assistance and guidance.

I am thankful to Mr Steve Swindells and other members of the Faculty of Dentistry technical laboratory for assistance with manufacturing the denture acrylic substrates used in the study.

I would also like to thank others at the University of Otago who have helped me, in particular Mr Andrew McNaughton, Department of Anatomy, who was extremely helpful with confocal microscopy, imaging and image analysis.

I gratefully acknowledge the Sir John Walsh Research Institute for the award of a Fuller Scholarship that funded this study.

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List of Abbreviations

°C	Degrees Celsius
3-D	Three-dimensional
µg	Microgram
µL	Microliter
µm	Micrometre
µM	Micromolar
ABC	ATP-binding cassette (pump)
BHI	Brain-heart infusion (growth medium)
CFU	Colony-forming unit
CV	Crystal violet
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
e.g.	For example
<i>et al</i>	<i>Et alii</i> (and others)
Fig.	Figure
FLC	Fluconazole
g	Gram
<i>g</i>	Gravitational (force)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
h	Hour
MFS	Major facilitator superfamily (transporter)
MIC	Minimum growth inhibitory concentration
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar
MOPS	3-(N-morpholino) propanesulfonic acid (buffering agent)
nm	Nanometre
OD	Optical density
pH	Measure of the concentration or activity of the (solvated) hydrogen ion

PBS	Phosphate-buffered saline
PI	Propidium iodide
R_a	Surface roughness
rpm	Revolutions per minute
RPMI 1640	Growth medium developed at Roswell Park Memorial Institute
RC21v3	Manually synthesised D-octapeptide ²¹ version 3
w/v	Weight per volume
YPD	Yeast extract peptone dextrose (growth medium)

Preface

This thesis was undertaken as part of the course requirements for the Doctor of Clinical Dentistry (Prosthodontics). Financial assistance was provided by the Sir John Walsh Research Institute with the award of a Fuller scholarship.

The body of this thesis is organized into four chapters. Chapter 1 gives an outline of the project and provides the scientific background and proposal for this research. Chapter 2 describes the materials and experimental protocols followed for each part of the study.

Results are collated into one chapter (Chapter 3). This is divided into sections that describe the results of the initial planktonic pilot study, the development and verification of a novel dental acrylic substrate pattern for biofilm growth, and finally the results of the preliminary biofilm investigation.

These results are discussed in the last chapter (Chapter 4), and limitations of the research are outlined before conclusions are presented with suggestions for future research.

1 Literature review

1.1 Introduction

This review focuses on the role that the fungus *Candida albicans* plays in clinical oral infection, in particular those related to acrylic dentures. The antifungal resistance mechanisms deployed by *C. albicans*, specifically with respect to azoles, and the development of strategies to overcome clinically observed azole resistance will also be discussed.

1.2 Oral fungal infection

Over the past 20 years, there has been a significant increase in the incidence of opportunistic fungal pathogens causing life-threatening mycoses (Pfaller and Diekema, 2010) with the more commonly isolated species comprising of the genus *Candida* (Lamagni, 2001).

In humans *Candida* species can be found as microbial commensals of the oral mucosa in approximately half the population (Thein, 2009), and *C. albicans* is the most commonly isolated species. The fungal infection candidiasis is thought to originate from cells in this endogenous microbial reservoir, which, through circumstances relating to host immunity and mechanisms of fungal pathogenicity, are able to override the normal host defences. Of particular importance to dental professionals are *Candida*-associated lesions such as angular cheilitis, median rhomboid glossitis, pseudomembranous candidiasis and denture-induced stomatitis, a commonly observed infection found in 11- 71% of denture wearers (Budtz-Jørgensen, 1974; Lund *et al.*, 2010; Gendreau and Loewy, 2011). Another group of clinically observed infections are those associated with primary keratinized lesions such as Lichen planus where 40% of cases are super-infected with *Candida*. Of significance in this group is Leukoplakia where the *Candida* super-infection has been linked with the initiation of epithelial dysplasia, and oral neoplasia (McCullough *et al.*, 2002). Although there are several species of *Candida* that cause oral infections including *Candida glabrata*, *Candida krusei* and *Candida dubliniensis*, the most common aetiological agent of oral candidiasis is *C. albicans* (Rautemaa and Ramage 2011).

1.2.1 *Candida albicans*

A gram-positive, eukaryotic organism, *C. albicans* multiplies asexually via mitotic cell division (ten Cate *et al.*, 2009) and is uniquely able to switch its morphology between yeast and hyphal modes of growth. This enhances the ability of cells to quickly adapt to new environmental niches, and contributes to the variation in expressed virulence attributes necessary to establish infection at various stages of colonisation and tissue penetration (Odds, 1994). *C. albicans* can therefore be found colonising areas as diverse as the mucosal surfaces of the mouth, digestive tract and genitourinary tract (Cannon *et al.*, 1995), and *Candida* infection may present clinically in many different forms.

The genetically dictated dimorphism of *C. albicans* is attributed to changes in the biosynthesis of the cell wall, which also mediates the interaction between the cell and its environment (Odds, 1988) connecting the cell wall directly or indirectly to the virulence of *C. albicans*.

The fungal cell wall is composed of polysaccharides β -1, 3-glucan, β -1, 6-glucan, chitin and, depending on the environmental conditions, a variable amount and composition of glycoproteins, (ten Cate *et al.*, 2009). β -1, 3-glucan and chitin are the structural components of the cell wall. The surface glycoproteins form the second major architectural component of the cell wall and are linked to the inner structure by a flexible layer of β -1, 6-glucan. The surface layer of cell wall proteins determine the physico-chemical properties of the cell and helps facilitate cellular interaction with the proteins found in the oral environment, for example in saliva, and on other microbial inhabitants (ten Cate *et al.*, 2009). For example the outer wall of *C. albicans* contains two surface proteins, Eap1 and Hwp1, that are involved in the adhesion of cells to biotic and abiotic surfaces, such as those found in the oral environment, and thus the formation of adhered colonies or biofilms (ten Cate *et al.*, 2009).

1.2.2 Biofilms in the oral environment

Biofilms are communities of microorganisms adhered to a surface and encased in a matrix, mostly of microbial origin (Cannon and Chaffin, 1999). The colonisation and subsequent biofilm formation by *C. albicans* is modulated by a number of environmental factors and can be an aetiological factor in oral disease.

The severity of oral fungal infections has been directly linked to increased numbers of yeast cells present at the site of mucosal infection (Theilade and Budtz-Jørgensen, 1988); (Budtz-Jørgensen *et al.*, 1996). Cell adhesion, colony growth and biofilm development can occur on abiotic surfaces such as dental acrylics, which can then act as microbial reservoirs. As with many biofilm infections, complete resolution of candidiasis can be difficult as the biofilms can demonstrate up to a 100-fold increase in resistance to the antifungal fluconazole (Chandra *et al.*, 2001a). This can result in chronic or recurrent infection that can lead to further problems such as impaired wound healing, acquired antifungal resistance, and spread of infection (Hall-Stoodley, 2009). There are multiple environmental factors that influence the colonisation of a surface by *C. albicans* and biofilm formation (Table 1).

Table 1. *Factors affecting the formation of C. albicans biofilms*

Factors affecting the development of <i>C. albicans</i> biofilms			
medium	inoculum	hydrodynamics	substrate
• composition e.g. mucin, glucose • temperature • presence of other micro-organisms, antifungal agents	• species, strain e.g. <i>Candida albicans</i> • number of cells • growth time/ biofilm age	• salivary and /or fluid flow rate • presence of shear forces	• roughness • composition, abiotic or biological • conditioning films (pellicles)

Factors that modify the attachment of *C. albicans* cells to a substrate include: variation in surface-texture; the chemical or biological composition of the substrate to which the microorganisms adhere; and hydrodynamic forces within the fluids surrounding the substrate that influence flow and shear forces across the surface.

The formation of a biofilm is the result of a number of physical, biological, and chemical processes that are modulated by the environmental conditions and stage of biofilm development itself (Bryers, 2008). In the oral environment the biofilm develops through a series of stages from the moment saliva first contacts the biological or

prosthetic surface through to the formation of complex adhered microbial communities (Fig. 1. adapted from Subramani, 2009).

Time	Event	Players
0-4hrs	Association	Salivary pellicle phosphoproteins, glycoproteins, albumin, lipids, lysozyme
	Adhesion	Bacterial colonisation mostly <i>Streptococcus</i> (e.g. <i>S. sanguinis</i> , <i>S. mitis</i>), <i>Actinomyces</i>
	Proliferation	
	Micro-colonies	
	Diversification	<i>Candida albicans</i> colonises
1-14 days	Biofilm formation	
	Growth maturation/ microbial succession	Anaerobic bacteria colonisation

Fig. 1. Development of an oral biofilm.

Examination of single species *C. albicans* biofilms has revealed that they are composed of an extracellular matrix, containing carbohydrates, protein, hexamine, phosphorus and uronic acids, and a mix of cell morphologies (Chandra *et al.*, 2001b). *In vitro* polymethylmethacrylate (PMMA) models have been used to study the development of *C. albicans* biofilms and show that they progress through three distinct developmental stages (Chandra *et al.*, 2001a) adhesion, germ-tube formation and maturation.

Germ-tube formation (the initial stage in hyphal growth) occurs 3-6 hours after the initial attachment of yeast cells, and is followed by an intermediate developmental stage characterized by the production of a matrix of extracellular material. Biofilm maturation continues, forming a dense network of yeasts, hyphae and pseudohyphae (Hawser and Douglas, 1994; Chandra *et al.*, 2001b). This mature biofilm is divided into two distinct layers. A layer of tightly packed yeast cells in contact with the substratum,

that seems to be important in anchoring the biofilm, and an overlying thicker, more open, hyphal layer (Baillie and Douglas, 1999; Kuhn *et al.*, 2002b). The confocal laser scanning microscope has allowed a detailed observation of the structure of the living biofilm to be observed, revealing a complex architecture comprising pedunculated biofilm islands of multiple aggregated cells with interspersed 'water', or nutrient, channels (Chandra *et al.*, 2001b).

1.2.3 Salivary pellicle

Saliva is a unique feature of the oral cavity and it is the polarity of its solubilised proteins, and those of the hard, non-shedding surfaces present in the oral environment that are key to the spontaneous adsorption of salivary proteins to oral surfaces and the formation of a salivary pellicle (Hannig and Hannig, 2009).

Saliva is composed of water and a variety of electrolytes, and proteins including immunoglobulins, enzymes and mucins. These components are present in low concentrations that vary with changes in salivary flow, and enable salivary functions such as modulation of pH and antibacterial action, as well as microbial aggregation. The parotid glands are the main producers of serous secretions that contribute most of the secretion volume and electrolyte content to both stimulated and unstimulated saliva, whereas the mucous secretions from the minor salivary glands, such as those found in the palate and cheeks, are produced at a relatively constant rate but contribute most of the mucins, immunoglobulins, and other proteins found in whole saliva (Humphrey and Williamson, 2001).

Almost immediately after cleansing, a tooth, restoration surface or inserted dental prosthesis, such as a denture, will develop a surface film via the selective adsorption of salivary glycoproteins or mucins (Tabak *et al.*, 1985). Depending on the contribution from major and minor salivary glands, the pellicle will vary in protein composition and therefore the adsorption pattern will vary (Jensen *et al.*, 1992). This salivary pellicle may act as a substrate lubricant, buffer or antibacterial barrier, but can also facilitate microbial adhesion and biofilm formation by incorporating specific microbial adhesion receptors such as mucin MG2, fibrinogen, lysozyme, proline-rich proteins and α -amylase, and providing a short term microbial nutrient source (Douglas, 1994; Nikawa *et al.*, 1996; Iontcheva, 1997). Within minutes of the formation of the salivary pellicle,

it has been observed that single cells of colonising microbial species from the normal oral commensals adhere to the film overlying the substratum.

1.2.4 Cell adhesion

The microbial biofilm, as a community of microorganisms may be single-species but is more commonly comprised of multiple species that participate in complex inter- and intra-species interactions. This interaction and communication can affect microbial adhesion and enhance the survival of specific species and the biofilm community as a whole. Cellular components and mechanisms specific to individual microbial species modulate their interaction with other microbial species and the environment, and instigate the chemical interactions with molecules within the environment that can lead to adhesion.

C. albicans can exist in both yeast and hyphal forms in the oral cavity, although planktonic cells are almost always in the yeast form, and initial surface contact is mediated by both specific cell-surface protein interactions and non-specific physico-chemical interactions such as hydrophobicity (Naglik *et al.*, 2011).

1.2.4.1 Physico- chemical interactions

The polarity, hydrophobicity and hydrophilicity, of both the substrate surface chemistry and saliva proteins can result in repulsive, or attractive forces between objects which can interact over a distance of 10-50 nm. Ionic and non-covalent electrostatic and acid-base interactions will also occur but only over a short distance of less than 5 nm. Non-covalent Van de Waals forces and Coulomb interactions will also impact on the adsorption of salivary proteins to oral surfaces and can act over a significant distance of up to 100 nm (Hannig and Hannig, 2009).

Some surface components of fungal cells have been identified as adhesins with a specific affinity for epithelial receptors (Calderone and Braun, 1991) facilitating the attachment of cells to the oral mucosa. At a molecular level, the adhesive forces between adhesins and receptors will be a combination of various physico-chemical interactions between atoms within the adhesin and receptor molecules.

Hard surfaces found in the oral environment can be both biotic (tooth structure) and abiotic (restorative or prosthetic materials). These surfaces differ to the epithelial

surfaces in that they are non-shedding and have characteristics specific to their chemical composition including the surface free energy.

Characteristics of the substrate such as polarity, hydrophilicity and wettability modulate the surface free energy and consequently the attraction and adhesion of the salivary proteins and formation of the pellicle and microbial biofilm (Teughels *et al.*, 2006).

In vitro studies also indicate that microbial adhesion onto materials used in dental restorative procedures is very dependent on the surface roughness and hydrophobicity of the material (Teughels *et al.*, 2006). The formation of a salivary pellicle, however, appears to have a masking effect, homogenising the characteristics of underlying substrate surfaces, and reducing the influences of features such as surface roughness and free surface energy (Göcke, 2002; Tanner *et al.*, 2003). This subsequently modulates the selective binding of bacteria to oral surfaces.

1.2.4.2 **Cellular mechanisms**

One to two hours following contact with host cells, almost all *C. albicans* yeast cells switch to the hyphal morphology (Dalle *et al.*, 2010). Hyphal-specific proteins Als3p and Hwp1p, members of the glycosylphosphatidylinositol (GPI) protein family found in the *C. albicans* cell wall, have been shown to be key adhesins that bind to specific epithelial cell receptors (Liu and Filler, 2011; Naglik *et al.*, 2006).

Part of the hyphal wall protein Hwp1p appears to be recognised by the host epithelial cells as a suitable substrate for the enzyme transglutaminase and the resultant cross-linked binding of *C. albicans* to epithelial cells appears to be irreversible. It is thought that this covalent binding may have some bearing on the ability of *C. albicans* biofilms present in the oral cavity to withstand the shear forces imposed by salivary flow (ten Cate *et al.*, 2009).

1.2.4.3 **Surface characteristics**

Early plaque formation is in part due to the mechanical retention of micro-organisms in irregularities on tooth surfaces (Lie, 1979) with initial colonization of enamel shown to start from the pits, grooves or abrasion defects in the surface (Nyvad and Ferjerskov, 1987).

Acrylic dentures and other restorative materials, like teeth, present as a non-shedding, hard surface to which the oral biofilm can adhere, and the surface topography of these

materials has been shown to affect the initial microbial adhesion (Nakazato *et al.*, 1989; Siegrist *et al.*, 1991). When considering the surface roughness and its effectiveness as protection from mechanical shear forces such as those caused by salivary flow or oral hygiene practices, several factors have importance. The average roughness (R_a) has been the most commonly reviewed, but the mean of maximal peak to valley heights or 'depth of defect' of a surface profile and the 'width' or average spacing between the profile peaks are also important in assessing the size of the irregularities on a surface.

A literature review in 1997 concluded that particular dental restorative materials such as gold, composite and acrylic (denture) resins, needed to be polished in specific ways, for example relative to their hardness, microscopic structure and particle size, in order to obtain a smooth surface (Bollen *et al.*, 1997).

Considering denture acrylic, the initial surface roughness ranges from an R_a of 0 μm for injection heat-polymerizing resin to 0.5 μm for autopolymerised resin. Use of a tungsten carbide bur, as per normal clinical protocol for denture adjustment, modified the surface roughness of auto-polymerised resin to 3.99 ± 1.14 , and injection heat-polymerizing resin to 2.86 ± 0.69 μm (Kuhar and Funduk, 2005). Normal lab protocol is to polish with a paste consisting of loose abrasives, such as aluminium oxide- Al_2O_3 , at 3000 rpm which gives an R_a 0.02 ± 0.01 μm for auto-polymerised resin, and 0.03 ± 0.01 μm (Kuhar and Funduk, 2005). Use of sandpaper has been assessed in only a few studies with 400grit producing an R_a of 0.75 ± 0.02 μm and a 1200 grit gave an R_a $0.26 \mu\text{m} \pm 0.01 \mu\text{m}$ for an undisclosed processing (Bollen *et al.*, 1997). Following a study in which the rate of microbial adhesion to implant abutments fabricated from machined titanium (R_a 0.2 μm) and ceramic (R_a 0.06 μm) where there was a significant increase in adhesion rate for surfaces with roughness over 0.2 μm , but not for smoother surfaces, it was proposed that a "surface roughness threshold" for microbial attachment could be set at 0.2 μm (Bollen *et al.*, 1996).

It has been suggested that the influence of surface roughness over-rides that of the surface-free energy. This was proposed following an *in vivo* study of plaque formation using resin strips glued to teeth (Quirynen and Bollen, 1995), the results of which were that an increase in the surface roughness of the resin strips above an R_a value of 2 μm resulted in a dramatic increase in the bacterial colonization in comparison to smooth strips ($R_a = 0.12$ μm).

An *in vitro* study of *C. albicans* adhesion to denture acrylic investigated the effect of surface roughness: from polished (mean R_a value of $0.23 \pm 0.17 \mu\text{m}$) to fine ($1.82 \pm 0.57 \mu\text{m}$) and coarse ($3.53 \pm 0.7 \mu\text{m}$) on adhesion. It was found that after one hour incubation with *C. albicans* cells the polished surface had only half of the surface coverage by cells compared to the fine surface finish but there was no significant difference between coverage of the fine and coarse surface finishes (Taylor *et al.*, 1998).

In another *in vitro* study, the presence of a salivary pellicle on *C. albicans* adhesion to roughened denture acrylic was investigated. In this study the salivary pellicle caused a reduction in adhesion of *C. albicans* to roughened denture material (Radford *et al.*, 1998), leading to the suggestion that the salivary pellicle somehow modified the influence of surface roughness on microbial adhesion.

1.2.4.4 **Environmental factors**

Salivary flow does not occur evenly throughout the mouth, and may be altered further by the presence of dental prostheses such as a maxillary upper denture, or implant bridge. Of particular relevance to microbial colonisation is the peripheral extension of the maxillary complete-denture prostheses, designed to enhance stability and create a seal to aid retention, that effectively isolates the palatal mucosa from the natural flushing effect and regulation of oral pH by the salivary flow from the parotid gland (Humphrey and Williamson, 2001). The lack of salivary flow under the denture, as well as the change to the environmental conditions (e.g. pH, nutrient availability), will affect biofilm formation and development.

The degree of fungal adhesion to hard surfaces within the oral cavity may also be modified by the adsorption of salivary components such as proteins and mucins onto the cell surfaces of already surface-adhered bacteria (O'Sullivan *et al.*, 2000) where they may act as ligands for *C. albicans* and facilitate their attachment (Calderone and Braun, 1991; Edgerton *et al.*, 1993; Nikawa *et al.*, 1993; Vasilas *et al.*, 1992).

1.2.4.5 **Interspecies interactions**

Although there is a predominance of certain species in specific locations within the oral cavity, such as *Streptococcus sanguinis* that colonizes only soft tissues, and *Simonsiella muelleri* which is present predominantly on the hard palate, some species such as *Granulicatella adiacens*, and *Streptococcus mitis* are common to all oral sites (Aas *et*

al., 2005). In the dentate population the predominant bacteria cultivated from samples of the palate are *Streptococcus* species (especially *mitis*), *Gemella* species (especially *haemolysans*), *Granulicatella* species (especially *elegans*) and *Neisseria spp.* (Aas *et al.*, 2005). It was originally thought that the loss of teeth in the edentulous patient would eliminate some bacteria from the mouth due to the lack of a suitable colonisation site, but microbiological analysis has revealed the contrary. Both *S. sanguinis* and *S. mutans*, although absent from the saliva of unrestored edentulous patients, return as prominent components of the denture biofilm (Carlsson *et al.*, 1969), and the predominant microbe in palatal samples again is *S. mitis* which contributes over 13.5% of the total microbial DNA in samples taken from edentulous subjects (Sachdeo *et al.*, 2008).

The pioneer species in the early colonisation of the salivary pellicle are primarily *Streptococcus* species, specifically *mitis*, *sanguinis* and *oralis* which make up 60-90% of the cultivated species (Nyvad and Kilian, 1987). These can metabolise carbohydrates producing glucans that allow the direct adhesion of microbes to surfaces (Kolenbrander and London, 1993). Although bacterial adhesion is initially weak and reversible it becomes irreversible with time.

The interaction between the primary coloniser bacteria and yeasts has been suggested as a modifying factor in the adherence of *C. albicans*, and the subsequent architecture and behaviour of the biofilm (van der Mei, 2000). Inter-generic co-aggregation of oral bacteria has been shown to involve protein-carbohydrate interactions and it is suggested that the interspecies co-aggregation that occurs between *C. albicans* and bacterial species may be a similar interaction (Jenkinson *et al.*, 1990). Adhesion studies have revealed that *C. albicans* is able to recognise polypeptides and cell wall polysaccharide receptors characteristic of certain streptococcal families (Holmes *et al.*, 1996). These findings suggest specificity in the coaggregation of oral commensal microbes and an interaction which is determined by recognition of complimentary surface components.

In vitro experiments have shown examples of this interspecies interaction with pre-treatment of experimental substrate surfaces with either *S. sanguis* (now *S. sanguinis*) or *Porphyromonas gingivalis* proving to have an inhibitory effect on any subsequent *C. albicans* cell adhesion, and adhesion is apparently enhanced when substrate surfaces are 'conditioned' with *Escherichia coli* (Nair and Samaranayake, 1996).

Modification of the growth medium in studies of *C. albicans* and *S. sanguis* biofilm formation have demonstrated that yeast cells subjected to glucose starvation have increased coaggregation with streptococcal species including *sanguis*, *gordonii*, and *salivarius* (Jenkinson *et al.*, 1990).

The impact of oral bacteria on the formation of *C. albicans* biofilms is not always favourable though, with *Lactobacillus acidophilus*, *Actinomyces israelii* and *P. gingivalis* having a variable suppressive effect on *C. albicans* growth (Thein *et al.*, 2006).

With more than 50% of the oral microbial taxa recognised by DNA analysis being, as yet, uncultivated (Aas *et al.*, 2005) it is possible the even more complex interspecies interactions exist within the oral biofilms that enhance and perpetuate the biofilm as a community.

Oral infections such as denture stomatitis and angular cheilitis have mixed-species aetiology with both *Candida* and bacteria such as *E. coli* and *Staphylococcus aureus* acting as co-infectors (Theilade and Budtz-Jørgensen, 1988). Increased numbers of yeast cells are present at the mucosal site of infection (up to 100 times that found on healthy mucosae) (Theilade and Budtz-Jørgensen, 1988) and there is a correlation between increasing population size and disease severity. Therefore the concentration of *C. albicans* cells within a biofilm is relevant to the initiation of the pathological processes associated with disease (Cannon *et al.*, 1995; Dalle *et al.*, 2010).

1.3 Infection and host defence

Some oral pathologies, such as candidiasis, may arise from opportunistic infections. The process that enables a normally benign commensal to become pathogenic to its host is linked to the complex interaction between the *C. albicans*, host epithelial cells and a reduced host tissue defence.

1.3.1 Clinical infection

A 2003 review described *Candida* -species as a major cause of hospital-acquired (nosocomial) infection, and the US National Nosocomial Infections Surveillance System ranked *Candida* as the fourth most common cause of bloodstream infection (Douglas, 2003). Although infection caused by biofilms formed on surgically implanted

devices, or as free forming plaques on damaged tissue such as heart valves is rare (Donlan, 2002), they can cause serious, and sometimes fatal, consequences. Biofilm contamination of dental prostheses in contrast, is frequent and has the propensity to recur making it difficult to treat.

As *C. albicans* is present as a normal human commensal, it is necessary to quantify the number of yeast present in order to diagnose a true *Candida* infection. Critical quantities of *C. albicans* colony forming units (CFU) in unstimulated saliva have been used to define the clinical categories of ‘carriers’ and infected individuals. A carrier is defined as someone with < 400 CFU/mL unstimulated whole saliva who is culture-positive only with no clinical signs or symptoms of candidiasis (Epstein *et al.*, 1980). Carriers are re-classified as having a chronic or acute infection if they have > 400 CFU/mL unstimulated whole saliva with a mean of ~1500 or ~3500 CFU/mL respectively, and clinical signs that occur with, or without, symptoms being described by the infected individual (Epstein *et al.*, 1980).

C. albicans is associated with, and can be the cause of, a spectrum of oral cutaneous lesions. The two most common forms of lesion are pseudomembranous, such as thrush, which presents as cream coloured, soft and friable mucosal plaques, and erythematous, for example denture stomatitis which is characterised by an area of mucosal erythema sharply delineated by the fitted surface boundaries of the denture or oral appliance (Cawson, 2008). Classification of *Candida* infection is according to the clinical presentation as a primary, or associated oral candidiasis, or as secondary when there is an oral manifestation of a systemic mucocutaneous candidiasis (Table 2). Primary candidiasis is further classified into the clinical variants; acute or chronic pseudomembranous (thick whitish coating); acute or chronic erythematous (inflammation); chronic plaque-like; and nodular (Axell, 1997).

Table 2. *Classification of C. albicans oral infections*

Infection	Classification	Examples
Primary oral candidiasis	Acute or chronic	▪ pseudomembranous, erythematous
	<i>Candida</i> - associated	▪ hyperplastic, erythematous, pseudomembranous ▪ e.g. denture stomatitis, angular cheilitis, median rhomboid glossitis
Secondary oral candidiasis	Oral manifestations of a systemic mucocutaneous disease	▪ e.g. thymic aplasia

Examples of *Candida*-associated lesions include denture stomatitis, angular cheilitis, and median rhomboid glossitis, with denture-induced stomatitis occurring in 11-67% otherwise healthy denture wearers (Budtz-Jørgensen, 1974). Another group of infections observed in the clinic, that may be classified separately, are the primary keratinized lesions that are secondarily infected with *C. albicans*. Lichen planus is a condition that lies within this classification, with 40% of cases having a secondary infection with *C. albicans*. Of significance in this group is leukoplakia in which the super-infection by *C. albicans* has been linked with the initiation of epithelial dysplasia, and oral neoplasia (McCullough *et al.*, 2002).

Tissue damage associated with *C. albicans* biofilms is the result of a distinctly-staged process. Initial adhesion needs to occur with the formation of germ-tubes, active penetration of tissue by hyphae and invasion via induced endocytosis occurs next, followed by necrosis and apoptosis of host cells (Naglik *et al.*, 2011; Wächtler *et al.*, 2011).

1.3.2 *Candida* invasion

The lack of epithelial penetration demonstrated by a hyphal-deficient mutant strain *C. albicans* (Korting *et al.*, 2003) indicates that, as with fungal adhesion, the hyphal surface contributes to *C. albicans* virulence and pathogenicity.

Contact between epithelial cells and *C. albicans* hyphae can trigger a response in both cells that instigates the invasion of the oral tissues by *C. albicans* cells (Dalle *et al.*, 2010). This can be as an induced endocytosis by the epithelial cells leading to the uptake of both viable and dead fungal cells into the epithelial cell (Naglik *et al.*, 2011), or a fungal-determined active penetration of the hyphae between or directly through the epithelial cells (Naglik *et al.*, 2011).

Endocytosis is a clathrin-mediated mechanism (Moreno-Ruiz *et al.*, 2009), and its induction is linked with several *C. albicans* cell proteins (Naglik *et al.*, 2011) but predominately by the interaction between host epithelial cell surface E-cadherin and hyphal-specific Als3p (Phan *et al.*, 2007). It occurs relatively early in the invasion process, within 4 hours of contact (Villar and Zhao, 2010), compared to active penetration, and internalization which occurs indiscriminately with both dead and vital fungal cells (Dalle *et al.*, 2010).

Active invasion of epithelial cells by fungal hyphae is suggested to be mediated by the secretion of lytic enzymes by *C. albicans*, specifically aspartyl proteinases (Ray and Payne, 1988). As it is a *C. albicans*-mediated process, it is likely that when a *C. albicans* biofilm overlies dead or functionally inactive epithelial cells such as in the stratified squamous epithelial layer of the oral mucosa, active penetration will still occur, followed by induced endocytosis when the hyphae reaches the underlying viable submucosal cell layer (Naglik *et al.*, 2011).

Although mucosal invasion may be facilitated by secreted hydrolytic enzymes, hyphal formation, and contact sensing, ultimately it is the host's immune competence that determines whether clearance, colonization, or candidiasis occurs (Cannon *et al.*, 1995).

1.3.3 Host defence

It has been suggested that the endogenous *C. albicans* population will proliferate to a pathological level (see section 1.3.1) when host and environmental conditions allow (Table 3. adapted from Odds, 1988), with evidence of a strong link between the level of host immune competence.

In the oral cavity, specific salivary components and the mechanical surface flushing created by the salivary flow provides an initial defence against *Candida* infection. Salivary glands are exocrine glands and their secretions contain immunologic proteins

including secretory IgA, IgG, and IgM that provide defence by directly damaging bacterial cells, and by aggregating or clumping micro-organisms inhibiting their attachment to host tissues. By reducing the ability of microbes to adhere to oral surfaces and reducing the bacterial population, fungal adhesion and subsequent colonization can be controlled (McNabb, 1981; Humphrey and Williamson, 2001). The salivary glands produce other secretions such as selected mucins, and peptides (e.g. histatins), which also have antifungal activity (Odds, 1994).

Table 3. *Factors that predispose for Candida infection*

Predisposing Factor	Explanation	Example
Infection or disease	infectious, idiopathic, congenital or other debilitating disease or disorder	compromised immunity, endocrine dysfunction e.g. Sjögrens syndrome , cell dysplasia e.g. lichen planus
Antibiotic or antibacterial, immunosuppressive therapy	treatment that alters the composition of endogenous micro flora, or the host immune response	antibiotics, corticosteroids, immunosuppressants, antidepressants
Malnutrition, malabsorption or dietary	excess or deficiency of foodstuffs that alters the host immune response, cell reserve, microbial environment	carbohydrate-rich diet, vitamin deficiencies
Mechanical	traumatic or environmental damage, or change due to the introduction of prostheses	dentures and maxillofacial obturators, surgery
Physiologic	digression from normal physiological state	pregnancy, infancy, elderly

Host epithelial cells have been shown to have a pro-inflammatory response to *C. albicans* cells in which the release of cytokines is dose-dependent and amplified by the presence of fungal hyphae (Moyes *et al.*, 2010). By recognising an alteration in the normal commensal load of *C. albicans*, and the presence and increase in the burden of

potentially cell damaging hyphae, oral epithelial cells initiate a direct host response to *C. albicans* infection.

Particularly relevant to the denture-wearing population, the aging mucosa shows a thinning of the epithelium and although there is an increase in collagen, there is a decrease in keratin, ground substance, specialized nerve endings, and vascularity (Hill, 1984). These physical changes that occur as a natural result of aging decrease the regenerative capacity and ability of the mucosa to resist injury and infection. This loss of regenerative capacity may also occur directly or indirectly subsequent to mechanical trauma, illness, disease or as a side-effect of pharmaceutical prescription (Odds, 1988).

It has been shown that host immune protection appears to be site-specific, and resistance to *C. albicans* infection at one particular anatomical site doesn't indicate an overall immunity to the species (Calderone and Fonzi, 2001). T-cell immune responses play an important role in protection against oropharyngeal, cutaneous and, perhaps, vaginal infections, however resistance to systemic disease is more often associated with a functional phagocytic response involving neutrophils or mononuclear phagocytes.

1.4 Antifungals and antifungal resistance

Infection by *C. albicans* usually responds to antifungal drugs if they are regularly applied topically to the affected mucosa, and to the dental appliance, if any is worn adjacent to the infected mucosa or, in cases of persistent infection, as a systemic medication. Difficulties in patient management occur with infection under fixed implant-supported complete denture prostheses where the option of repeated application of a topical antifungal is not so easily achieved, and in the case of chronic resistant infection.

1.4.1 Antifungals

There are relatively few drug targets that are specific for the fungus *C. albicans*, as these organisms, like their human host, are eukaryotes. Antifungal agents used for the treatment of systemic fungal infections are listed in Table 4.

Table 4. *Antifungal agents used for treatment of systemic fungal infections*

Class	Drug	Mode of action	Static/cidal*
Polyene	amphotericin B nystatin	affect cell membrane directly	fungicidal
Azole		inhibit synthesis of cell membrane ergosterol	
Imidazole	miconazole ketoconazole		fungistatic
Triazole	fluconazole itraconazole posaconazole voriconazole		fungistatic
Echinocandin	caspofungin micafungin anidulafungin	inhibit synthesis of cell wall β -1,3 glucan	fungicidal to yeast, fungistatic to molds
Fluoropyrimidine	5-fluorocytosin	inhibit synthesis of nucleic acid	fungistatic

* Whether the drug is fungistatic (inhibits fungal growth) or fungicidal (kills fungal cells)

There are contraindications for each drug type which may include side effects such as hepatotoxicity (especially polyenes), drug interactions (e.g. voriconazole), and restrictions in administration due to formulation (echinocandins) (Sable *et al.*, 2008). Fluconazole is a bis-triazole that works as an antifungal by binding and inhibiting Erg11p, a cytochrome P₄₅₀ enzyme 14 α -lanosterol demethylase, vital in the biosynthetic pathway of ergosterol. By inhibiting the biosynthesis of a major sterol in fungal cell membranes, there is a resultant decrease in ergosterol content in the cell membrane, and an accumulation of toxic ergosterol precursors, in particular 14-methylergosta-8,24(28)-dien-3,6-diol, which inhibit cell growth (Akins, 2005). As ergosterol is not present in animal cells this explains why azoles are relatively free from any serious host toxicity. Unfortunately, azoles have the limitation of being only fungistatic, inhibiting growth rather than killing fungal cells, and infection-related inflammation can relapse 2-4 weeks after treatment (Budtz-Jorgensen *et al.*, 1988). Nevertheless, fluconazole is the antifungal most commonly administered (Horn *et al.*, 2009) and it has been suggested that this widespread use of azoles has contributed to the development of

significant drug resistance in some *C. albicans* strains reducing the drug's therapeutic efficacy (White *et al.*, 1998a).

1.4.2 Antifungal resistance

As with bacteria, when *C. albicans* grows as a biofilm it becomes many times more resistant to antibiotics than when it grows planktonically –as a cell suspension in liquid media (Chandra *et al.*, 2001a; Donlan and Costerton, 2002). More concerning are the data indicating that the antifungal resistance of *C. albicans* is a trend now starting to be seen in populations other than the institutionalised patient who has a propensity to suffer from chronic or recurring candidiasis (Pfaller *et al.*, 2011). There have been a number of suggestions for the mechanisms by which the biofilms increase microbial resistance to antimicrobial agents (Table 5).

Table 5. *Proposed modes of biofilm resistance to antibiotics.*

Mode of resistance
• restricted penetration of drugs through the biofilm matrix (Seneviratne <i>et al.</i>, 2008) • phenotypic changes resulting from a decreased growth rate or nutrient limitation (Donlan and Costerton, 2002) • expression of 'resistance genes' induced by contact with a surface (Ramage <i>et al.</i>, 2002) • target modification by mutation or enzymatic changes (Levy, 2004) • target substitution (Levy, 2004) • antibiotic modification or destruction (Bryers, 2008) • expression of drug efflux pumps during the early phase of biofilm formation (Niimi, 2010) • alterations in membrane sterol composition (Mukherjee <i>et al.</i>, 2003) • 'persister' cells responsible for resistance (Lewis, 2007)

1.4.3 Azole resistance

1.4.3.1 Cellular mechanisms

The molecular mechanisms for azole resistance include over-expression, or mutation of the drug target, a mutation in other components of cellular metabolism resulting in altered drug degradation, or as a result of alteration in cellular transporter function and/or membrane permeability. With *C. albicans*, the azole drug target, Erg11p, can be over-expressed, as can the major facilitator superfamily (MFS)- type transporter Mdr1p, or the ATP-binding cassette (ABC) pumps (White *et al.*, 1998a) (Fig. 2. from Niimi, 2004a).

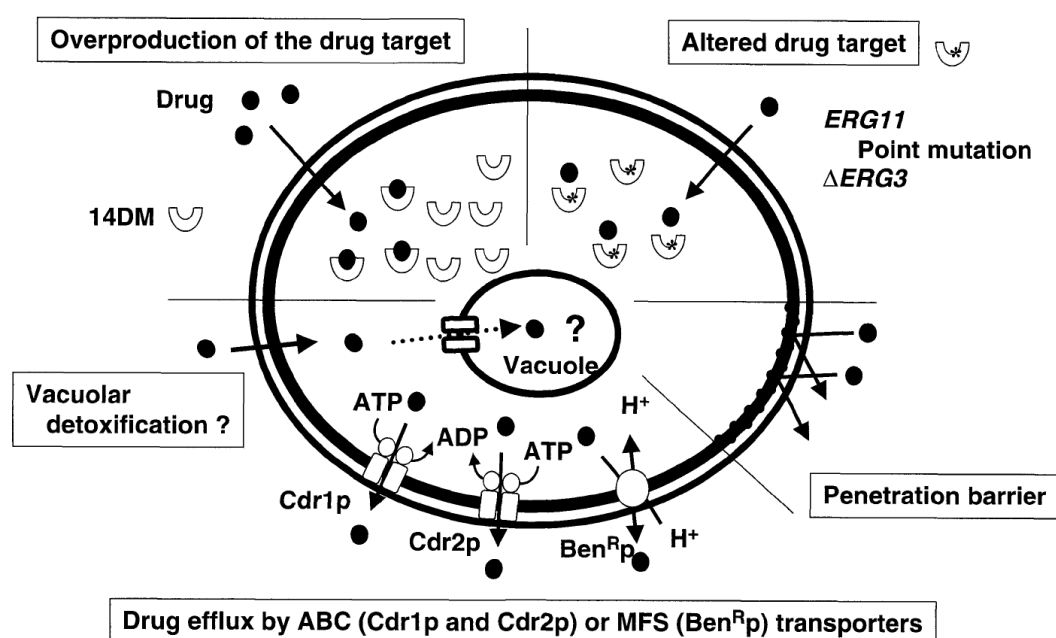


Fig. 2. Possible azole resistance mechanisms in *C. albicans*.

Molecular genetic studies suggest that azoles enter fungal cells by facilitated diffusion and that high-level azole resistance is most often associated with an energy-dependent drug efflux (Albertson *et al.*, 1996; Cannon *et al.*, 2009; Mansfield *et al.*, 2010). This mode of resistance has been further defined as an over-expression of plasma membrane transporters, specifically the ABC proteins Cdr1 and Cdr2 (Sanglard *et al.*, 1996;

Sanglard *et al.*, 1997; Holmes *et al.*, 2008) (Fig. 2). Genetically engineered strains of *C. albicans* with *CDR1*, or both *CDR1* and *CDR2*, genes deleted have increased susceptibility to azoles, and it has been shown that Cdr1p contributes more to *C. albicans* azole resistance than does Cdr2p (Holmes *et al.*, 2008).

The increased antifungal resistance seen in early stage biofilms has been suggested as linked to the expression of these ABC transporters (Mukherjee *et al.*, 2003), and an increase in metabolic activity of developing *C. albicans* biofilms (Chandra *et al.*, 2001a). The decrease in the ergosterol content of biofilm cells may also be of significance in the resistance of *C. albicans* to azole antifungals (Mukherjee *et al.*, 2003).

1.4.3.2 **Genetic factors**

At the molecular genetic level, analysis of the antifungal phenotype of biofilms has indicated that intrinsic cellular mutations lead to the development of a resistance phenotype of *Candida* cells. It has been shown that mutations altering cellular metabolic mechanisms have more influence on biofilm resistance than any biofilm-associated factors (Ramage, 2002). Genetic resistance that arises from mutation may manifest as a normal distribution of primary resistance levels within a population, or there may be secondary, induced, mutations that occur after long-term exposure to an environmental pressure such as antifungal drugs, where selective pressure results in the growth of more resistant strains (Perumal *et al.*, 2007).

The link between *Candida* azole resistance and an increased cell density (Perumal *et al.*, 2007) supports the theory that the increased antifungal resistance of biofilm pathogens occurs primarily as a result of the increased probability of mutation that occurs with the increased cell density.

1.4.3.3 **Biofilms and microbial interaction**

C. albicans cells growing in a biofilm show a ~ 100-fold increase in resistance to the antifungal fluconazole (Chandra *et al.*, 2001b). As indicated in Table 5, the increased drug resistance seen in biofilm-associated microbes is considered to be associated with properties of the biofilm matrix, altered cellular growth and metabolic rates seen in biofilms, or by patterns in gene expression that are specific to the biofilm cell. Although it has been observed that the presence of bacteria and bacterial matrix

material in mixed *C. albicans* biofilms retards diffusion of the antifungal agents, as does the matrix material in single species biofilms, it does not account fully for acquired or increased drug resistance seen in biofilm *C. albicans* cells (Baillie and Douglas, 1999; Al-Fattani and Douglas, 2004). It has also been suggested that the binding of antifungals to biofilm matrix component β -1,3-glucan, found at elevated levels within the biofilm, may account for the increased resistance to fluconazole seen in *C. albicans* biofilms as compared to planktonic cells (Nett *et al.*, 2007).

Extracellular signalling molecules can modulate the behaviour of *C. albicans* through direct cell-to-cell communication, or indirectly by secreted metabolic by-products and extracellular molecules that are present within the biofilm. An example of this ‘quorum-sensing’ occurs when the mature biofilm, and aging planktonic *C. albicans* cells, secrete farnesol which inhibits the morphological transition of *C. albicans* yeast cells to the filamentous or hyphal form (Ramage, 2002). Secretion of compounds from bacterial species within the biofilm also act in quorum-sensing cell regulation processes that affect *C. albicans* (Hogan, 2004). By modifying the behaviour and morphological transitions of *C. albicans* cells these messengers modify biofilm antifungal resistance (Thein *et al.*, 2006).

1.5 Overcoming azole resistance

1.5.1 Measurement of antifungal susceptibility

It is important to quantify the susceptibility of fungi to antifungal drugs in order to guide antifungal therapy and evaluate the efficacy of drugs. The measure of fungal susceptibility to a drug is the minimum growth inhibitory concentration (MIC). This is the minimum concentration of a drug that will inhibit growth, typically in a liquid (planktonic) culture. Measuring the resistance of fungal pathogens to drugs with a standardized *in vitro* antifungal susceptibility testing protocol accepted by the Clinical and Laboratory Standards Institute (formally the NCCLS), and the European Committee for Antimicrobial Susceptibility Testing (EUCAST), has allowed for valid-cross referencing of multicentre studies, and for the development of a library of MIC values for a number of antifungal agents. This is extremely important when validating new drugs, and in planning treatment of patients with fungal infections. Biofilm cells are phenotypically different and numerically at a greater cell density than in the standardised planktonic NCCLS assays. Therefore, for biofilm-related infections such

as oro-pharyngeal candidiasis, there is limited correlation between *in vitro* liquid MIC results and therapeutic outcomes. To enable a closer correlation, assays need to be modified to include testing the drug susceptibility of the developing biofilm. A commonly used model for *in vitro* testing is to use colorimetric assay of *C. albicans* biofilm grown on the walls of a polystyrene microtitre plate, or as sessile *C. albicans* cells grown on substrate discs on the well-floor of microtitre plates (Jin *et al.*, 2003; Nett *et al.*, 2011a).

Crystal violet (CV; hexamethyl pararosaniline chloride), also known as Gentian violet, is a triarylmethane dye that is taken into the cell nucleus and its intensity of colour is proportional to the amount of dye taken into the cells. Assuming it penetrates all cells equally, a relatively simple method to quantify the cell numbers within biofilm samples is by measuring the colour subsequently released by stained cells. Although the CV assay allows a quantitative measure of the cell density, staining cannot be used to distinguish between healthy cells, cells that are alive but losing cell functions, and dead cells.

Care must also be made when using optical density readings with regards to the pH of the liquid culture or biofilm being tested as the colour of the dye is pH dependant. When dissolved in water the dye has a blue-violet colour with an absorbance maximum at 590 nm, but at a pH of 1.0 the dye is green with absorption maximum at 420 nm and 620 nm, and in a strongly acidic solution (pH of -1), the dye is yellow with an absorption maximum at 420 nm (Adams, 1914).

Another way of measuring the susceptibility of *C. albicans* to drugs is to do viable cell counts where cells are plated on solid media, incubated, and colony forming units (CFU) counted. Although viable cell counts have problems with enumeration when applied to biofilms due to the high variability of *Candida* morphological forms, especially the presence of lengthy hyphae which give rise to only one colony when cultured, they are still considered as a valid way of measuring antifungal susceptibility.

1.5.2 Chemosensitization

One way to overcome the azole drug resistance of *C. albicans* is to inhibit the drug efflux pumps that reduce the intracellular azole concentration (Fig. 3. from Cannon *et al.*, 2009). This approach has been validated by the demonstration that the combination of immunosuppressant FK506 with azoles was fungicidal for strains of *C. albicans*

expressing *CDR1* (Schuetzer-Muehlbauer *et al.*, 2003). Modification of the calcineurin-mediated stress tolerance of the *C. albicans* cells with the calcineurin inhibitor FK506 removes the growth dependence on plasma membrane efflux pumps such as those encoded by *CDR1* and *CDR2*. Similarly, a combination of cyclosporine A and fluconazole has proven to be fungicidal to a fluconazole-resistant *C. albicans* strain (Marchetti *et al.*, 2000; Marchetti *et al.*, 2003; Uppuluri *et al.*, 2008).

Although these synergies have shown success in overcoming fluconazole resistance and proved to be fungicidal to azole-resistant *C. albicans* strain, the effect of prescribing immunosuppressants such as cyclosporine A or FK506 on an already immunocompromised host could lead to unpredictable and undesirable clinical effects. Reinstating the antifungal potency of the specifically targeted azoles is of considerable interest as it potentially provides an ideal, low-risk, solution to the treatment of resistant *Candida* infections in polypharmic, and severely debilitated patients such as the immunocompromised.

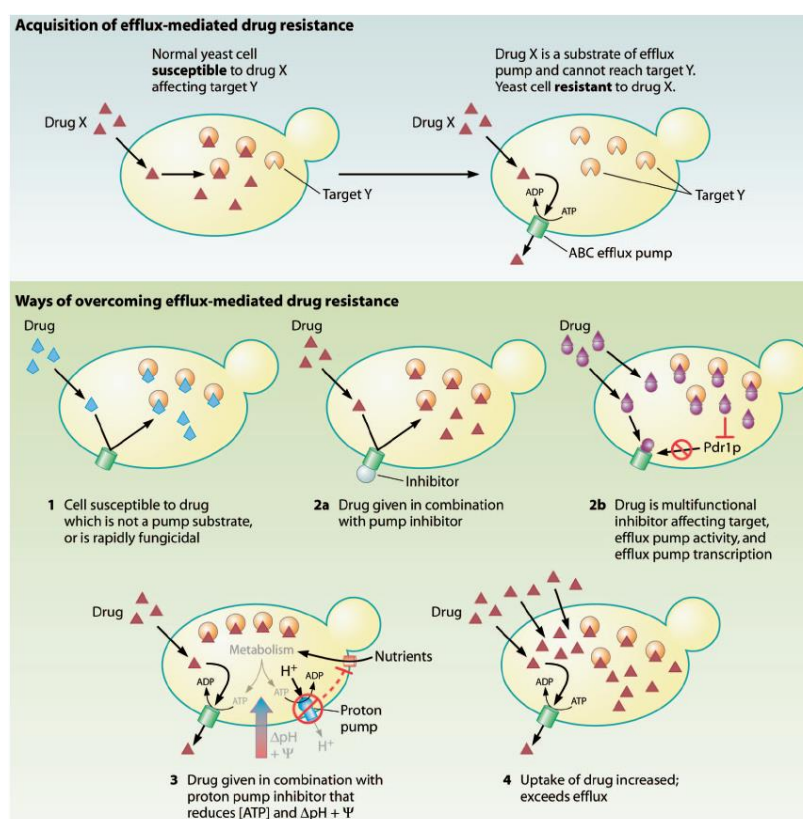


Fig. 3. Efflux-mediated fungal drug resistance

1.6 Efflux pump inhibitor RC21

1.6.1 Development of RC21v3

Within the Molecular Microbiology Laboratory (MML) of the Sir John Walsh Research Institute there is a D-octapeptide combinatorial drug library. This library consists of octapeptides chemically synthesised from D-amino acids to prevent degradation of the peptides by human proteases thereby increasing their biological half-life when used in animal infection models, or when applied to humans. The peptides have the general formula: D-NH₂-ABX₃X₂X₁RRR-CONH₂ where A and B are two specified amino acids and X₁, X₂, and X₃ individually can be any one of 18 D-amino acids (cysteine and glycine are not present for chemical synthesis reasons). The library is ordered into 324 (18²) peptide pools according to the possible combinations of the 18 amino acids in the A and B positions. Within each pool there are theoretically 5,832 (18³) peptides due to all possible combinations of 18 amino acids at the X₁, X₂, and X₃ positions. This means that the library potentially contains 1.89 x10⁶ (18⁵) different D-octapeptides that can be screened to identify peptides with particular properties. For example the library has been screened in the MML to find inhibitors of *Saccharomyces cerevisiae* drug efflux pump Pdr5p (Niimi *et al.*, 2004b), *S. cerevisiae* plasma membrane ATPase Pma1p (Monk *et al.*, 2005) and, more recently, *C. albicans* drug efflux pump Cdr1p (Niimi, 2012). The inhibitor of Cdr1p, octapeptide RC21, was modified to a more potent derivative RC21v3 which is a highly specific inhibitor of the CaCdr1p-mediated multidrug efflux pump in *C. albicans* (Niimi, 2012).

RC21v3 is able to chemosensitize *C. albicans* cells to fluconazole by inhibiting Cdr1p (Fig. 4. courtesy R. Cannon). The observation that it doesn't penetrate the fungal cell suggests that it is less likely that RC21v3 will be degraded by intracellular detoxification pathways and it has been proposed that it would remain biologically active for some time (Niimi, 2012). Additionally, it has been shown that at concentrations of up to 100 µM, RC21v3 has no deleterious effect on the viability of a human epithelial or red blood cells (Niimi, 2012).

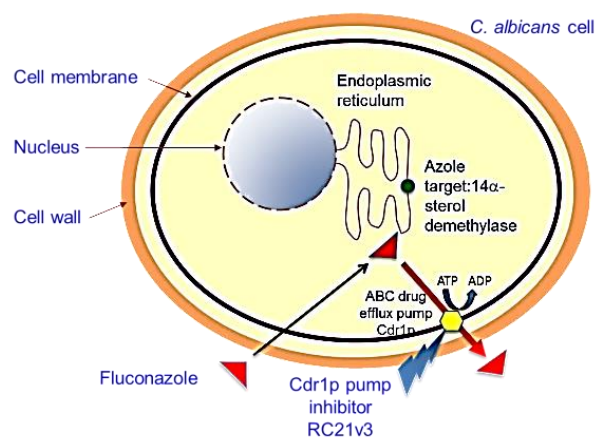


Fig. 4. Synergistic action of the peptide derivative RC21v3 and antifungal fluconazole on *C. albicans*.

Importantly, RC21v3 has been shown to be effective in combination with fluconazole in overcoming azole-resistance in an animal model of candidiasis (Hayama *et al.*, 2012). Although RC21v3 has no antifungal activity by itself, there was a promising dose-dependent enhancement of fluconazole activity that reduced both the clinical symptoms of oral candidiasis in mice and the viable cell count for both resistant and susceptible strains of *C. albicans* (Hayama *et al.*, 2012).

Development of this peptide in combination with azoles would be of considerable benefit to the prosthodontist in the treatment of patients with persistent *Candida* infection under fixed or removable dental prostheses.

1.7 Research aims and hypothesis

1.7.1 Aims

Further studies are needed to confirm that the use of RC21v3 makes the fungistatic azole fluconazole fungicidal. As it is azole-resistant *C. albicans* strains that have the most clinical significance, the study will include both fluconazole-susceptible and fluconazole-resistant *C. albicans* strain where the resistance is due primarily to expression of efflux pump Cdr1p.

It is evident that oral candidiasis is linked to the development and growth of the microbial biofilm. The behaviour of biofilm *C. albicans* cells and their morphology is

different to planktonic cells, so to study clinically-relevant antifungal efficacy a biofilm model must be used and the effect of RC21v3 in combination with fluconazole on growth and viability of *C. albicans* biofilms developed on denture acrylic measured.

When using *in vitro* analysis of the behaviour of *C. albicans* with a view to extrapolating the results to the dental patient, it is important to use a model that closely resembles the biofilm associated with the disease that develops within the oral environment. The common protocol for measuring biofilm growth uses acrylic discs submerged in the wells of standard microtitre plates that are then incubated statically (Kuhn, 2002a). Gravity and settling results in a layer of cells resting on top of the substrate which is not representative of the oral environment where oral surfaces are subject to the flow and forces associated with the movement of saliva. A suspended substrate may more closely approximate the *in vivo* denture environment with agitation during the assay mimicking the biodynamic movement of fluid in the oral cavity. Therefore one of the aims of the study is to confirm that RC21v3 acts synergistically with azoles to both inhibit growth and kill planktonic *C. albicans* isolates.

A clinically relevant substrate model will be developed to enable biofilms to be transferred with minimal disturbance through the experimental stages. This novel biofilm model will then be used to investigate the RC21v3 acts synergistically with azoles to both inhibit growth and kill *C. albicans* isolates grown as a biofilm.

1.7.2 Hypotheses

The hypotheses underlying this study are that:

1. A modified biofilm growth assay involving suspended acrylic substrate can be developed that will more closely mimic biofilms on denture surfaces and can be used to measure the synergism of drugs against *C. albicans* biofilms;
2. RC21v3 will act synergistically with fluconazole to inhibit the growth of, and kill, *C. albicans* planktonic cells; and
3. RC21v3 will act synergistically with fluconazole to inhibit the growth of, and kill, *C. albicans* biofilm cells.

2 Materials and Methods

2.1 Materials

2.1.1 Strains and growth media

Candida albicans

C. albicans strains used in this study are listed in Table 6. They include ATCC 10261, a well-studied fluconazole-susceptible laboratory strain; MML610, a fluconazole-susceptible clinical isolate; and MML611 a fluconazole-resistant isolate derived from MML610 and chosen specifically as its resistance is due primarily to over-expression of efflux pump Cdr1p (Holmes *et al.*, 2008).

Table 6. *C. albicans* strains used in this study

Strain	Phenotype	Reference/source
ATCC 10261	Wild type laboratory strain Azole-susceptible	American Type Culture Collection
MML610	Azole-susceptible	(Holmes et al., 2008; Marr, 2001), White, T. (Seattle, WA)
MML611	Azole-resistant	(Holmes et al., 2008; Marr, 2001), White, T. (Seattle, WA)

Growth media

Cells were maintained on yeast extract peptone dextrose (YPD) agar plates containing 1% (w/v) Bacto yeast extract (Becton, Dickinson and Company, Sparks, MD, USA), 2% (w/v) Bacto peptone (Becton, Dickinson and Company), 2% (w/v) glucose and 2% (w/v) agar at pH 5.5. Agar plates were stored at 4 °C until required.

Liquid susceptibility assay growth medium was made by dissolving 10.3 g of RPMI 1640, containing glucose 0.2% (w/v), from Sigma (St. Louis, MO) in 500 mL of water and adding 0.165 mol (34.53 g) MOPS buffering agent (3-(N-morpholino)

propanesulfonic acid, sodium acetate trihydrate, EDTA) from Sigma (St. Louis, MO). The solution was adjusted to pH7 with addition of NaOH prior to the total volume made up to 1 L with water before autoclave sterilisation.

2.1.2 Saliva

Whole saliva (14 mL) was collected from a healthy subject with no recent history of medication or illness into a sterile universal glass bottle kept on ice. The saliva was then centrifuged in a Corex glass centrifuge tube at 12,000 g at 4°C for 15 min to remove oral microorganisms and debris. Portions of the supernatant (5 mL) were put into fresh sterile universal glass bottles, and mixed with an equal volume of KCl buffer. KCl buffer (1 L) was made up of solution A containing in 800 ml water: 0.27 g KH_2PO_4 (final concentration in complete buffer 2 mM), 0.46 g K_2HPO_4 (final concentration in complete buffer 2.6 mM) 0.373 g KCl (final concentration in complete buffer 5 mM), pH 6.5 and solution B containing, in 200 mL water: 0.15 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (final concentration in complete buffer 1 mM); solutions A and B were autoclaved separately before mixing (Cannon *et al.*, 2010).

One tablet of protease inhibitor ('cOmplete mini', EDTA-free Roche Diagnostics, Germany) was then added to each 10 mL of diluted saliva prior to division into assay aliquots and storage at -20°C until used (Ramage *et al.*, 2004).

2.1.3 Chemicals

Antifungal

Fluconazole (FLC) was purchased from Pharmaceuticals Limited (Auckland, New Zealand) at a stock concentration of 2 mg/mL and diluted in water to a working concentration of 128 µg/mL.

RC21v3

D-octapeptide RC21v3, D-NH₂-FFKWQRRR-CONH₂[Mtr], (Niimi *et al.*, 2012) was manually synthesised, purified by high performance liquid chromatography (HPLC) and characterized by mass spectrometry at the Centre for Separation Science at Massey University (Palmerston North, New Zealand). The peptide was dissolved in dimethyl sulfoxide (DMSO) and, as the peptide contained a single tryptophan, the concentration was determined by measuring its absorbance at 280 nm (11.78 mM). For this series of

experiments the concentration according to its absorbance at 280 nm was 12.9 mM confirming the RC21 peptide as version 3 (RC21v3).

Acetic acid

10% (v/v: 5 mL glacial acetic acid (Merck, Darmstadt, Germany) in 45 mL distilled water).

Crystal violet

0.5% (w/v) crystal violet (Sigma-Aldrich, St Louis, Missouri, U.S.A.) was dissolved in water, filtered through a 0.44 µm filter and diluted with distilled water to a final concentration of 0.1% (w/v)

Live-dead BacLight stain

A Live-dead *BacLight* cell viability staining kit (Life Technologies, Auckland, New Zealand) that includes the dyes SYTO-9 (green fluorescence) and propidium iodide (PI) (red fluorescence) was used. Prior to use, fluorescent stains were dissolved in 30µL dimethyl sulfoxide (DMSO) to give a concentration of 0.3% (w/v).

2.1.4 Biofilm substrate

To enable biofilm susceptibility testing, several substrate designs were developed to identify a suitable model which was then investigated to assess reliability as an experimental model (see results section pages 52-55).

Test substrates were fabricated from polymethylmethacrylate (PMMA), an acrylic resin material used in the construction of denture bases. Conventional injection-moulding processing techniques (vacuum flask and heat cure) were used with Vertex™ Regular Crystal Clear dental acrylic (Vertex-Dental B.V., Zeist, the Netherlands).

Substrates were then polished with 400 grit sandpaper followed by 800 grit sandpaper before decontamination by immersion in chlorine solution for 15min (as per removable denture pre-delivery clinical protocols) using 2 x 500 mg tablets of anhydrous sodium dichloro-1,3,5-triazinetriene (Guest Medical Ltd. Aylesford. U.K.) in 40 mL filtered de-ionized (ultra-pure) water. Substrates were then stored in sterile ultra-pure water at room temperature until needed.

2.2 Methods

2.2.1 Biofilm development

C. albicans cells from single colonies on YPD agar plates were used to inoculate 1 ml RPMI 1640 (0.2% w/v glucose) and incubated at 35°C, with agitation at 150 rpm for 18 h using a shaking incubator (Bio-Line, Edwards Instrument Company, Narellan, NSW, Australia).

The concentration of cells was calculated by measuring the optical density at 600 nm (OD₆₀₀) using a spectrophotometer (Ultrospec 6300pro Amersham Biosciences, NJ, USA), and diluted with standard RPMI 1640 to give a cell concentration of 1×10^7 cells/mL (Seneviratne *et al.*, 2009).

Prior to biofilm formation, acrylic substrates were re-sterilized by immersion in chlorine solution (2 x 500 mg tablets of anhydrous sodiumdichloro-1, 3, 5-triazinetriene (Guest Medical. U.K.) in 40 mL filtered de-ionized water) for 15 min followed by quick immersion in absolute ethanol (100%) and brief flaming

Sterile substrates were pre-treated by submersion in 200 µL prepared saliva and incubation at 37°C for 30 min. The saliva-coated acrylic substrates were then rinsed with growth medium (RPMI 1640) before being suspended in the microtitre plate wells containing 200 µL of RPMI 1640 inoculated with a standardized concentration of *C. albicans* cells.

Initial incubation was at 37°C with agitation (150 rpm, shaking incubator) for 12 h to create an even dispersion of cells through the medium and to ensure maximum contact of the inoculum with the substrate. Then the substrate was transferred to microtitre plates containing fresh growth medium (RPMI 1640 supplemented with 1.8% w/v glucose) where they were incubated with a more gentle agitation (50 rpm) (Nett *et al.*, 2011b). These aerobic, dynamic conditions were chosen to mimic the oral environment and have been shown to modify and increase the biomass of *C. albicans* biofilms (Thein *et al.*, 2007)

The substrates were transferred to microtitre plate wells containing 200 µL fresh growth medium (RPMI 1640 supplemented with 1.8% w/v glucose) every 24 h to ensure a continuous nutrient supply for the developing biofilm.

The growth of biofilms was assessed with the crystal violet assay at 12, 24, 36, 48, 60 and 72 h by transferring substrates to a fresh microtitre plate prepared for the quantification assays (see section 2.2.4).

2.2.2 Liquid MIC susceptibility assay

Sterile 96-well, flat-bottom microtitre plates (Nunc, Thermo Fisher Scientific, North Shore, New Zealand) were used for the liquid MIC determinations. The peripheral microtitre plate wells contained 200 μ L RPMI, to act as a negative no-drug, no-inoculum control, and to help prevent desiccation of the inner microtitre plate wells during incubation.

A two-fold dilution series of fluconazole was made in the growth medium RPMI 1640 (0.2% w/v glucose) with thorough mixing of each dilution before transfer of 100 μ L to the new dilution series of wells (containing 100 μ L RPMI 1640).

MIC assays for *C. albicans* strains were carried out in triplicate on the same microtitre plate. *C. albicans* cell suspension (100 μ L of 1×10^4 cells/mL) was added to the wells in triplicate rows on the microtitre plate. One column of the microtitre plate contained 100 μ L of the top concentration of fluconazole (20 μ g/mL), and one column of the microtitre plate contained no fluconazole, only growth medium (RPMI), thereby acting as a positive cell growth control (Fig. 5).

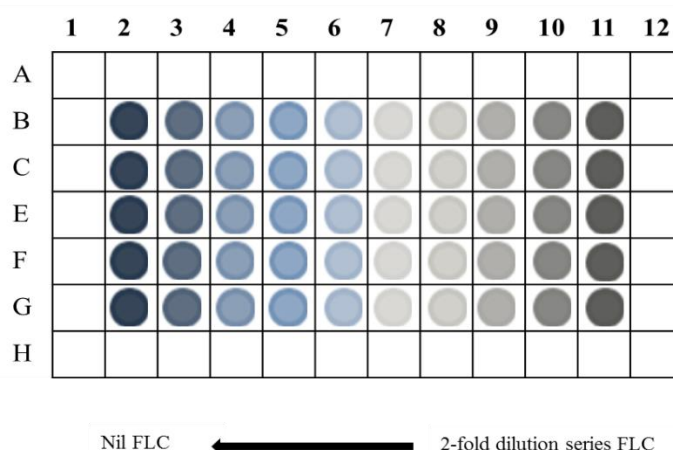


Fig. 5. Microtitre plate layout for liquid MIC assay. A 2-fold dilution series of fluconazole was prepared in a microtitre plate to which a standardised cell culture was added (● RPMI only; ● FLC 10 μ g/mL top concentration).

Microtitre plates were then incubated at 37°C with agitation (200 rpm) for 24 h. The growth was measured with a spectrophotometer (OD₆₀₀) and the minimum growth inhibitory concentration was recorded as the minimum concentration of drug resulting in >80% growth inhibition relative to the no-drug control (MIC₈₀).

Minimum fungicidal concentrations were determined for wells with no visible cell growth by taking 5 µL samples from these wells and plating them on YPD agar plates. Plates were then incubated at 37°C for 24 h prior to counting the number of colonies that grew (colony-forming units). The lowest drug concentration resulting in no growth on the agar plate was recorded as the minimum fungicidal concentration.

The reading of growth in MIC assays was repeated at 48 h using a Synergy 2 (Biotek Instruments Inc., Vermont, U.S.A.) microtitre plate reader and results recorded using Gen5 Microtitre Data Collection Analysis software (Biotek Instruments Inc., Vermont, U.S.A.).

The MIC assay was repeated twice on separate days using fresh yeast cell suspensions.

2.2.3 Checkerboard liquid MIC susceptibility assay

A checkerboard assay was carried out using sterile 96-well, flat-bottom microtitre plates to assess the susceptibility of *C. albicans* cells to drug combinations (Niimi *et al.*, 2012; Holmes *et al.*, 2008).

Planktonic cell drug sensitivity assay

As with the liquid MIC assay, the peripheral microtitre plate wells contained 200 µL RPMI 1640, to act as a negative, no-drug, no-inoculum control and to help prevent desiccation of the inner microtitre plate wells during incubation. Aliquots (200 µL) of RC21v3 (80µM) were distributed to 6 wells in column 11 of the microtitre plate and a two-fold dilution series (100 µL + 100 µL RPMI 1640) made horizontally, right to left, with thorough mixing of each dilution.

Fluconazole LC, at a concentration of 40 µg/mL (for strains ATCC 10261, MML610) or 160 µg/mL (for the strain MML611) was distributed to 10 wells in row B of separate microtitre plates. A two-fold dilution series (100 µL + 100 µL RPMI 1640) was then

made vertically in the growth medium with thorough mixing of each dilution before transfer to the next row of wells in the dilution series (Fig. 6).

Portions (50 μ L) of the fluconazole dilution series were then transferred to the corresponding wells of the microtitre plate containing the RC21v3 dilution series to give a total volume of 150 μ L (one row contained the top concentration of fluconazole and one row contained no antifungal, one column of the microtitre plate contained 150 μ L of the top concentration of RC21v3 and one column contained no peptide). Cells suspensions were prepared for MIC assays and 50 μ L of *C. albicans* cells (2×10^4 cells/mL) was added to each well (excluding the peripheral wells). Plates were then incubated at 37°C with agitation (100 rpm) for 48 h and the MIC was determined using the Synergy 2 (Biotek Instruments Inc., Vermont, U.S.A.) microtitre plate reader to measure the OD₆₀₀. Results were recorded using Gen5 Microtitre Data Collection Analysis software (Biotek Instruments Inc., Vermont, U.S.A.).

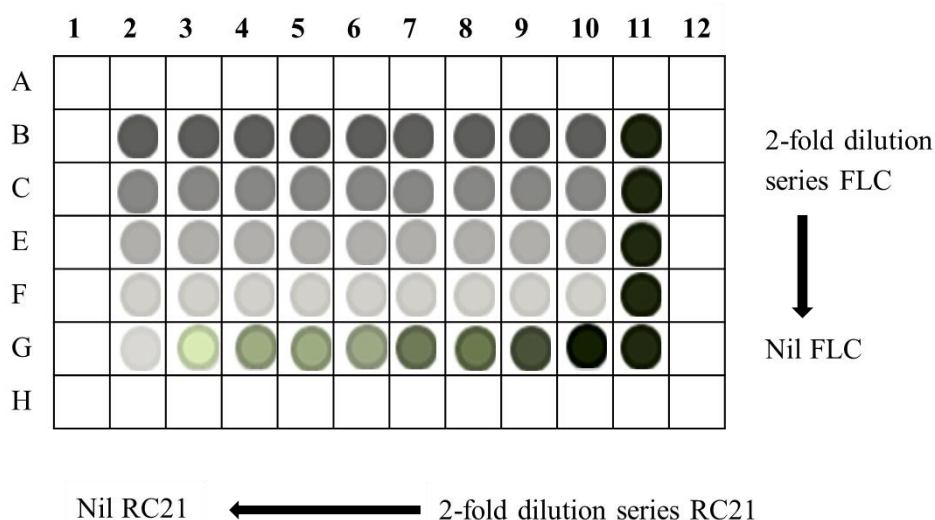


Fig. 6. Microtitre plate layout for checkerboard liquid MIC assay. A 2-fold dilution series of fluconazole and RC21v3 was prepared in two separate plates and then combined to make the checkerboard plate. (● FLC 10 μ g/mL top concentration; ● RC21 20 μ g/mL top concentration)

Biofilm cell drug sensitivity assay

For the biofilm assay the fluconazole dilution series (100 μ L) was carried out using supplemented RPMI 1640 (2% w/v glucose) before being transferred to the

corresponding wells of the plate containing the RC21v3 dilution series, also carried out in RPMI 1640 (2% w/v glucose), to give a total volume of 200 μ L (one row contained the highest concentration of fluconazole and one row contained no antifungal, one column of the microtitre plate contained 200 μ L of the highest concentration of RC21v3 and one column contained no peptide). Biofilms of a single *C. albicans* strain were grown for 48 h on portable substrates before being added to the wells on the checkerboard plate. Plates were then incubated at 37°C with agitation (50 rpm). At 24 and 48 h the MIC was determined using the crystal violet growth assay (see section 2.2.4) (Nett *et al.*, 2011a).

2.2.4 Biofilm formation quantification using crystal violet

Biofilm formation was assessed through quantification of total biomass by crystal violet (CV) staining. Biofilms were grown on substrates which were then transferred to the CV assay microtitre plates. Biofilms were fixed by immersing the substrates in wells containing 200 μ L of 99% (v/v) methanol. They were removed after 15 min, and the substrates and attached biofilms allowed to air dry at room temperature. The substrates were then immersed in CV (200 μ L) 0.1% (w/v) in the wells of a fresh microtitre plate and incubated at 37°C for 20 min. The substrates and adhered biofilms were then removed from the wells, gently washed by dipping (2 x) in sterile, ultra-pure water and air dried. Dye was released from the stained biofilm by immersing the preparation in microtitre wells containing 200 μ L of acetic acid 10% (v/v) for 30 min. Substrates were removed from the acid and the solution in the microtitre plate wells subjected to agitation to mix the contents of each well thoroughly before the absorbance of the resultant solution was measured in a Synergy 2 (Biotek) microtitre plate reader at OD₅₉₀ (Adams and Rosenstein, 1914). Results were recorded using Gen5 Microtitre Data Collection Analysis software.

When comparing biofilm MIC results between strains or to the initial planktonic assays, the 48 h incubation period required to establish the biofilm prior to the checkerboard assay means that the extent of the biofilm when drugs are added may vary from experiment to experiment. Triplicate assays were carried out to reduce the error associated with variation in biofilm size.

2.2.5 Confocal microscopy

Non-invasive examination of the biofilm as a three-dimensional structure, was made using the Live-dead *BacLight* cell viability staining kit (Life Technologies), with SYTO-9 remaining within the metabolically active cells as a green fluorescence, and propidium iodide (PI) cancelling out the SYTO-9 in non-viable (dead) cells thus staining the cells red. The fluorescence of the stained biofilm was recorded with confocal microscopy. Following biofilm formation and exposure to fluconazole and/or RC21v3, or neither drug, selected biofilms were prepared for confocal examination.

Adhered biofilms were dipped sequentially three times in fresh PBS to remove traces of growth medium (da Silva *et al.*, 2010).

In a dark-room each fluorescent stain was dissolved in 3 mL dimethyl sulfoxide (DMSO) to give a concentration of 0.3% (w/v) before equal volumes (100 μ L) were combined in the wells of a fresh microtitre plate and mixed thoroughly. Biofilms were transferred to the wells and the plates were covered with aluminium foil to exclude UV-light. The plates were incubated at 37°C for 20 min, followed by sequential washing three times in RPMI (0.2% glucose) before confocal microscopy. Safety precautions were taken to avoid operator exposure to the dyes (Seneviratne *et al.*, 2009).

Visualisation of the fluorescently stained biofilm was by confocal laser scanning microscopy (Zeiss LSM 710; Carl Zeiss Microscopy GmbH, Carl Zeiss, Jena, Germany. Software: Zeiss ZEN 2009, running on Windows Vista) undertaken in the University of Otago Centre for Confocal Microscopy using a 10 x PlanApo 0.45 NA objective lens and rhodamine-fluorescein iso-thiocyanate protocol with excitation at 488 nm (argon laser), with an emission range of 493-541 nm (live) and 585-656 nm (dead) beam-splitting. Biofilms were assessed with a dry lens to reduce distortion to the biofilm that would occur with a slide cover.

A series of images were obtained over 60 μ m in the z axis (cuts of 3.59 μ m) for a three dimensional view of the biofilm.

3 Results

3.1 Susceptibility of planktonic *C. albicans* cells to fluconazole and to RC21v3

3.1.1 Aim

With denture-induced stomatitis occurring relatively frequently in denture wearers, often as a recurrent or persistent infection, candidiasis is of importance to the dental clinician. The aim of this set of experiments was to carry out the standard liquid MIC protocol to test the hypothesis that RC21v3 works synergistically in combination with the azole fluconazole to reduce the number of viable cells in planktonic cultures of both azole-resistant and azole-susceptible *C. albicans* strains.

3.1.2 Results

The fluconazole MIC₈₀ for *Candida* strain MML611 was 20 µg/mL, which was 32-fold higher than that for the control azole-susceptible strains ATCC 10261 and azole-susceptible clinical isolate parental strain MML610 (MIC = 0.625 µg/mL) (Table 7).

Table 7. Susceptibility of *C. albicans* strains to fluconazole (FLC) by liquid MIC

Strain	MIC ₈₀ (µg/mL)*
ATCC 10261	0.625
MML610	0.625
MML611	20

* The experiment was carried out three times and the MIC results are from a representative experiment. The MIC values for the three separate experiments did not vary more than one doubling dilution.

The fluconazole MIC data for all *C. albicans* strains showed partial inhibition, or trailing growth, at high drug concentrations of 15-20% of the no-drug controls (Fig 7). Preliminary assays using a greater range of concentrations of fluconazole (64 µg/mL)

showed that the trailing growth of the ATCC 10261 strain persisted up to at least 64 $\mu\text{g/mL}$.

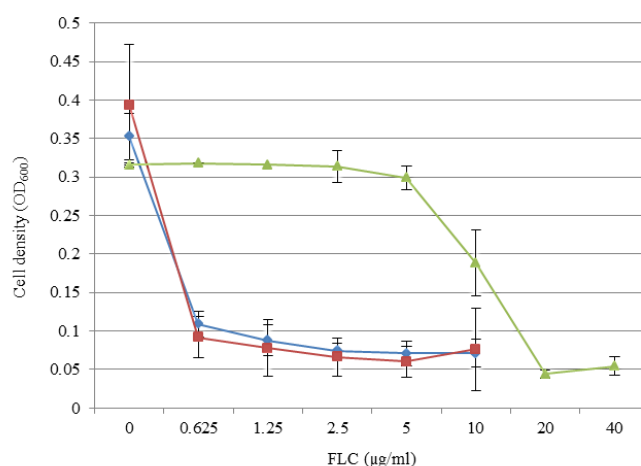


Fig. 7. Fluconazole liquid MIC profiles for three *C. albicans* strains. Blue line, ATCC 10261; red line, MML610; green line, MML611. Cell density (OD_{600}) was measured after incubation at 37°C for 24 h

Susceptibility of the *C. albicans* strains to RC21v3 was also tested using the same liquid microdilution method. The results of these assays indicated that RC21v3 alone (up to 40 μM) did not have a significant effect on cell growth of any of the strains tested (Fig. 8; Table 8).

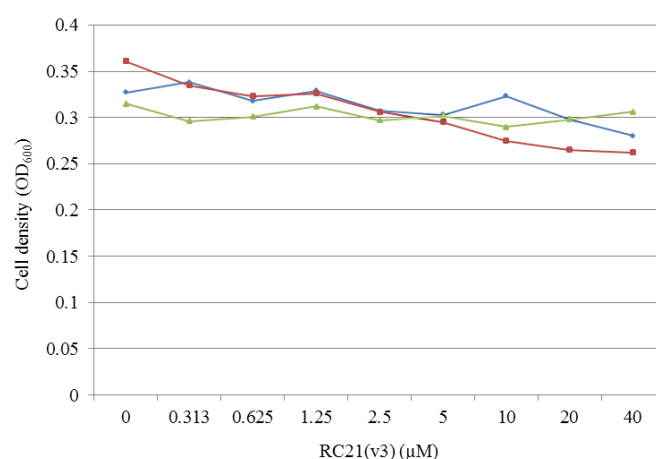


Fig. 8. Effect of RC21v3 on the density of three *C. albicans* strains. Blue line ATCC 10261; red line MML610; green line MML611. Cell density (OD_{600}) was measured after incubation at 37°C for 24 h

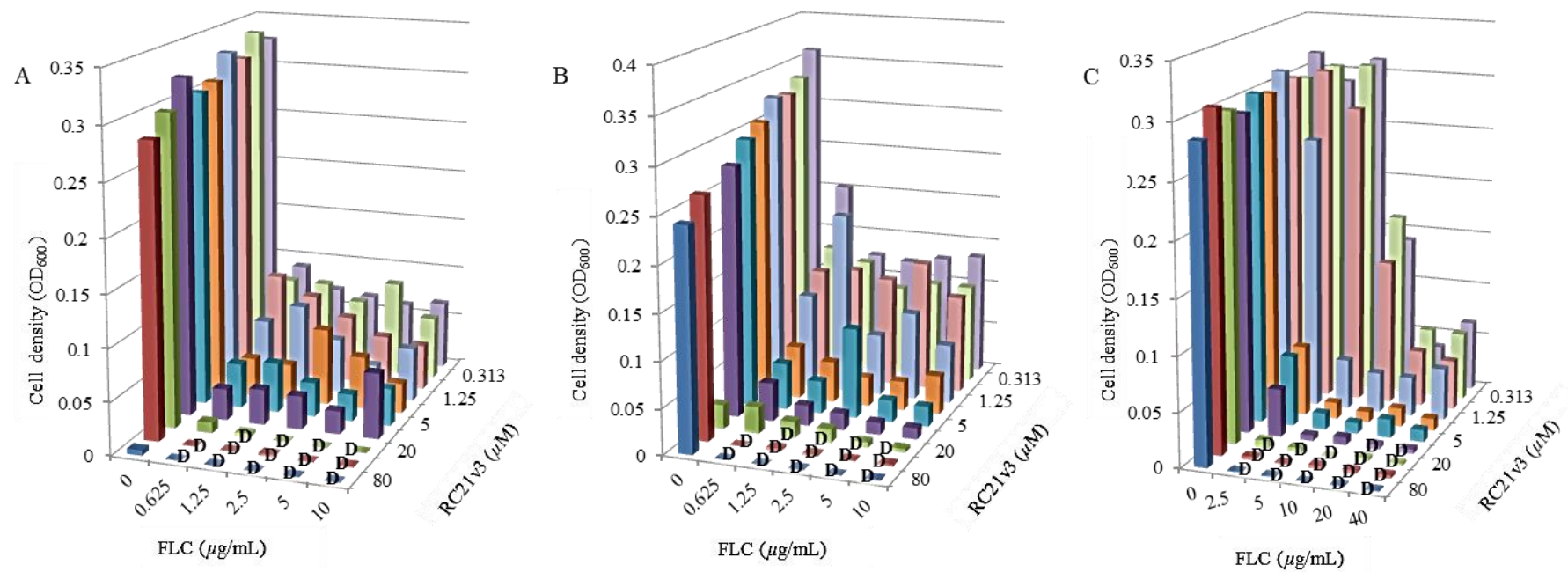


Fig. 9 Planktonic checkerboard MIC assay showing the synergy between RC21v3 and FLC for *C. albicans* strains.

A, ATCC 10261; B, MML610; C, MML611. 'D' (dead) indicates fungicidal effect of drug combination.

Checkerboard liquid MIC assays were carried out to see if RC21v3 acted synergistically with fluconazole to inhibit the growth of *C. albicans*. The results of the checkerboard planktonic MIC assays showed that in the presence of 20 – 80 µM RC21v3, the trailing growth of ATTC 10261 (Fig. 9A) and MML610 (Fig. 9B) was completely eliminated and fluconazole became fungicidal.

The fluconazole -resistant isolate MML611 demonstrated a significant reduction in growth in the presence of 10 – 80 µM RC21v3 (Fig. 9C), and fluconazole proved to have a fungicidal effect with RC21v3 eliminating the trailing growth of MML611 as was seen with the fluconazole-susceptible strains.

The interaction between fluconazole and RC21v3 was assessed using the fractional inhibitory concentration index (FICI), which is calculated by the following equation: $FICI = FIC \text{ (fractional inhibitory concentration) of compound A} + FIC \text{ of compound B}$, where the FIC of A is defined as the MIC of A in combination with B, divided by the MIC of A alone, and the FIC of B is defined as the MIC of B in combination with A, divided by the MIC of B alone. The FICI values can then be used to classify the interaction between drugs A and B. Synergistic interactions give FICI values ≤ 0.5 , antagonistic interactions give FICI values > 4.0 , and if there is no interaction between drugs A and B the FICI values lie between 0.5 and 4.0 (Odds, 2003).

Table 8. Drug interaction relation between FLC and RC21v3 for *C. albicans* planktonic cells

Strain	FLC MIC (µg/mL)			RC21v3 MIC (µM)			
	Alone	Combined	FIC*	Alone	Combined	FIC	FICI**
ATCC 10261	0.625	0.625	1	>80	20	<0.25	<1.25
MML610	0.625	0.625	1	>80	20	<0.25	<1.25
MML611	20	2.5	0.125	>80	20	<0.25	<0.375

* The fractional inhibitory concentration

** The fractional inhibitory concentration index

FICI calculations showed that when fluconazole and RC21v3 were co-administered to *C. albicans* strain MML611 they acted synergistically, whereas there was no obvious synergy observed between the two compounds when applied to fluconazole-susceptible ATCC 10261 or MML610, although trailing growth was eliminated (Table 8).

3.2 A novel *in vitro* biofilm model

3.2.1 Aim

The aim of this set of experiments was to design an experimental substrate that will support a reproducible biofilm similar to that grown on dentures *in vivo*. The experimental system is hypothesised to be more user-friendly than the conventional microtitre plate well-floor model and should protect the biofilm during experimental procedures preserving the fragile biofilm architecture for confocal analysis.

3.2.2 Results

To ensure relevancy to the clinical situation, the commonly used dental restorative material Vertex™ Regular Crystal Clear PMMA was chosen for the construction of the biofilm substrate.

To ensure ease of manufacture, the fabrication of substrates was kept simple by employing conventional injection-moulding processing techniques (vacuum flask and heat cure) that are used in the construction of denture bases. Substrates were then polished to a uniform finish with 400 grit sandpaper followed by 800 grit sandpaper only so as to mimic an older denture and to preserve some surface roughness ($> 0.2 \mu\text{m}$) to ensure cell adhesion and biofilm development.

For the trial models a wax template made using an impression of a form invested in stone prior to casting in PMMA. Once the trial had identified the optimal form, a metal template was cut to ensure uniform duplication of the substrate.

When designing the optimum form, a number of features were considered.

It was decided to use a suspended, vertical substrate rather than discs on the bottom of wells employed in other assays. This ensured that any non-adherent cells would not be incorporated within the biofilm as they would have fallen to the floor of the microtitre well during incubation periods. This was important as it meant the biofilm would be

composed of strongly adherent cells, as will occur with *in vivo* biofilms. This design also precluded the damage that could be caused by washing the biofilm to eliminate any non-adherent cells that are required for other protocols. Another feature of the suspended substrate is that biofilm will form on all sides of the ‘flag’, whereas with discs on the bottom of wells the base does not support biofilm growth as it is in contact with the floor of the microtitre well.

The design also provides a large surface area available for the formation of the *C. albicans* biofilm. A number of shapes were considered, a cone (Fig. 10A) or cylinder within an assay well provided the largest surface area, but they were difficult to process to a standard shape. Therefore a flat form was chosen. This proved to be the best shape for a number of reasons. It was much easier to fabricate, duplicate and finish to uniformity than other shapes. The flat surface also allowed the use of microscopy to visualise the biofilm architecture.

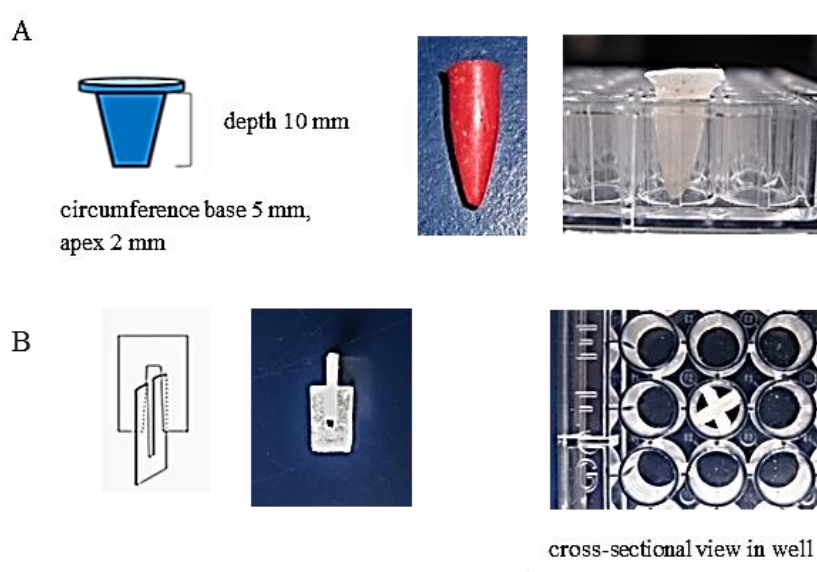


Fig. 10. Prototype models of acrylic subject for *in vitro* biofilm formation. A, the conical design; B, two-piece cross-tab design.

When considering the biofilm assay procedure, the substrate must only cause minimal disruption to the fluid movement that mimics the oral fluid flow and distributes the cells to the substrate surface during the attachment phase. Some of the form designs, such as the conical design (Fig. 10A), were discarded because trial immersions carried

out in the standard 96-well microtitre plates to be used in the biofilm assays indicated that there was insufficient space around the form for the experimental fluid volumes. The two-piece individual cross-tab design (Fig. 10B) had a large surface area, but was discarded because it impeded fluid flow around the form, and was also more difficult to fabricate.

Standard biofilm growth and analysis protocols require the movement of the biofilm through multiple steps and different solutions. Therefore another consideration was whether substrates could be linked together so that several biofilms could be moved through the various solutions at the same time. This would also help minimise disturbance of the biofilms.

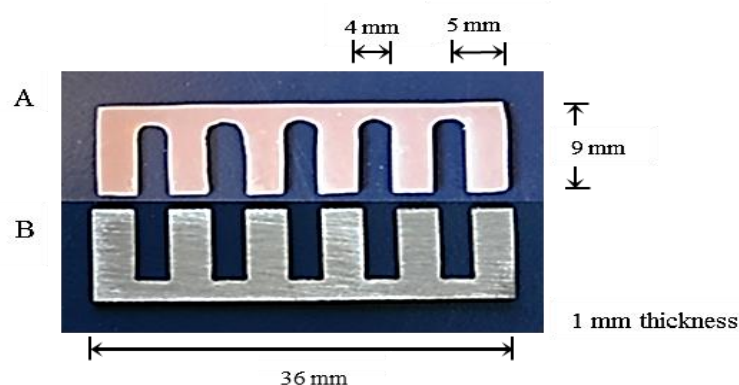


Fig. 11. Multiple 'flag' model chosen for in vitro biofilm formation assays. A, Acrylic assay substrate; B, Metal template

A "flag" model was therefore developed with several flags of acrylic linked together in a strip (Fig. 11). The bar at the top suspended the flags in the microtitre plate wells, so they did not touch the well bottom and the biofilm assay liquid extended 4 mm up the acrylic flag. This gave each flag a surface area of 53 mm² on which the biofilm could form. This design was adopted as it gave a large surface area for biofilm growth without restriction of fluid flow or volume and six biofilms could be assayed simultaneously. The bar at the top proved to be an efficient means to transfer the biofilms through the experiment stages with minimal disturbance (Fig. 12). The bar was narrow enough, however, to still allow the microtitre plate lid to fit and maintain sterility within the microtitre plate (Fig. 12B).

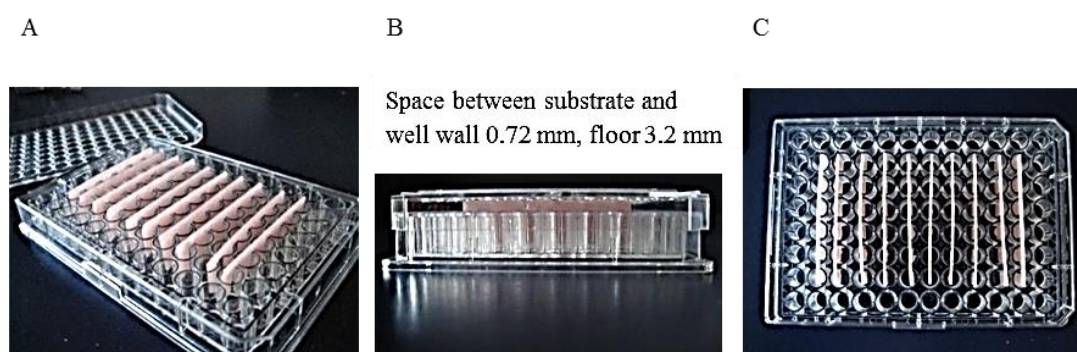


Fig. 12. Multiple 'flag' model suspended in a 96-well microtitre plate. A, view from superior angle; B, from side and C, from directly above the plate.

3.3 *C. albicans* growth

3.3.1 Aim

The aim of this series of experiments was to optimise *C. albicans* biofilm formation on the multiple flag substrate.

3.3.2 Results

3.3.2.1 Disinfection procedure

Both the saliva and substrates are possible sources of microbial contamination. In order to remove microorganisms from saliva and the substrate, the following treatments were performed and microbial contamination was tested.

Whole saliva was collected and centrifuged at 12,000 *g* at 4°C for 15 min to remove oral microorganisms and debris. Approximately 5mL aliquots of the supernatant were transferred into fresh sterile universal glass bottles, and mixed with an equal volume of 100 mM KCl buffer (see section 2.1.2) before storage at -20°C until used.

Two substrate disinfection procedures were tested: 10 min immersion in absolute ethanol followed by brief flaming; and 10 min chlorine treatment as per technician denture disinfection protocols, NZDA Code of Practice 2008, using 2 x 500 mg tablets of anhydrous sodiumdichloro-1,3,5-triazinetriene [Guest Medical U.K.] in 40 mL filtered de-ionized water). Following sterilisation, substrates were tested for microbial contamination by incubation for 48 h in RPMI medium followed by the CV assay.

The CV assay showed minimal growth of microorganisms when the substrate was treated with either, ethanol and brief flaming (OD_{600} 0.028 - 0.046), or with chlorine (OD_{600} 0.073 - 0.08) prior to 48 h incubation in RPMI.

It was decided to use both substrate disinfection methods to minimise the risk of contamination. Chlorine was used prior to storage in sterile ultra-pure water, and substrates were further disinfected with ethanol and brief flaming prior to pre-treatment with saliva.

Sterilized substrate flags were pre-treated with saliva and then incubated in 200 μ L RPMI (0.2% glucose) at 37°C for 60 h. There was no microbial growth in the RPMI medium indicating that the saliva was sterile.

3.3.2.2 *Effect of saliva pre-treatment of substrate on biofilm formation.*

Biofilm formation of *C. albicans* ATCC 10261 on the substrate was quantified using the CV biomass assay using biofilms formed with and without pre- treatment of the substrate with saliva. It was found that after 48 h the substrate pre-treated with saliva had higher biofilm mass than the untreated substrate (Fig. 13).

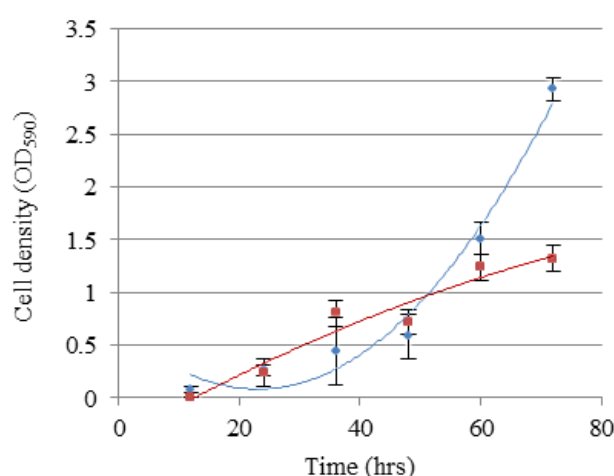


Fig. 13. *Effect of saliva pre-treatment of substrate on biofilm formation.*
The biofilm formation on six flag substrates with or without pre-treatment with saliva prior to immersion in RPMI medium containing *C.albicans* ATCC10261 at 1×10^7 cells/mL was measured with the CV assay;
Red trend line, without saliva; Blue trend line, with saliva.

It was decided, therefore, to include pre-treatment of the substrates with saliva in the experimental protocol not only to generate larger biofilms, but to also mimic more closely the *in vivo* clinical situation where dentures are quickly coated with salivary pellicles. The nature of the *C. albicans* biofilm that developed on saliva-coated substrate was investigated by CV staining (Fig. 14).

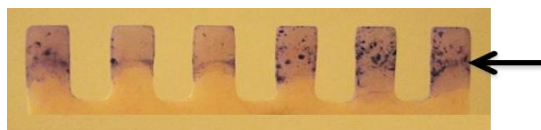


Fig. 14. Crystal violet staining of biofilm formed on the surface of the multiple 'flag' substrate *C. albicans* ATCC 10261 biofilm (arrow) after 48 h incubation in RPMI medium.

The CV staining revealed that the biofilm was not confluent across the flags and instead was clumped. This indicated that there may have been insufficient growth over the 48 h incubation and the possibility of improving biofilm growth by increasing the glucose concentration in the growth medium was investigated.

3.3.2.3 **Effect of glucose concentration in growth medium on biofilm formation.**

The growth medium often used for biofilm formation, RPMI, is nutritionally poor with only 0.2% (w/v) glucose. Such a low concentration of soluble carbohydrate doesn't accurately reflect the oral environment where there is regular exposure to soluble sugars. Also biofilm formation in RPMI (0.2% glucose) was insufficient for subsequent experiments to measure the effect of drugs on the inhibition of biofilm growth (Fig. 15A). To improve the relevance to *in vivo* biofilms and to facilitate greater biofilm growth, the RPMI growth medium was supplemented with glucose to give a final concentration of 2% (w/v). In addition, the substrate flags were transferred to fresh RPMI (2% glucose) after the 12 h cell adhesion period, then each subsequent 24 h to provide optimal growth conditions. These conditions resulted in increased biofilm growth, especially for strain ATCC 10261 (Fig. 16).

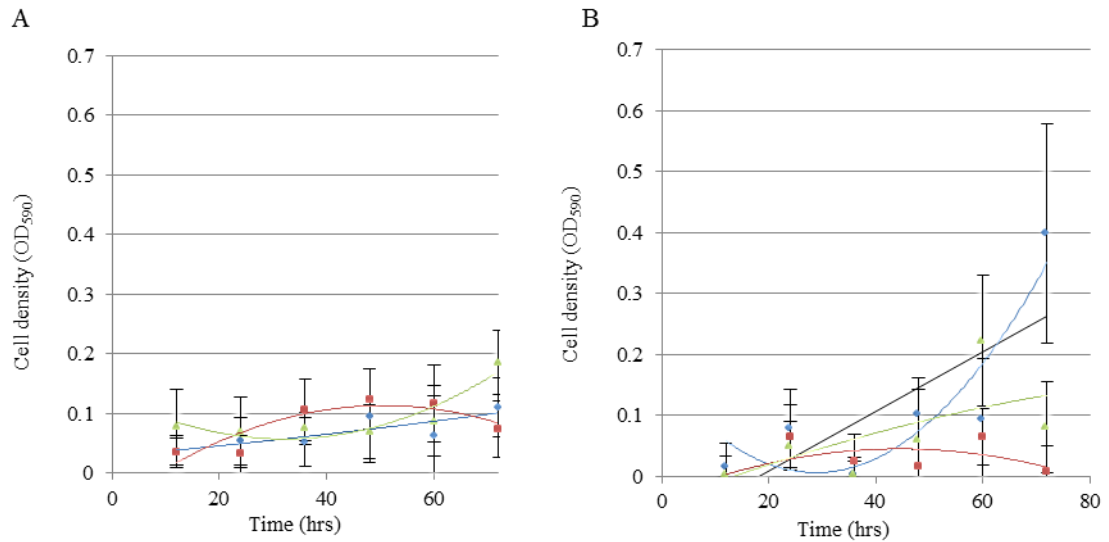


Fig. 15. Effect of RPMI glucose concentration on biofilm formation. The biofilm formation on substrate in media inoculated with 1×10^7 cells/mL and different concentrations of glucose was measured with the CV assay. The values represent the means $\pm$ SD of three separate experiments.

A. RPMI with 0.2% glucose, B. RPMI with 2% glucose (RPMI plus additional 1.8% glucose). Blue trend line ATCC 10261; red trend line MML610; green trend line MML611.

3.3.2.4 Strain variation in biofilm formation.

Of the three *C. albicans* strains tested, ATCC 10261 formed the most consistent, and greatest biofilm mass over the sample substrates when dyed with CV (Fig. 16), and this strain was selected for further study.

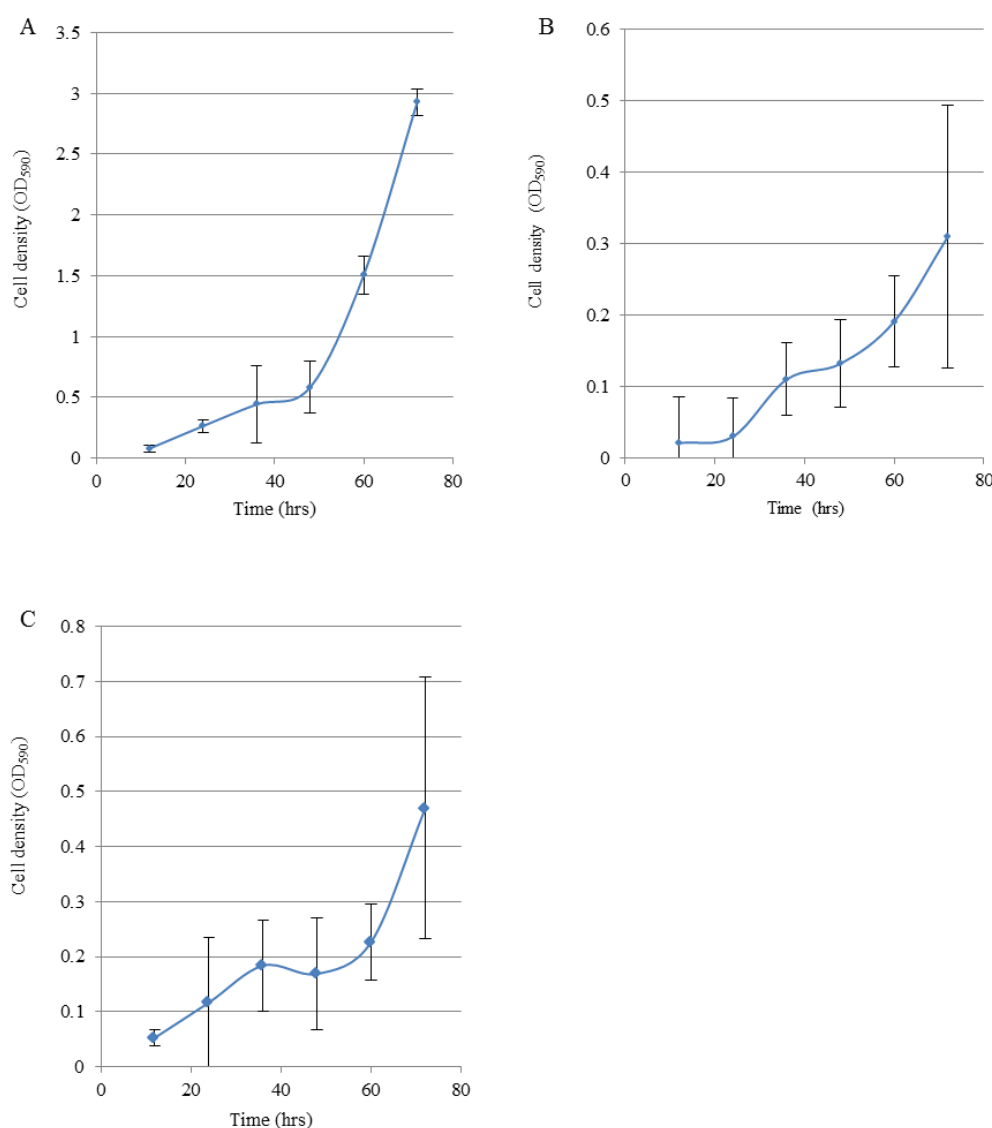


Fig. 16. Biofilm formation of three *C. albicans* strains determined with CV assay. A, ATCC 10261; B, MML610; C, MML611. Initial inoculum was 10^7 cells/mL. The values represent the means $\pm$ SD of three separate experiments.

3.3.2.5 Effect of inoculum cell concentration in the adhesive phase of the assay on subsequent biofilm formation.

The effect of changing the concentration of cells of *C. albicans* ATCC 10261 in the initial adhesion phase of the assay on subsequent biofilm formation was tested. It was found that at least 1×10^6 cells/mL were required for biofilm formation (Fig.17).

However, an inoculum concentration of 1×10^7 cells/mL in the adhesion phase gave the

most reproducible, and extensive, biofilm formation, and this inoculum concentration was used in subsequent assays.

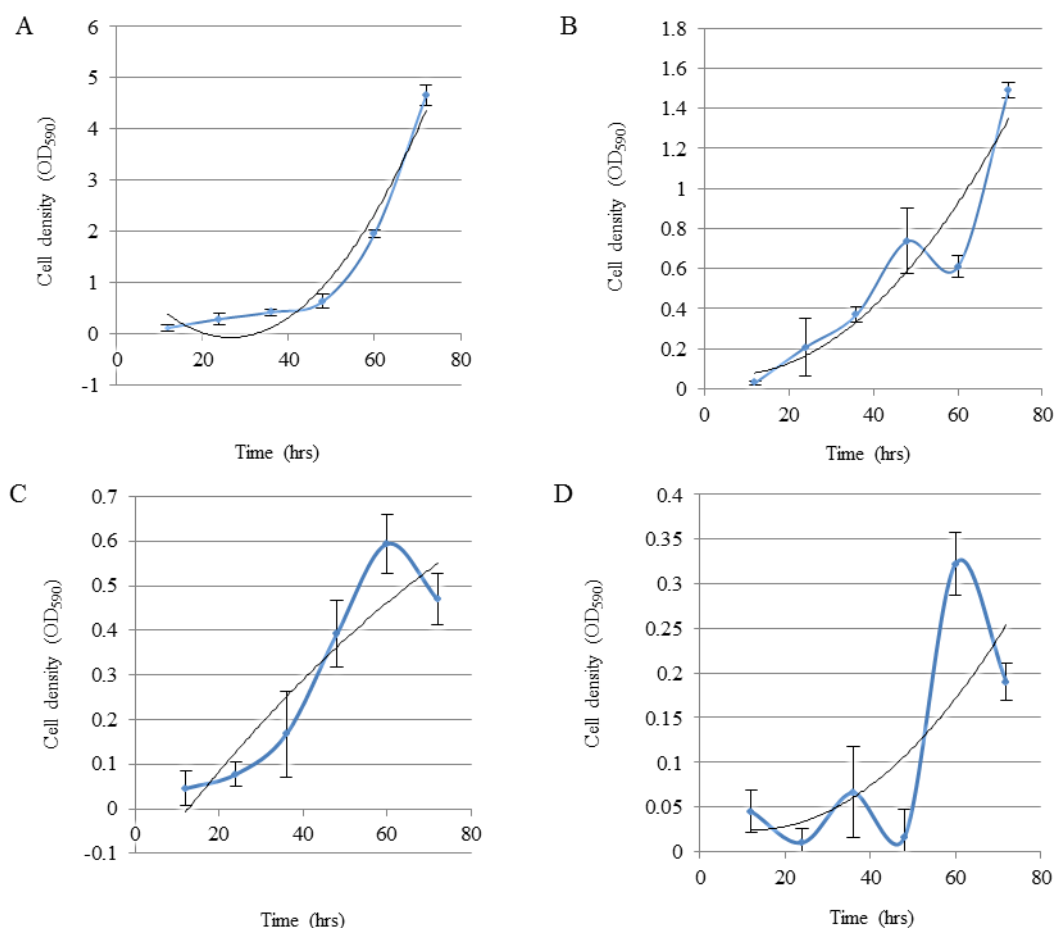


Fig. 17. Effect of initial cell concentration on subsequent biofilm formation. Biofilms formed from ATCC 10261 cells at the following inoculum concentrations in the adhesion phase of the assay. A, 10^7 cells/mL; B, 10^6 cells/mL; C, 10^5 cells/mL; D, 10^4 cells/mL. Growth medium was replenished after the 12 h cell adhesion period, then each 24 h.

3.4 Antifungal susceptibility of *C. albicans* biofilms

3.4.1 Aim

The susceptibility of the *C. albicans* biofilms developed in section 3.3.2 to fluconazole in the presence or absence of RC21v3, and whether the combination of RC21v3 and fluconazole is fungicidal for biofilm cells will be determined quantitatively using the CV staining method.

3.4.2 Results

3.4.2.1 *Determination of the MIC of FLC for C. albicans biofilms*

Substrates were sterilized (see section 3.3.2.1) before pre-treating with clarified saliva at 35°C for 30 min. After rinsing with fresh RPMI, the substrates were suspended in microtitre plate wells containing 200 µL of ATCC 10261 cell suspension (1×10^7 cells/mL in RPMI containing 2% (w/v) glucose; RPMIG).

Initial incubation was at 35°C with agitation at 150 rpm in a shaking incubator (Bio-Line, Edwards Instrument Company) for 12 h to maintain an even dispersion of cells through the medium and to ensure maximum contact with the substrate for adhesion before transfer of the substrate to microtitre plates containing fresh RPMIG. The substrates were then incubated under a more gentle agitation at 50 rpm (Nett et al., 2011b).

After 48 h incubation, substrates were transferred to a new plate containing fresh RPMIG with or without fluconazole. Four separate plates were prepared to contain different concentrations of fluconazole to determine a biofilm MIC. Plate 1 was a no-drug control, plate 2 contained fluconazole at 1/4 MIC (0.156 µg/mL: determined from planktonic MICs – Table 7), plate 3 contained fluconazole at the MIC (0.625 µg/mL) and plate 4 contained fluconazole at 4 x MIC (2.5 µg/mL) to enable determination of a concentration appropriate for biofilm chemosensitization assays.

Biofilm growth was quantified with the CV assay at 12, 24, 36, 48, 56 and 72 h time points.

Results

There was a dose-dependent response of biofilm growth to fluconazole (Fig. 18). After 12 h exposure to the antifungal, a fungistatic effect could be seen at fluconazole concentrations of 0.625 µg/mL and 2.5 µg/mL. This decrease in growth became an almost complete biofilm eradication at the highest fluconazole concentration (2.5 µg/mL), but at a fluconazole concentration of 0.625 µg/mL the biofilm growth appeared to recover after 24 h exposure to the antifungal (Fig. 18). It was evident that a fluconazole concentration of 0.156 µg/mL was sub MIC and had a minimal effect on biofilm growth. This concentration of fluconazole was therefore selected to determine whether RC21v3 would have a synergistic effect on biofilm growth by checkerboard CV assay and for confocal microscopy analysis.

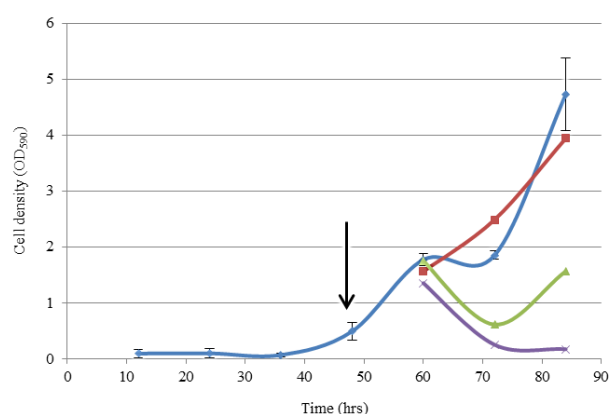


Fig. 18. *Effect of FLC on C. albicans ATCC 10261 biofilm growth.* Initial cell inoculum was 1×10^7 cells/mL. Growth medium was replaced at 12, 36, 48 and, 72 h time points, and FLC was added at 48 h (indicated by vertical arrow). Blue line, no FLC added; red line, 0.156 µg/mL FLC added; green line, 0.625 µg/mL FLC added; purple line, 2.5 µg/mL FLC added.

3.4.2.2 *Effect of fluconazole and RC21v3 on C. albicans biofilms*

The previously optimised biofilm growth protocol was used to generate *C. albicans* ATCC 10261 biofilms on flag substrates.

After 48 hr incubation, substrates were transferred to a new microtitre plate that had been prepared as follows. The peripheral microtitre plate wells contained 200 µL RPMIG to help prevent desiccation of the inner wells during incubation. RC21v3 (top concentration 80 µM) was distributed to 6 vertical wells of the microtitre plate and a 2-fold dilution series was made horizontally in RPMIG with thorough mixing of each dilution (final volume 100 µL). Fluconazole (top concentration 40 µg/mL) was distributed to 10 horizontal wells of a separate microtitre plate, and a 2-fold dilution series was then made vertically in RPMIG with thorough mixing of each dilution before transfer to the next row of wells in the dilution series.

For the chemosensitisation assays, the fluconazole dilution series (100 µL) was transferred to the corresponding wells of the plate containing the RC21v3 dilution series to give a total volume of 200 µL (and combination of the two drugs). Substrates with adhered biofilms were transferred to this assay plate. The plate was then incubated

at 37°C with gentle agitation (50 rpm) and the medium changed after 24h. The MIC was determined at 24 h and 48 h using the CV assay.

Results

The results are given as a mean of three separate experiments which showed minimal variance of not more than 5% (Fig. 19). *C. albicans* cells growing as a biofilm were shown to be more resistant to fluconazole than planktonic cells in agreement with the literature (Chandra et al., 2001a). The MIC of FLC for ATCC 10261 biofilms was greater than 10 µg/mL, which was 16-fold higher than for planktonic cells (Fig. 19; Table 7).

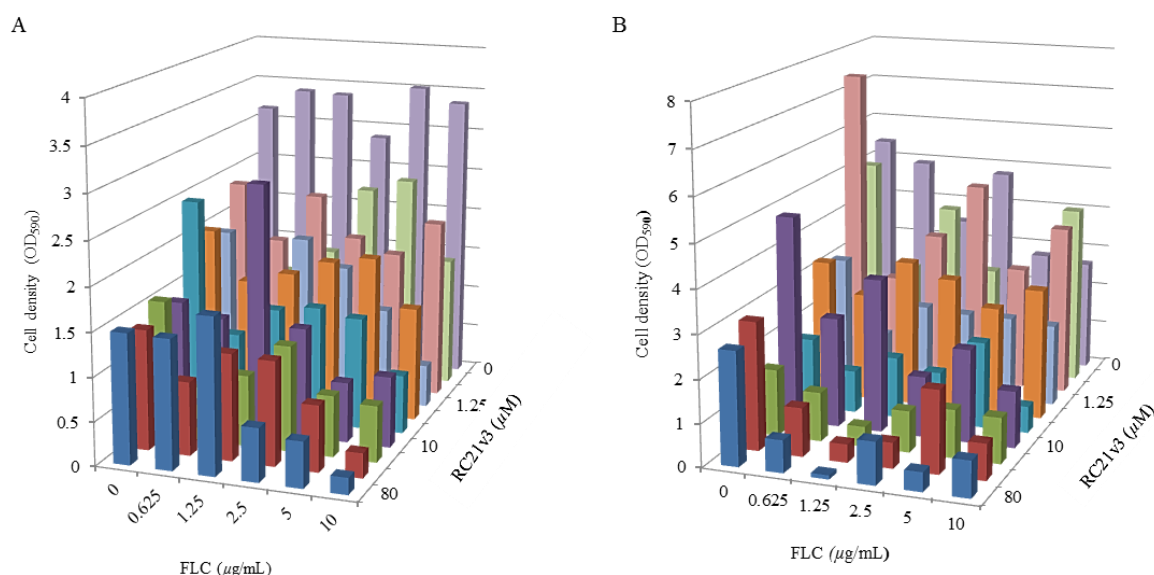


Fig. 19. Biofilm checkerboard assay to investigate the interaction between RC21v3 and FLC on *C. albicans* ATCC 10261.

A, density measured after 24 h and B, 48h. The results are given as a mean of three separate experiments which showed a variance of not more than 5%.

Allowing for the variation in adhered biofilm and small sample size, RC21v3 seems to enhance the antifungal effect of fluconazole on *C. albicans* in a biofilm.

The results of the checkerboard assays after 24 hr showed that in the presence of RC21v3, the *C. albicans* ATCC 10261 biofilm became chemosensitized to fluconazole. Fluconazole at concentrations of 2.5 - 10 µg/mL showed a significant inhibitory effect

on biofilm growth in the presence of RC21v3 at concentrations of 20 μM and above (Fig.19A). Prolonged exposure of the biofilm to fluconazole and RC21v3 (48 h Fig. 19B) produced inconsistent results (Fig. 19B).

The FICI was calculated from the checkerboard MIC data (Table 9). There was a synergistic interaction between RC21v3 and FLC against *C. albicans* ATCC 10261 biofilms, as the FICI was <0.5 (Table 9).

Table 9. Drug interaction relation between FLC and RC21v3 against *C. albicans* ATCC 10261 biofilms

FLC MIC ($\mu\text{g/ml}$)			RC21v3 MIC (μM)			FICI
Alone	Combined	FIC	Alone	Combined	FIC	
>10	2.5	0.25	>80	20	<0.25	<0.5

FICI was calculated using 24 h results.

3.4.2.3 Confocal visualisation of *C. albicans* biofilms

Confocal microscopy was used to enable preliminary non-invasive investigation of the biofilm formed on the flag substrate as a three-dimensional structure. Live-dead BacLight cell viability staining (Life Technologies) was used, in which SYTO-9 remained within the metabolically active cells as a green fluorescence, and propidium iodide (PI) cancelled out the SYTO-9 in non-viable cells staining the cells red. Following biofilm formation and exposure to fluconazole alone (10 $\mu\text{g/mL}$), RC21v3 alone (80 μM), or a combination of fluconazole and RC21v3 (10 $\mu\text{g/mL}$ and 80 μM respectively) for 24 h, representative biofilms from each treatment group and a no drug control were subjected to confocal examination.

Adhered biofilms were washed in PBS to remove traces of growth medium (da Silva et al., 2010) and prepared for confocal microscopy (see section 2.2.4)

Confocal microscopy of the fluorescently stained biofilm was carried out to give a series of images obtained over 60 μm in the z axis for a three dimensional view of the *C. albicans* ATCC 10261 biofilm.

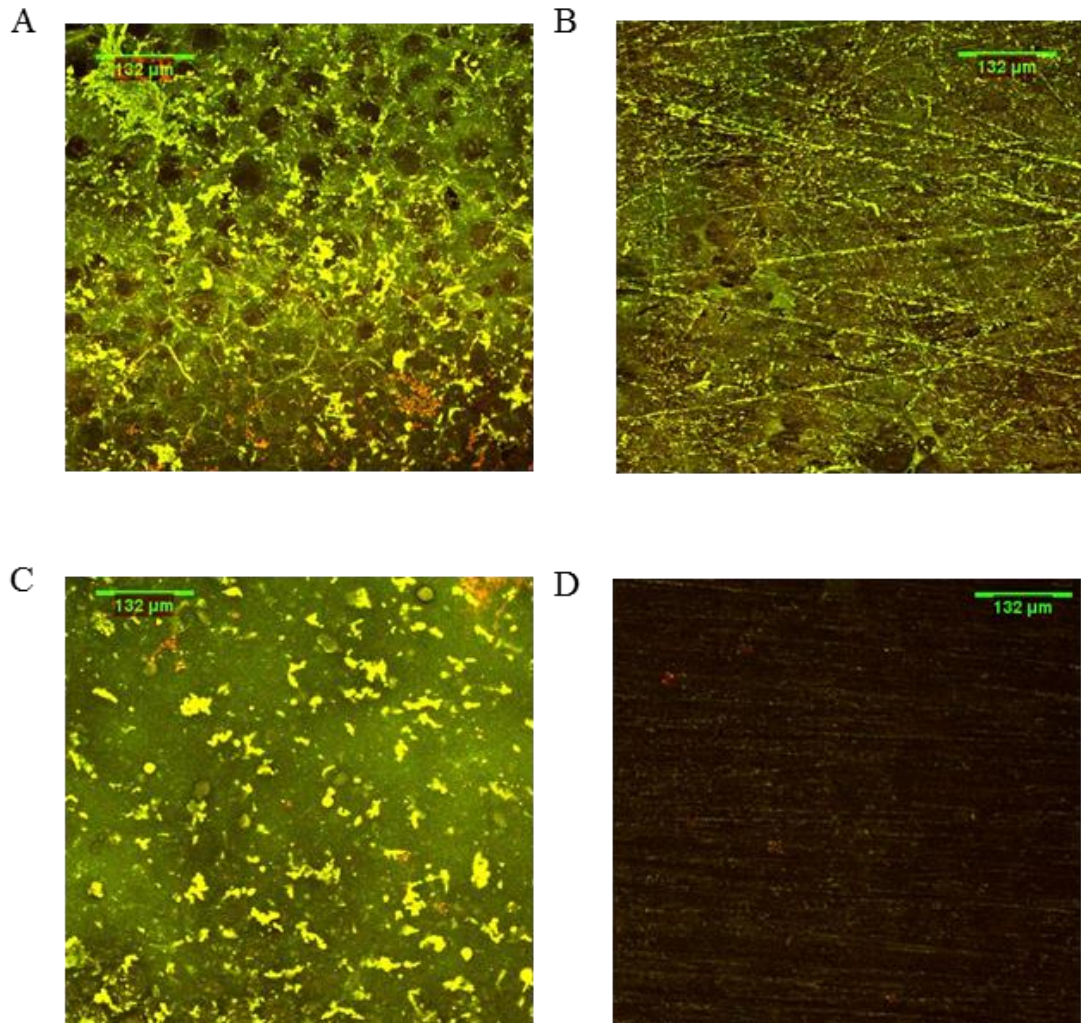


Fig. 20. *C. albicans* ATCC 10261 biofilms stained with propidium iodide (PI) and SYTO-9 fluorescent dyes. A, no drug control; B, biofilm exposed to 10 µg/mL FLC; C, biofilm exposed to 80 µM RC21v3; D, biofilm exposed to the combination of 10 µg/mL FLC and 80 µM RC21v3. Composite 'z' axis stack of live/dead imaging is shown.

Results

The live/dead imaging of propidium iodide (PI) and SYTO-9 fluorescent stains provided a visual image of the biofilm following various drug exposures (Fig. 20).

Exposure to the combination of fluconazole (10 µg/mL) and RC21v3 (80 µM) resulted in a dark biofilm image (Fig.20D). This dark colour may indicate damage to the matrix and biofilm structure resulting in a decreased uptake of both fluorescent dyes. Exposure to either fluconazole (10 µg/mL) or RC21v3 (80 µM) resulted in a modification of the

biofilm structure (Fig 20B and C), with the fluconazole-exposed biofilm appearing as small colonies well distributed over the substrate but definitely aggregating in the substrate surface irregularities caused by finishing techniques (Fig. 20B). The biofilm exposed to RC21v3 appears to have developed more into clumps of cells surrounded by a very thin, unstructured matrix (Fig. 20C) compared to the biofilm not exposed to any drug (Fig. 20A).

Although it was thought that the concentrations of both fluconazole and RC21v3 used in the assay were below the MICs for the *C. albicans* strain chosen, there still appeared to be some effect on biofilm growth as well as the architecture. The thickness of the biofilm exposed to a combination of fluconazole (10 µg/mL) and RC21v3 (80 µM) was reduced to 6% that of the no drug control, and to 14% that of that exposed to fluconazole only (Table 10).

Table 10. *Effect of drug exposure on C. albicans biofilm thickness*

Treatment	Biofilm thickness		
	Mean (µm)	SD* (µm)	Max** (µm)
No drug control	121	47.5	213
FLC (10 µg/mL)	54.2	21.4	93.3
RC21v3 (80 µM)	33.9	19.0	81.5
FLC (10 µg/mL) + RC21v3 (80 µM)	7.8	1.2	16.0

Thickness analysis was performed using 8-bit composite 'z' axis stack.

*Standard deviation

** Maximum biofilm thickness

The extent of biofilm coverage of the substrate was measured at different distances from the substrate surface ('z' axis) (Fig. 21). It was found that the biofilm attached to the substrate over a reduced surface area before it spread out with increased distance from attachment (Fig. 21). This may indicate the biofilm has a pedunculated structure, with the superficial layers containing matrix material that is dispersed over a wider area than the adherent basal cell layer.

As indicated by the 'z' axis stack images (Fig. 21), the exposure of biofilms to either fluconazole or RC21v3 affected the area of the substrate covered. Exposure to fluconazole resulted in flatter biofilms with greater coverage nearer the substrate surface. RC21 did not appear to have as large an effect as fluconazole on either the biofilm thickness or spread over the substrate surface. A combination of both drugs reduced the area of substrate covered by the biofilm to a maximum of 43% from a maximum 61% area covered for the control biofilm left unexposed (Fig. 21).

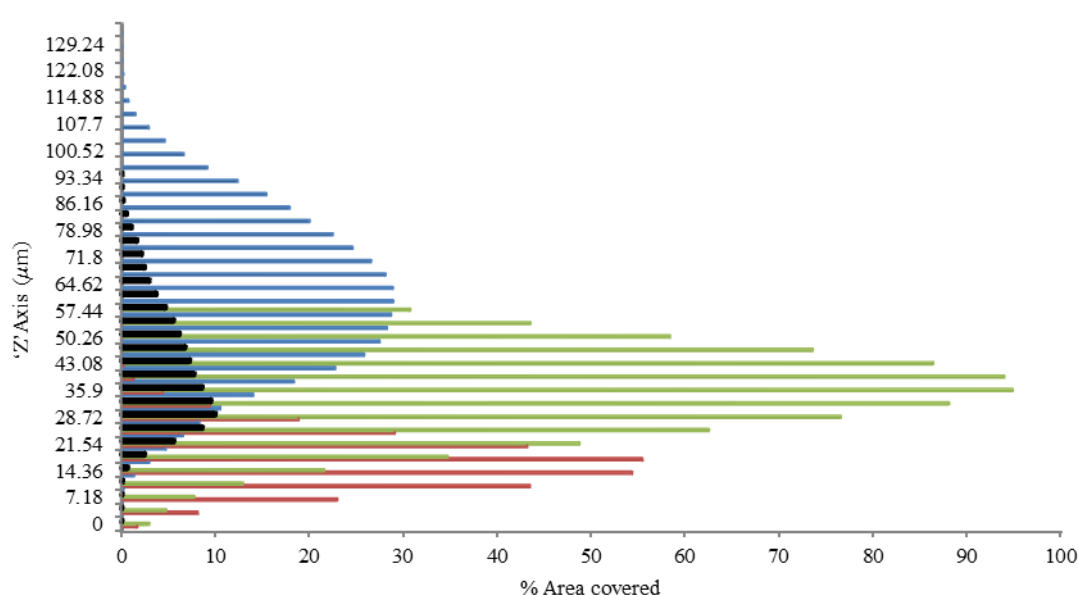


Fig. 21. Percentage area of sample substrate covered by the *C. albicans* ATCC 10261 biofilm as a function of distance from the substrate surface ('z' axis). Blue colour, no-drug control biofilm; red colour, FLC-exposed biofilm; green colour, RC21v3-exposed biofilm; black colour, biofilm exposed to FLC plus RC21v3.

The confocal image data can be presented to give a view of the 'z' axis biofilm, or as it is in cross section lying on the substrate (Fig. 22). The biofilm exposed to RC21v3 showed clumping of cells in micro-colonies (Fig. 22C) whereas exposure to fluconazole resulted in thin biofilms of uniform thickness (Fig. 22B). This view also demonstrated the irregularities and sloping nature of the substrate surface which complicates analysis, necessitates multiple measurements over the sample, and may be

the cause of the large standard deviation seen for the thickness measurements (Table 10).

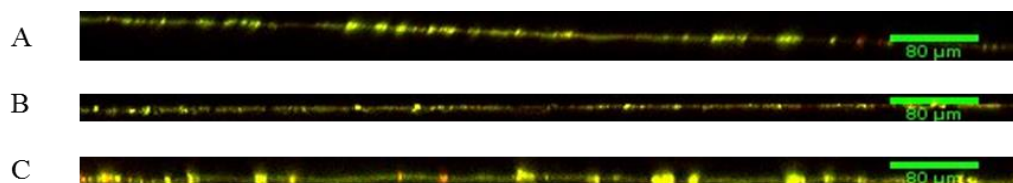


Fig. 22. Adhered biofilm viewed in cross-section or as 'z' axis sample along the 'y' axis A, no drug control; B, biofilm exposed to FLC; C, biofilm exposed to RC21v3.

Analysis of the confocal image data using a split-colour histogram allowed a comparison of the coverage by live and dead cells through the biofilm over the 'z' axis (Fig. 23). This showed a significant increase in the number of dead cells after exposure to the fluconazole alone (Fig. 23B, Table 11) and RC21v3 alone (Fig. 23C). Taking into consideration the different scales used on the x-axes in Figure 23, the area covered can be compared to that of the complete biofilm (Fig. 21). It can be seen that the dead cells covered a larger area than the live cells did in all biofilms analysed, except the biofilm exposed to RC21v3 (Fig. 23C), confirming that RC21v3 on its own does not affect cell viability.

The confocal data was also used to calculate the total biofilm volume, or bio-volume (Table 11). The bio-volume calculations indicated that although RC21v3 had some effect on the architecture of the biofilm, there was little effect on its growth, as the bio-volume increased in some instances relative to the no drug control. This is in contrast to the fluconazole-exposed biofilm which had reduced growth compared to the no drug control, as well as a modification in architecture. When exposed to the combination of fluconazole (10 $\mu\text{g/mL}$) and RC21v3 (80 μM) the biofilm was reduced in volume to 25% that of the no drug control (Table 11). The percentage area covered and thickness was also greatly reduced in the biofilm exposed to both fluconazole and RC21v3 (Table 11).

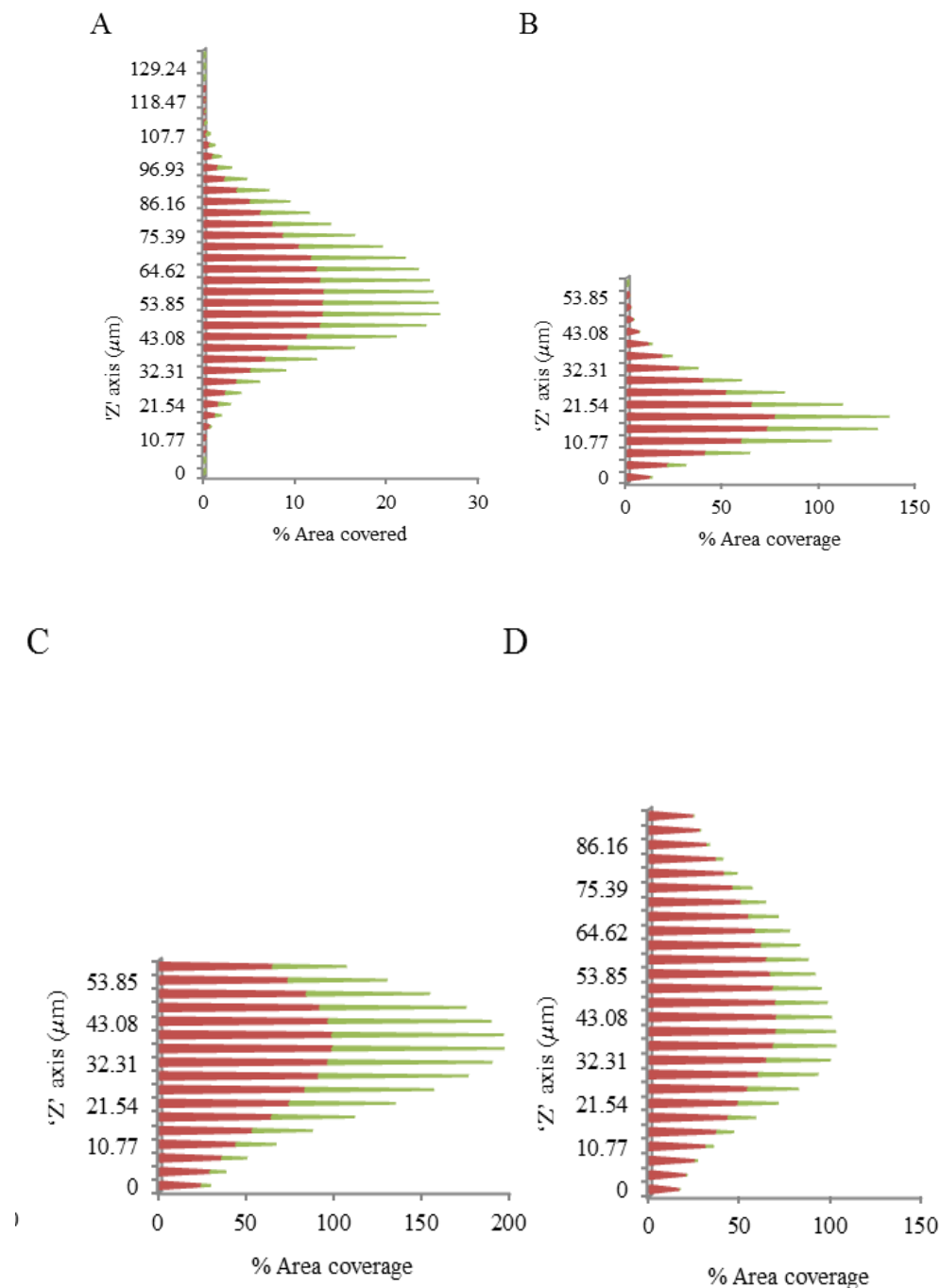


Fig. 23. Live and dead components of *C. albicans* biofilms.
Mean area over the 'z' axis covered by *C. albicans* ATCC 10261 biofilm where live cells are coloured green and dead cells are coloured red.
A, no-drug control; B, biofilm exposed to FLC only; C, biofilm exposed to RC21v3 only; D, biofilm exposed to FLC and RC21 v3. N.B. scale of 'x' axis varied between treatment groups.

Table 11. *Summary of confocal analysis of C . albicans ATCC 10261 biofilms exposed to FLC and/or RC21v3*

Treatment	Bio- volume (x 10⁶ µm³)	Area biofilm coverage (%)	Mean thickness ± SD (µm)	Live/dead cell ratio
No drug control	207	11.8	121 ± 47.5	27 : 73
FLC (10 µg/mL)	121	18.3	54.2 ± 21.4	46 : 54
RC21v3 (80 µM)	371	49.5	33.9 ± 19.0	28 : 72
FLC (10 µg/mL) + RC21v3 (80 µM)	68.2	3.6	7.8 ± 1.2	23 : 77

4. Discussion

The severity of oral fungal infections has been shown to be related to increased numbers of yeast cells at the site of infection (Theilade *et al.*, 1983; Theilade and Budtz-Jørgensen, 1988). Furthermore the concentration of yeast cells that occurs with the formation of a *Candida* biofilm can overwhelm the host immune defences providing an aetiological link between the biofilm and the initiation of the pathological processes associated with disease (Cannon *et al.*, 1995; Dalle *et al.*, 2010).

In the oral environment *Candida* cell adhesion, colony growth and biofilm development can occur on soft or hard, biotic and abiotic surfaces such as dental acrylics, which can then act as microbial reservoirs from which a chronic fungal infection, candidiasis, can emanate. Microbial biofilms regularly form on dental prostheses and they have the propensity to re-form following debridement making associated infections difficult to treat. A reflection of this is the reporting of candidiasis in 11 to 71% of denture wearers (Budtz-Jørgensen, 1974; Lund *et al.*, 2010; Gendreau and Loewy, 2011).

Therefore it was important to investigate the antifungal susceptibility of *C. albicans* biofilms that form on denture acrylic (PMMA), as this is of relevance to the treatment of patients with chronic denture stomatitis, and those with biofilm infections associated with fixed dental appliances.

4.1 Design of the biofilm model

When designing an *in vitro* assay relevant to the behaviour of *in vivo* oral biofilms it is important to use a model that closely resembles the biofilm associated with the disease that develops within the oral environment. *In vitro* assays are, by necessity, simplifications, as only a few aspects of the oral environment can be simulated without protocols and experimental set-ups becoming too complex. Therefore in designing *in vitro* assays a balance must be made between simplicity and inclusion of enough variables to adequately model the oral environment.

4.1.1 Environmental factors

There are a number of important factors specific to oral biofilms to consider in the development of *in vitro* assays including the presence of saliva and the related

characteristics such as shear and attractive forces found in this fluid environment. Cell adhesion, colony growth and biofilm development on dental acrylics has been observed to be enhanced by the surface conditioning provided by the saliva pellicle (Nikawa *et al.*, 1996). It has been demonstrated that the composition of the saliva affects the degree of adhesion of *C. albicans* to dental acrylic (Vasilas *et al.*, 1992; Edgerton *et al.*, 1993), with evidence for both high- and low-molecular-mass salivary mucins within the salivary pellicle being involved (Edgerton *et al.*, 1993). It is also thought that salivary proteins adsorbed onto prosthetic surfaces may act as ligands for *C. albicans* adhesion (Calderone and Braun, 1991; Edgerton *et al.*, 1993; Nikawa *et al.*, 1993). For the series of biofilm assays in the present study, unstimulated whole saliva from a single individual was used to pretreat the acrylic substrate before exposure to the cell suspension. Unstimulated whole saliva contains a greater percentage of minor salivary gland secretions than stimulated whole saliva and therefore is similar to the fluid present under maxillary full denture prosthesis. Although saliva from a single individual is not ideal as it may be atypical, for the purpose of this study it is more clinically relevant than artificial saliva and the single collection divided into aliquots added consistency to the sequence of biofilm assays.

A comparison of several *in vivo* and *in vitro* analyses of the effect of various components of media on the growth and behaviour of *Candida* species indicates that dietary carbohydrates stimulate the initial adhesion and persistent colonisation of oral surfaces (Samaranayake *et al.*, 1980; Samaranayake and MacFarlane, 1982; Samaranayake, 1986). Therefore, to improve the cell adhesion and biofilm formation, the growth medium RPMI was supplemented with glucose.

4.1.2 Substrate factors

The substrate on which the *in vitro* biofilm is grown needs to have relevance to the dental restorative materials associated with clinical biofilm development. A limitation of many *C. albicans* biofilm assays is that they use biofilms grown on the walls of polystyrene microtitre plates. Polystyrene is unlikely to be found in the oral cavity as a prosthetic material so has little relevance. Although the spontaneous adsorption of salivary proteins, and the formation of a pellicle, on oral surfaces appears to have a masking effect that reduces the influences of material characteristics, such as surface roughness and free-surface-energy, on microbial adhesion, and subsequently modulates

the selective binding of microbes to oral surfaces (Göcke, 2002; Tanner *et al.*, 2003), it is still important to maintain authenticity and use as a substrate a dental restorative material that clinically is associated with candidiasis.

The use of a simply processed, reproducible substrate template for *in vitro* biofilm assays will reduce the errors associated with processing variables, and allow for a comparison of different restorative materials which is important in prosthetic material development. A common protocol for measuring biofilm growth on PMMA uses discs submerged in the wells of standard microtitre plates that are then incubated statically (Kuhn, 2002a). Two aspects of this model reduce its efficacy and relevance. Firstly gravity and settling results in a layer of cells resting on top of the substrate, rather than cells adhering and the subsequent biofilm formation on the substrate surface. Secondly it is not representative of the oral environment where surfaces are subject to the flow and forces associated with the movement of saliva.

A suspended substrate more closely approximates the *in vivo* situation with gentle agitation during the assay allowing the growth medium to mimic the biodynamic movement of fluids that occurs in the oral cavity. The suspended substrate model also increases the surface area available for biofilm growth.

The outer, more open, hyphal layer of the *C. albicans* biofilm have been described as only loosely adhered to the underlying layer of the mature biofilm (Kuhn *et al.*, 2002b). The use of standard washing protocols to remove planktonic cells from the discs on the bottom of microtitre plate wells in other biofilm assays is likely to dislodge this surface layer. The biofilm may also be damaged by handling with tweezers when samples are moved through successive steps in test protocols. Therefore the use of a suspended, transportable substrate with multiple flag tokens on one strip developed in this project helps limit the damage to the biofilm by eliminating the need to wash non-adherent cells from the biofilm as these will have fallen to the floor of the microtitre well during incubation.

The development of this novel substrate model resulted in a simple assay protocol based on previous 96-well microtitre plate models but modified to allow the analysis of biofilms growing on clinically relevant prosthetic materials. In order to accurately replicate a denture biofilm the substrate design was assessed for its ability to meet the criteria outlined in the following points.

1. Use gravity to remove non-adherent cells from the biofilm;
2. Prevent disturbance of the biofilm during assay steps and preclude any damage that could be caused by standard biofilm washing protocols;
3. Allow ease of manipulation through the steps of standard biofilm growth protocols;
4. Use appropriate fluid volumes for biofilm growth;
5. Allow fluid flow and introduce shear forces to mimic the oral fluid flow *in vivo*;
6. Provide an adequate surface area for biofilm growth;
7. Be simply processed to ensure a standard dimension, and uniform surface finish;
8. Allow the use of microscopy to visualise the biofilm architecture.

The suspended “flag” model developed provides a relatively large surface area for biofilm growth without restriction of fluid flow. The design allows multiple test wells (biofilms) to be assayed simultaneously, and provides a simple method of biofilm transfer through the steps of the protocol with minimal disturbance. Multiple assays demonstrated that the final biofilm model assay resulted in a reproducible *C. albicans* ATCC 10261 biofilm of sufficient density for further analysis.

In vitro *C. albicans* biofilms have been reported with thicknesses ranging from 25 to more than 45µm (Ramage, 2005). Although the biofilms achieved for *C. albicans* strains MML610 and 611 covered only a small area and were too small to allow further assays to be carried out in this study, it may be that biofilms of a greater volume could be obtained by using a flow chamber incubator (Kaneko *et al.*, 2013). The parallel-plate flow chamber is a sealed flow displacement system that allows controlled delivery of fluids (media) at specified flow rates and shear forces. *C. albicans* biofilms grown in these flow chambers on silicone substrates have been shown to have an average growth rate of 17.2 ± 1.3 µm/h becoming approximately 270 µm thick after 12h growth (Kaneko *et al.*, 2013). Therefore with increased funding and time, other strains of *C. albicans* could be assessed for the antifungal synergy of RC21v3 and fluconazole.

The use of computer aided milling (CAM) to prepare the acrylic suspended flags could reduce the variation in the substrates as human error and processing errors are eliminated, and finishing of surfaces should be more consistent.

4.2 Fluconazole and RC21v3 synergy

There are relatively few drug targets that are specific to the fungus *C. albicans*, and it has been reported that fluconazole, which targets the biosynthesis of membrane ergosterol is the antifungal that is most commonly administered to patients (Horn *et al.*, 2009). Unfortunately, azoles have the therapeutic limitation that they are fungistatic, inhibiting growth rather than killing fungal cells, and denture stomatitis-related inflammation can relapse 2-4 weeks after fluconazole treatment (Budtz-Jorgensen *et al.*, 1988).

It has also been proposed that the widespread use of azoles has contributed to the development of significant drug resistance in some *C. albicans* strains thereby reducing the drug's therapeutic efficacy (White *et al.*, 1998b), a trend no longer confined to the institutionalised patient, a group that have been shown to have a propensity to suffer from chronic or recurring *Candida* infection (Pfaller *et al.*, 2011). The specificity of azoles as an antifungal ensures relatively little host toxicity, and their solubility and bioavailability makes the azole group of antimycotics valuable in the treatment of candidiasis. Therefore there is merit in investigating a possible solution to resolve the clinical problem of azole resistance, and to review any potentiating effects that may render the drugs fungicidal.

It has been suggested that one way to overcome the azole drug resistance of *C. albicans* is through inhibition of the drug efflux pumps that cause high-level resistance in clinical isolates (Cannon *et al.*, 2009), specifically the ATP-binding cassette (ABC) proteins Cdr1p and Cdr2p, with Cdr1p having greater importance (Sanglard *et al.*, 1996; Sanglard *et al.*, 1997; Holmes *et al.*, 2008). Following this line of research the D-peptide derivative RC21v3 has been identified as a highly specific inhibitor of the Cdr1p multidrug efflux pump in *C. albicans* (Niimi *et al.*, 2012).

The peptide RC21v3 does not penetrate the fungal cell due to the positive charge on the three arginine and the one lysine amino acids and, as such, was considered unlikely to be degraded by intracellular detoxification pathways and remain active as a pump inhibitor for longer. An *in vitro* growth assay also showed the peptide chemosensitized fluconazole-resistant *C. albicans* strains to fluconazole (Niimi *et al.*, 2012). Further investigation into the effect of RC21v3 and its possible synergistic action with the azole

fluconazole could provide important information relevant to resolution of azole-resistant candidiasis.

4.2.1 Synergism in planktonic growth assay

The Clinical and Laboratory Standards Institute (formally the NCCLS), and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) have standardized the protocol (M27-A2) for susceptibility testing of fungal strains as planktonic cultures. Previous work has shown that *C. albicans* strains exposed to azoles exhibit a ‘trailing growth’ phenotype, or partial inhibition of growth, that occurs over an extended range of antifungal concentrations indicating a dose-dependant susceptibility to the drug (Rex *et al.*, 1997). This results in a degree of resistance after 24 h incubation with viable cells remaining that can re-establish fungal biofilms in chronic or persistent infections (Revankar *et al.*, 1998). This has meant that rather than measuring an absolute MIC which is the minimum concentration at which all growth is inhibited, the standard value measured is the MIC₈₀, the minimum concentration needed to inhibit 80% of the culture growth. This value is used to indicate susceptibility of *C. albicans* to azole drugs (Rex *et al.*, 1997).

In the preliminary checkerboard assays of fluconazole in combination with RC21v3 carried out on planktonic *C. albicans*, the ‘trailing growth’ seen when all three test strains had been exposed to fluconazole only, was eliminated. Specifically, exposure of *C. albicans* strains to RC21v3 at a concentration of 20 µM in conjunction with fluconazole at a concentration of 0.625 µg/mL or more, resulted in a marked reduction in the trailing growth of strain MML611, eliminating the trailing growth in both MML610 and ATCC 10261 strains. This suggested that there was a fungicidal effect of the co-administration of the two drugs which was confirmed by plating the cells at the end of the MIC assay on agar without fluconazole. All *C. albicans* strains express Cdr1p at low levels (Holmes *et al.*, 2008) which may explain the fungistatic nature of fluconazole - the pump may prevent fluconazole reaching the intracellular concentration needed to kill the cells. The inhibition of Cdr1p by RC21v3 allows the intracellular concentration of fluconazole to cross the threshold and become fungicidal to *C. albicans*.

Calculation of the FICI indicated that RC21v3 and fluconazole acted synergistically to inhibit *C. albicans* growth (FICI value <0.5). The degree of synergism was greatest for the azole-resistant *C. albicans* strain MML611 (Table 8).

4.2.2 Synergism in biofilm growth assay

It has been shown that pathology associated with *C. albicans* infections is linked to the development and growth of the microbial biofilm (Douglas, 2003; Ramage, 2005). In addition, biofilm-related infections, such as oro-pharyngeal candidiasis, have been demonstrated to be associated with large numbers of cells phenotypically and behaviourally different to planktonic cells. Therefore, to allow a closer approximation to the antifungal behaviour of *in vivo* biofilms, *in vitro* assays should include testing the susceptibility of the developing biofilm (Jin *et al.*, 2003); (Nett *et al.*, 2011a).

The increased drug resistance of biofilm-associated microbes has been linked to a number of factors. The biofilm matrix may restrict the penetration of antifungal drugs into the biofilm, shielding the basal cells. The matrix may also simply bind the antifungal drug preventing its entry into fungal cells. Altered cellular growth, increased metabolic activity or changed patterns of gene expression that are specific to the biofilm cells, such as those that result in reduced ergosterol content of membranes, may also play a role in the increased drug resistance seen in *C. albicans* biofilms. Metabolic by-products and extracellular signalling molecules within the biofilm mediate cellular interactions, cell regulation, and phenotypic changes of yeast cells (Ramage, 2002) that may enhance the survival of the biofilm. However, it has been shown that the azole resistance of early-stage *C. albicans* biofilms is most often associated with an energy-dependant drug efflux (Mukherjee *et al.*, 2003; Ramage *et al.*, 2002). Therefore it was important to measure whether RC21v3 could overcome the fluconazole-resistance of early biofilms.

Although RC21v3 was discovered only recently (Niimi *et al.*, 2012), its efficacy in an *in vivo* mouse infection model in overcoming oral candidiasis caused by fluconazole-sensitive and fluconazole-resistant *C. albicans* strains has been determined (Hayama *et al.*, 2012). The results for both strains were a drop in viable *C. albicans* numbers, a reduction of clinical lesion scores, and indicated a dose-dependent enhancement of the antifungal effect of fluconazole with concurrently administered RC21v3. There was also a promising RC21v3 dose-dependent enhancement of topical itraconazole

treatment of *in vivo* murine oral lesions (Hayama *et al.*, 2012). However, the effects of RC21v3 on biofilms will need further investigation. A major aim of this project, therefore, was to determine whether RC21v3 in combination with fluconazole is synergistic and/or fungicidal to *C. albicans* cells growing as biofilms.

Crystal (gentian) violet can be used in a relatively simple assay to quantify the amount of yeast cell material in biofilms, and so give a measure of cell growth, or growth inhibition. The crystal violet (CV) is a basic dye which binds to negatively charged surface molecules and polysaccharides, and staining cannot be used to distinguish between extracellular matrices, healthy cells, cells that are alive but losing cell functions, and dead cells (Peeters *et al.*, 2008). Several biofilm assays use the compound XTT [2, 3-bis (2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] to measure the metabolic activity of cells, and thus growth inhibition. However, that results from XTT-reduction and CV colorimetric assays indicate a statistically significantly positive correlation (Li *et al.*, 2003; Jin *et al.*, 2003). Therefore the CV assay was used in these preliminary studies to further decrease assay complexity and reduce cost enabling multiple assays to be carried out within the time and funding constraints of the project.

Results of the checkerboard assays indicated that for biofilm growth, as for planktonic growth, RC21v3 and FLC acted synergistically to inhibit the growth of *C. albicans* ATCC10261.

Although the MIC values can be affected by the methodology used, such as growth conditions, MIC endpoint definition, reading method, and inoculum size used, the adoption of CLSI standard assay conditions helps reduce variation. Another point to consider is that the FICI calculation is based on MICs carried out when the other drug was at a particular concentration. It is possible that different FICI values would be obtained if this drug was at a different concentration. In addition the ‘trailing growth’ shown by *C. albicans* in the presence of FLC creates an artificial FIC and an FICI that is to be interpreted with care (Cuenca-Estrella, 2004).

The production of extracellular material, such as polysaccharides, by the biofilm cells occurs as the biofilm matures (Chandra *et al.*, 2001b). Uptake of the CV dye by this extracellular material may have contributed to the increased optical density of the CV assay as the incubation time increases, and not indicate a true increase in cell number.

A sudden decline in optical density may indicate a shearing away of part of the biofilm as it reaches a size, or shape that is affected by fluid movements. The basal cell layer that is left behind firmly attached to the substrate is able to regenerate the biofilm.

4.3 Confocal microscopy

Confocal microscopy is a relatively non-invasive technique for examining biofilm structure, compared with scanning electron microscopy which involves dehydration steps. The fluorogenic dye SYTO-9 used in confocal microscopy is a nucleic acid stain that binds to DNA which is found within the biofilm extracellular matrix, and is present in both viable and dead cells (Boulos *et al.*, 1999) thereby providing a measure of total biofilm biomass (Peeters *et al.*, 2008). When used in conjunction with propidium iodide (PI) which binds to the nucleotide pair of guanine and cytosine in both RNA and DNA, the green fluorescence of SYTO-9 is quenched in non-viable cells causing them to appear fluorescent red under laser excitation. This allows for a split analysis of the biofilm into live and dead components, not possible with the CV assay.

Although the confocal analysis revealed a biofilm profile similar to the typical pedunculated or ‘mushroom-shaped’ architecture of fungal biofilms (Baillie and Douglas, 1999), the layer of yeast cells in contact with the substratum appears to be not as dense, or tightly packed as in other confocal studies (Baillie and Douglas, 1999; Kuhn *et al.*, 2002b). This may be due to the initial elimination of non-adherent cells on the suspended substrate due to gravity.

Of interest in the present study was the modified architecture of the biofilm exposed to RC21v3. Cell clumping occurred and there was less definition in the structure of the extracellular matrix. Further study is required to understand if RC21v3 triggers changes in morphology, metabolism, or production of extracellular material.

Of note, the live:dead ratio of the biofilm exposed to only RC21v3 was similar to that of the no-drug control, confirming the lack of antifungal effect of RC21v3 on its own (Niimi, 2012; Hayama *et al.*, 2012).

4.4 Future directions

This project has opened up a number of avenues for further study. It was interesting that the *C. albicans* strains MML610 and MML611 did not form biofilms that were

extensive enough to test the synergism between RC21v3 and fluconazole. In future studies the growth conditions could be modified, for example, either providing more nutrients or extending the biofilm development phase, to improve biofilm growth with these strains.

All biofilm growth measurements were made with the CV assay, but it would be valuable to confirm the synergism effects using an alternative measure of growth such as the XTT colourimetric assay. The XTT (2,3-Bis(2-Methoxy-4-nitro-5-Sulfophenyl)-5-[9Phenyl-amino)Carbonyl]-2H-Tetrazolium Hydroxide) colorimetric assay is a fungal susceptibility test (Hawser *et al.*, 1998) that is widely used to measure the drug resistance of fungi in biofilms (Chandra *et al.*, 2001 b). The mitochondrial dehydrogenases of the live fungal cells convert the tetrazolium salt (XTT) into a water-soluble reduced formazan product that is yellow in colour. This provides a colorimetric indication of viable cell numbers that has been shown to be directly proportional to cell numbers and comparative to the conventional liquid assay (M27-A) (Hawser *et al.*, 1998; Ramage *et al.*, 2001).

Additional research is warranted into the effect that RC21v3 has on the growth and metabolism of *C. albicans* and its consequential modification of the biofilm to help identify mechanisms by which *C. albicans* might develop resistance to the synergism with fluconazole.

Denture stomatitis has a mixed-species aetiology (Theilade and Budtz-Jørgensen, 1988), and the interaction between the primary colonising bacteria and yeasts has been suggested as a modifying factor in the behaviour of the biofilm *C. albicans* (van der Mei, 2000). To bring the *in vitro* biofilm assay closer to the true *in vivo* situation, there is a need to investigate the effect of RC21v3 in combination with fluconazole on mixed species biofilms containing *C. albicans* and oral bacteria.

4.5 Concluding remarks

This study has developed a novel, reproducible, biofilm model that mimics biofilm formation on prosthetic surfaces and which can be used to measure the susceptibility of biofilms to antifungal drugs.

The study confirmed the synergistic combination of RC21v3 and fluconazole against planktonic *C. albicans* cells and that the combination was fungicidal.

The study showed, for the first time, that the combination of RC21v3 and fluconazole inhibited the growth of *C. albicans* biofilms and severely affected biofilm architecture.

In conclusion, RC21v3 in combination with fluconazole may have potential as a therapeutic modality for persistent oral and oesophageal candidiasis, including *Candida*-associated denture stomatitis.

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APPENDIX

6.1 EXCEL SPREAD SHEETS

6.1.1 Mean area of sample covered by biofilm over 'z' slices

(thickness of 'z' slice $3.59\mu\text{m}$)

[illegible]

(thickness of 'z' slice 3.59 μ m)

96

6.1.3 Split image local biofilm thickness

Random sampling of values (high intensity-low intensity), 36 samples within each random sample
NIL

	Label	Area1	Mean1	StdDev1	Area2	Mean2	StdDev2	Area3	Mean3	StdDev3	Area4	Mean4	StdDev4		Area5	Mean5	StdDev5	Area6	Mean6	StdDev6	Area7	Mean7	StdDev7	Area8	Mean8	StdDev8		Area9	Mean9	StdDev9	Area10	Mean10	StdDev10	Area11	Mean11	StdDev11	Area12	Mean12	StdDev12	
1	Projectior	84	52.929	36.647	24	60.958	31.944	90	55.022	35.269	56	73.821	17.722		72	55.653	48.802	40	65.25	47.628	60	70.35	49.17	42	83.833	42.545		45	157.489	43.796	40	143.9	46.466	56	168.071	18.592	117	130.974	60.575	
2	Projectior	84	55	34.994	24	75.708	16.126	90	65.844	24.708	56	74.25	14.419		72	87.486	29.744	40	85.95	40.92	60	66.133	40.623	42	94.619	36.169		45	151.689	37.974	40	163	26.028	56	161.696	32.52	117	105.145	78.64	
3	Projectior	84	69.571	21.105	24	53.917	38.946	90	72.311	42.127	56	69.036	21.815		72	71.375	24.076	40	55.3	47.254	60	13.6	27.43	42	87.929	41.131		45	28.778	56.538	40	158.675	24.174	56	150.036	46.727	117	2.154	13.334	
4	Projectior	84	60.024	29.292	24	10.375	28.04	90	44.744	52.288	56	39.679	37.273		72	54.347	42.585	40	37.8	36.413	60	0	0	42	59.595	53.186		45	19.933	31.627	40	107.125	62.645	56	125.446	64.616	117	10.41	28.358	
5	Projectior	84	16.452	30.614	24	68.083	35.677	90	2.978	13.885	56	9.107	24.314		72	3.556	14.763	40	7	21.268	60	0	0	42	24.976	40.643		45	15.911	28.29	40	55.525	32.466	56	90.25	73.427	117	24.88	40.725	
6	Projectior	84	3.381	15.211	24	22.25	39.367	90	18.611	28.59	56	0	0		72	3.264	13.552	40	1.625	10.277	60	0	0	42	19.048	34.486		45	11.933	24.137	40	18.05	31.662	56	34.107	56.105	117	6.564	24.334	
7	Projectior	84	13.56	27.081	24	20	39.826	90	17.933	26.156	56	10.768	24.83		72	0	0	40	12.05	24.408	60	0	0	42	32.881	40.358		45	0	0	40	66.825	15.533	56	13.143	30.307	117	0	0	
8	Projectior	84	30.095	32.513	24	0	0	90	0	0	56	28.982	29.246		72	0	0	40	0	0	60	0	0	42	17.905	34.704		45	0	0	40	74.125	30.84	56	62.625	49.161	117	0	0	
9	Projectior	84	0	0	24	0	0	90	0	0	56	0	0		72	0	0	40	0	0	60	0	0	42	67.643	40.791		45	0	0	40	0	0	56	116.25	40.847	117	0	0	
10	Projectior	84	0	0	24	0	0	90	0	0	56	0	0		72	0	0	40	0	0	60	0	0	42	0	0		45	0	0	40	0	0	56	50.911	49.534	117	0	0	
11	Projectior	84	4.857	21.852	24	0	0	90	0	0	56	0	0		72	0	0	40	0	0	60	0	0	42	20.214	32.352		45	0	0	40	92.85	29.843	56	48.482	40.703	117	0	0	
12	Projectior	84	27.012	41.749	24	0	0	90	0	0	56	47.25	46.007		72	0	0	40	104.1	96.599	60	0	0	42	35.024	37.18		45	0	0	40	108.075	56.595	56	0	0	117	0	0	
13	Projectior	84	33.952	51.886	24	13.125	26.136	90	28.189	43.301	56	40.018	53.259		72	0	0	40	48	46.24	60	0	0	42	64.714	38.838		45	13.2	34.034	40	6.3	22.407	56	2.679	14.044	117	0	0	
14	Projectior	84	28.619	45.134	24	0	0	90	15.989	34.578	56	40.268	51.642		72	29.653	43.586	40	67.925	55.104	60	0	0	42	72	29.399		45	39.178	46.349	40	0	0	56	5.411	19.686	117	4.692	16.325	
15	Projectior	84	0	0	24	0	0	90	11.444	29.342	56	0	0		72	7.361	24.586	40	87.55	40.445	60	0	0	42	50	45.325		45	11.733	30.252	40	0	0	56	5.429	19.75	117	9.034	22.797	
16	Projectior	84	0	0	24	0	0	90	23.911	37.726	56	0	0		72	9.389	26.743	40	2.05	12.965	60	0	0	42	20.31	37.434		45	0	0	40	0	0	56	8.143	23.719	117	10.718	25.245	
17	Projectior	84	0	0	24	0	0	90	8.022	24.202	56	0	0		72	1.139	9.664	40	44.9	45.021	60	16.8	33.884	42	12.833	29.044		45	83.333	74.042	40	0	0	56	6.857	22.098	117	22.419	55.258	
18	Projectior	84	0	0	24	0	0	90	4.333	17.967	56	0	0		72	9.875	26.31	40	37.825	52.415	60	0	0	42	9.167	25.238		45	143.4	36.657	40	0	0	56	4.125	17.495	117	84.863	80.845	
19	Projectior	84	0	0	24	0	0	90	5.067	19.064	56	0	0		72	8.444	24.052	40	34.2	47.338	60	0	0	42	9.286	25.566		45	134.778	42.592	40	0	0	56	4.143	17.571	117	87.35	83.809	
20	Projectior	84	0	0	24	0	0	90	12.267	27.583	56	0	0		72	1.014	8.603	40	29.85	37.026	60	2.4	13.033	42	9.286	25.566		45	89.089	67.556	40	0	0	56	4.179	17.722	117	85.085	83.516	
21	Projectior	84	0	0	24	0	0	90	16.567	30.198	56	0	0		72	7.764	22.114	40	3.6	15.892	60	0	0	42	17.524	34.306		45	0	0	40	0	0	56	5.571	20.27	117	16.684	46.16	
22	Projectior	84	13.202	31.567	24	0	0	90	21.156	31.657	56	28.054	43.509		72	4.583	16.895	40	59.675	39.213	60	0	0	42	40.333	46.053		45	7.556	21.612	40	0	0	56	9.768	26.078	117	18.376	35.403	
23	Projectior	84	6.667	20.671	24	0	0	90	14.4	27.092	56	11.232	25.901		72	16.236	27.309	40	49.125	43.939	60	0	0	42	70.786	39.914		45	34.133	32.29	40	0	0	56	11.286	27.894	117	27.65	41.653	
24	Projectior	84	10.298	24.211	24	3.833	18.779	90	0	0	56	11.696	25.314		72	0	0	40	19.075	29.51	60	0	0	42	79.095	32.62		45	5.244	16.98	40	31.5	35.271	56	9.893	26.411	117	61.923	66.518	
25	Projectior	84	39.964	48.39	24	4.125	20.208	90	0	0	56	40.607	46.728		72	0	0	40	17.4	26.918	60	0	0	42	43.476	47.305		45	0	0	40	31.8	33.865	56	7.089	22.847	117	0	0	
26	Projectior	84	0	0	24	0	0	90	0	0	56	0	0		72	0	0	40	0	0	60	0	0	42	10.119	27.86		45	0	0	40	59.575	27.916	56	6	21.829	117	0	0	
27	Projectior	84	0	0	24	0	0	90	0	0	56	0	0		72	0	0	40	0	0	60	0	0	42	29.381	44.43		45	0	0	40	0	0	56	114.732	26.812	117	0	0	
28	Projectior	84	0	0	24	0	0	90	0	0	56	0	0		72	0	0	40	0	0	60	0	0	42	0	0		45	0	0	40	0	0	56	21.964	28.73	117	0	0	
29	Projectior	84	0	0	24	0	0	90	0	0	56	0	0		72	0	0	40	0	0	60	0	0	42	26.31	33.949		45	0	0	40	30.3	46.874	56	54.804	41.278	117	0	0	
30	Projectior	84	61.131	47.036	24	0	0	90	0	0	56	71.107	43.4		72	0	0	40	0	0	60	0	0	42	17.262	31.255		45	0	0	40	47.35	57.993	56	26.821	38.249	117	0	0	
31	Projectior	84	39.893	45.233	24	4.917	16.657	90	35.189	47.64	56	56.661	44.288		72	0	0	40	11.75	31.484	60	0	0	42	33.571	37.387		45	0	0	40	74.075	36.239	56	60.107	64.898	117	3.829	14.195	
32	Projectior	84	0	0	24	0	0	90	26.778	42.249	56	0	0		72	4	19.318	40	17.825	36.107	60	0	0	42	49.929	45.335		45	14.622	34.454	40	56.375	39.618	56	110.536	60.015	117	15.915	27.218	
33	Projectior	84	7	23.356	24	45.375	32.776	90	3.911	18.237	56	1.5	11.225		72	0	0	40	2.15	13.598	60	0	0	42	66.333	48.765		45	5.889	22.284	40	46.65	40.618	56	139.536	46.473	117	8.017	21.839	
34	Projectior	84	11.726	28.896	24	0	0	90	30.211	40.901	56	7.321	23.594		72	85.444	46.779	40	50.475	44.858	60	0	0	42	92.19	35.499		45	9.533	27.269	40	94.375	68.864	56	155.429	30.575	117	1.821	11.271	
35	Projectior	84	56.786	37.188	24	54.958	32.415	90	89.533	54.083	56	34.696	40.427		72	63.889	37.814	40	14.175	31.169	60	21.133	36.914	42	99.667	28.388		45	54.422	83.194	40	145.175	61.227	56	165.161	20.018	117	81.65	71.97	
36	Projectior	84	69.643	25.756	24	71.958	15.33	90	70.8	25.176	56	64.946	30.556		72	95.486	29.761	40	98.575	34.119	60	93.55	34.438	42	93.738	35.85		45	169.8	37.123	40	163.45	29.601	56	166.018	26.767	117	130.957	55.116	
			711.762	32.4		509.582	36.4		695.21	29		760.999	38.1			619.958	31																							

FLC

Label	Area1	Mean1	StdDev1	Min1	Max1	Median1	Area2	Mean2	StdDev2	Min2	Max2	Median2	Area3	Mean3	StdDev3	Min3	Max3	Median3	Area4	Mean4	StdDev4	Min4	Max4	Median4	Area5	Mean5	StdDev5	Min5	Max5	Median5	Area6	Mean6	StdDev6	Min6	Max6	Median6
1 Projector	56	98.857	60.336	0	136	135	60	112.6	50.782	0	136	135	84	102.762	57.791	0	135	135	56	96.625	61.665	0	136	135	40	108.8	55.093	0	136	136	45	102.756	59.107	0	136	136
2 Projector	56	87.804	63.49	0	133	133	60	111.667	50.36	0	134	134	84	54.548	63.365	0	128	0	56	90.446	65.401	0	137	137	40	102.95	60.197	0	138	137	45	24.533	53.357	0	138	0
3 Projector	56	51.071	64.065	0	130	0	60	94.6	59.983	0	132	132	84	5.714	25.709	0	120	0	56	66.589	69.636	0	139	0	40	104.425	61.059	0	140	139	45	15.667	44.813	0	141	0
4 Projector	56	2.286	17.105	0	128	0	60	69.317	65.387	0	131	130	84	0	0	0	0	0	56	37.411	62.411	0	140	0	40	84.85	70.163	0	142	141	45	28.778	58.206	0	144	0
5 Projector	56	0	0	0	0	0	60	27.683	53.082	0	129	0	84	0	0	0	0	0	56	35.679	62.357	0	143	0	40	17.875	47.895	0	143	0	45	19.6	50.535	0	147	0
6 Projector	56	4.25	22.284	0	120	0	60	39.467	58.466	0	125	0	84	0	0	0	0	0	56	38.875	64.854	0	146	0	40	0	0	0	0	0	45	23.644	55.712	0	152	0
7 Projector	56	8.071	29.365	0	113	0	60	25.917	49.696	0	121	0	84	0	0	0	0	0	56	48.768	71.5	0	152	0	40	0	0	0	0	0	45	0	0	0	0	0
8 Projector	56	0	0	0	0	0	60	32.717	50.404	0	112	0	84	0	0	0	0	0	56	71.536	80.4	0	164	0	40	70.475	83.028	0	169	0	45	0	0	0	0	0
9 Projector	56	0	0	0	0	0	60	0	0	0	0	0	84	0	0	0	0	0	56	0	0	0	0	0	40	0	0	0	0	45	0	0	0	0	0	
10 Projector	56	0	0	0	0	0	60	0	0	0	0	0	84	0	0	0	0	0	56	0	0	0	0	0	40	0	0	0	0	45	0	0	0	0	0	
11 Projector	56	0	0	0	0	0	60	0	0	0	0	0	84	0	0	0	0	0	56	18.125	36.995	0	95	0	40	0	0	0	0	45	0	0	0	0	0	
12 Projector	56	32.857	67.06	0	169	0	60	64.65	77.143	0	157	0	84	0	0	0	0	0	56	33.911	51.847	0	114	0	40	18.95	41.67	0	109	0	45	0	0	0	0	0
13 Projector	56	2.768	20.713	0	155	0	60	24.85	56.035	0	150	0	84	0	0	0	0	0	56	36.339	55.543	0	121	0	40	28.975	50.83	0	118	0	45	66.289	54.738	0	111	110
14 Projector	56	2.643	19.777	0	148	0	60	26.183	55.729	0	144	0	84	0	0	0	0	0	56	50.714	61.299	0	125	0	40	15.125	40.527	0	121	0	45	0	0	0	0	0
15 Projector	56	0	0	0	0	0	60	70.1	70.693	0	141	139	84	0	0	0	0	0	56	20.339	46.901	0	127	0	40	0	0	0	0	0	45	0	0	0	0	0
16 Projector	56	0	0	0	0	0	60	75.917	69.249	0	139	138	84	0	0	0	0	0	56	9.214	33.523	0	129	0	40	0	0	0	0	0	45	0	0	0	0	0
17 Projector	56	34.5	60.297	0	138	0	60	77.15	68.036	0	137	136	84	0	0	0	0	0	56	0	0	0	0	0	40	0	0	0	0	45	22.933	49.877	0	129	0	
18 Projector	56	63.143	68.44	0	136	0	60	71.85	67.778	0	135	134	84	18.333	47.512	0	140	0	56	0	0	0	0	0	40	0	0	0	0	45	2.911	19.528	0	131	0	
19 Projector	56	62.071	67.28	0	134	0	60	66.5	67.061	0	133	133	84	11.083	36.98	0	133	0	56	0	0	0	0	0	40	3.35	21.187	0	134	0	45	14.867	42.525	0	134	0
20 Projector	56	32.75	57.238	0	131	0	60	65.683	66.239	0	132	131	84	34.381	56.328	0	126	0	56	0	0	0	0	0	40	13.5	41.016	0	135	0	45	15.089	43.16	0	136	0
21 Projector	56	0	0	0	0	0	60	68.883	64.979	0	130	129	84	0	0	0	0	0	56	0	0	0	0	0	40	3.4	21.503	0	136	0	45	3.067	20.572	0	138	0
22 Projector	56	0	0	0	0	0	60	53.017	63.26	0	128	0	84	0	0	0	0	0	56	0	0	0	0	0	40	0	0	0	0	45	24.933	54.227	0	141	0	
23 Projector	56	4.357	22.845	0	122	0	60	10.417	34.84	0	125	0	84	0	0	0	0	0	56	12.554	40.456	0	141	0	40	0	0	0	0	45	0	0	0	0	0	
24 Projector	56	31.089	51.865	0	117	0	60	20	45.099	0	120	0	84	0	0	0	0	0	56	51.214	69.335	0	144	0	40	0	0	0	0	45	0	0	0	0	0	
25 Projector	56	17.482	40.313	0	110	0	60	51.433	57.342	0	115	0	84	0	0	0	0	0	56	52.25	70.739	0	145	0	40	37.375	65.561	0	150	0	45	27.444	59.69	0	156	0
26 Projector	56	20.036	38.714	0	95	0	60	27.25	45.573	0	104	0	84	0	0	0	0	0	56	54.982	74.44	0	156	0	40	114.375	71.353	0	160	156	45	18.556	53.076	0	167	0
27 Projector	56	0	0	0	0	0	60	0	0	0	0	0	84	0	0	0	0	0	56	37.518	72.494	0	177	0	40	0	0	0	0	45	0	0	0	0	0	
28 Projector	56	0	0	0	0	0	60	0	0	0	0	0	84	0	0	0	0	0	56	0	0	0	0	0	40	0	0	0	0	45	0	0	0	0	0	
29 Projector	56	0	0	0	0	0	60	0	0	0	0	0	84	0	0	0	0	0	56	37.732	49.174	0	103	0	40	2.475	15.653	0	99	0	45	0	0	0	0	0
30 Projector	56	0	0	0	0	0	60	67.467	80.504	0	163	0	84	0	0	0	0	0	56	59.696	58.132	0	117	113	40	0	0	0	0	45	16.444	38.748	0	106	0	
31 Projector	56	0	0	0	0	0	60	10.067	37.984	0	151	0	84	0	0	0	0	0	56	44.071	59.665	0	125	0	40	0	0	0	0	45	20.533	44.658	0	116	0	
32 Projector	56	18.875	50.39	0	151	0	60	19.6	50.393	0	148	0	84	0	0	0	0	0	56	29.304	53.777	0	127	0	40	37.825	58.515	0	127	0	45	10.822	35.04	0	122	0
33 Projector	56	42	67.009	0	147	0	60	45.467	67.354	0	144	0	84	0	0	0	0	0	56	46.339	62.734	0	130	0	40	96.375	56.355	0	130	128	45	16.8	43.316	0	126	0
34 Projector	56	0	0	0	0	0	60	35.217	61.512	0	142	0	84	0	0	0	0	0	56	77.286	65.107	0	132	131	40	114.35	43.774	0	132	131	45	0	0	0	0	0
35 Projector	56	15	43.693	0	140	0	60	67.117	69.978	0	139	0	84	7.095	31.921	0	149	0	56	85.357	64.198	0	133	133	40	99.6	58.238	0	133	133	45	70.356	66.556	0	132	131
36 Projector	56	80.732	68.009	0	137	137	60	98.183	62.255	0	137	137	84	50.667	68.385	0	142	0	56	95.732	61.094	0	135	134	40	107.325	54.347	0	135	134	45	101.244	58.238	0	134	134

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Label	Area1	Mean1	StdDev1	Area2	Mean2	StdDev2	Area3	Mean3	StdDev3	Area4	Mean4	StdDev4	Area5	Mean5	StdDev5	Area6	Mean6	StdDev6	Area7	Mean7	StdDev7	Area8	Mean8	StdDev8	Area9	Mean9	StdDev9	Area10	Mean10	StdDev10	Area11	Mean11	StdDev11	Area12	Mean12	StdDev12
1 Projector	80	60.013	37.696	54	48.778	26.639	130	35.885	21.209	60	6.1	18.578	70	80.4	41.088	72	96.347	22.271	72	87.264	31.121	72	85.25	35.537	126	112.913	46.686	105	158.448	51.214	56	148.5	44.934	90	82.867	65.076
2 Projector	80	75.012	33.105	54	53.815	24.429	130	43.377	20.284	60	41.55	30.458	70	78.629	40.836	72	96.972	22.632	72	91.542	22.058	72	91.792	28.48	126	127.825	16.151	105	177.048	17.879	56	146.536	46.968	90	119	47.059
3 Projector	80	67.012	45.82	54	52.722	25.743	130	54.515	24.361	60	0	0	70	73.043	42.271	72	97.778	20.221	72	92.736	19.168	72	0	0	126	104.587	23.38	105	176.676	17.305	56	147.071	47.052	90	126.911	47.317
4 Projector	80	46.263	28.216	54	23.056	30.761	130	0	0	60	0	0	70	56.243	48.544	72	98.917	14.404	72	75.861	40.288	72	0	0	126	35.46	41.075	105	91.79	82.325	56	140.554	45.143	90	25.911	53.582
5 Projector	80	10.137	21.236	54	42.093	48.431	130	0	0	60	0	0	70	31.457	44.711	72	93.764	24.147	72	33.264	44.569	72	0	0	126	0	0	105	5.61	15.899	56	150.125	40.304	90	0	0
6 Projector	80	0	0	54	5.463	17.523	130	0	0	60	0	0	70	1.286	7.551	72	71.722	7.511	72	71.111	16.014	72	0	0	126	6.2	15.630	105	22.3	39.415	56	151.827	24.487	90	12.856	44.847
7 Projector	80	0	0	54	1	7.348	130	0	0	60	0	0	70	11.671	24.117	72	46.069	49.147	72	8.75	17.77	72	0	0	126	0	0	105	7.638	18.8	56	154.018	31.436	90	21.433	34.854
8 Projector	80	0	0	54	77.87	39.082	130	0	0	60	0	0	70	38.986	38.856	72	43.056	33.251	72	2.111	8.165	72	0	0	126	0	0	105	54.724	34.297	56	155.5	27.769	90	0	0
9 Projector	80	0	0	54	0	0	130	0	0	60	0	0	70	13.886	17.65	72	37.083	23.376	72	30.042	24.711	72	0	0	126	0	0	105	0	0	56	155.821	26.556	90	0	0
10 Projector	80	0	0	54	0	0	130	0	0	60	0	0	70	0	0	72	20.528	38.952	72	0	0	72	0	0	126	0	0	105	0	0	56	163.125	6.672	90	0	0
11 Projector	80	0	0	54	0	0	130	0	0	60	0	0	70	99.6	19.017	72	19.653	19.258	72	0	0	72	0	0	126	0	0	105	0	0	56	160	19.033	90	0	0
12 Projector	80	0	0	54	29.63	9.571	130	0	0	60	0	0	70	45.657	34.524	72	23.542	26.462	72	64.375	50.917	72	0	0	126	0	0	105	6.438	12.939	56	159.607	17.126	90	0	0
13 Projector	80	0	0	54	0.685	5.035	130	0	0	60	0	0	70	68.529	23.354	72	37.264	26.596	72	6.375	16.985	72	0	0	126	0	0	105	45.038	44.378	56	159.286	14.756	90	16.989	20.928
14 Projector	80	0	0	54	32.944	15.86	130	0	0	60	0	0	70	13.7	26.074	72	18.764	27.114	72	0	0	72	0	0	126	10.508	22.327	105	0	0	56	152.464	33.541	90	19.111	19.81
15 Projector	80	6.825	14.299	54	0	0	130	0	0	60	0	0	70	0	0	72	0.611	5.185	72	5.333	15.191	72	0	0	126	11.492	22.095	105	21.657	34.877	56	139.161	49.809	90	0	0
16 Projector	80	7.312	15.318	54	0	0	130	7.354	15.312	60	0	0	70	0	0	72	0	0	72	0.667	5.657	72	0	0	126	0	0	105	4.009	17.405	56	151.929	51.929	90	0.478	4.533
17 Projector	80	5.775	14.555	54	0	0	130	8.615	19.431	60	0	0	70	0	0	72	0	0	72	0	0	72	0	0	126	0	0	105	0	0	56	105.821	70.732	90	2.444	10.135
18 Projector	80	4.525	14.942	54	0	0	130	0.338	3.859	60	0	0	70	0	0	72	0	0	72	0	0	72	0	0	126	0	0	105	4.286	13.273	56	89.339	73.413	90	3	11.288
19 Projector	80	9.5	21.963	54	0	0	130	1.415	7.975	60	0	0	70	0	0	72	0	0	72	0	0	72	0	0	126	0	0	105	4.381	13.568	56	74.446	70.46	90	2.556	10.596
20 Projector	80	5.938	18.143	54	0	0	130	1.885	9.46	60	0	0	70	0	0	72	0	0	72	0	0	72	0	0	126	0	0	105	2.686	10.962	56	70.607	68.922	90	3.133	11.175
21 Projector	80	6.875	17.327	54	0	0	130	11.415	24.464	60	0	0	70	0	0	72	0	0	72	0	0	72	0	0	126	0	0	105	0	0	56	71.429	68.337	90	2.178	10.155
22 Projector	80	11.15	21.601	54	0	0	130	10.8	21.684	60	0	0	70	0	0	72	0	0	72	0.611	5.185	72	5.417	13.582	126	0	0	105	11.352	29.9	56	74.607	68.049	90	0	0
23 Projector	80	6.875	18.304	54	0	0	130	0	0	60	0	0	70	0	0	72	0	0	72	0	0	72	0	0	126	4.643	12.68	105	15.552	32.095	56	85.268	64.982	90	0	0
24 Projector	80	0	0	54	38.944	22.139	130	0	0	60	0	0	70	3.129	11.362	72	6.25	19.292	72	0	0	72	0	0	126	1.762	7.911	105	0	0	56	85.929	63.648	90	17.267	26.015
25 Projector	80	0	0	54	1.963	10.103	130	0	0	60	0	0	70	37.1	32.855	72	31.125	31.886	72	0.569	4.832	72	0	0	126	1.31	6.648	105	87	63.557	56	90.357	63.547	90	15.189	29.085
26 Projector	80	0	0	54	51.796	18.494	130	0	0	60	0	0	70	57.486	19.401	72	43.167	38.349	72	43.167	31.314	72	0	0	126	105	6	105	97.029	53.648	90	0	0	0	0	0
27 Projector	80	0	0	54	0	0	130	0	0	60	0	0	70	62.886	16.696	72	25.042	26.896	72	37.222	29.301	72	0	0	126	0	0	105	0	0	56	99.129	52.024	90	0	0
28 Projector	80	0	0	54	0	0	130	0	0	60	0	0	70	0	0	72	0	0	72	0	0	72	0	0	126	0	0	105	0	0	56	100.946	53.899	90	0	0
29 Projector	80	0	0	54	0	0	130	0	0	60	0	0	70	30.614	28.3	72	28.444	17.206	72	48.264	49.593	72	0	0	126	0	0	105	0	0	56	108.018	58.557	90	0	0
30 Projector	80	0	0	54	15.019	17.9	130	0	0	60	0	0	70	47.057	47.061	72	25.417	21.039	72	13.5	23.547	72	0	0	126	0	0	105	26.695	20.312	56	121.339	49.729	90	0	0
31 Projector	80	0	0	54	22.13	27.491	130	0	0	60	0	0	70	19.257	29.028	72	29.681	40.024	72	8.333	18.765	72	0	0	126	0	0	105	33.952	47.018	56	131.375	47.024	90	41.744	24.704
32 Projector	80	0	0	54	0	0	130	0	0	60	0	0	70	28.343	40.225	72	55.528	44.801	72	2.722	11.303	72	0	0	126	6.905	21.157	105	27.181	38.547	56	137.982	47.284	90	1.644	7.668
33 Projector	80	16.688	20.464	54	46.463	40.08	130	0	0	60	0	0	70	53.929	48.572	72	77.722	36.289	72	0	0	72	0	0	126	7.331	27.242	105	0	0	56	149.089	33.644	90	3.567	12.548
34 Projector	80	31.475	21.616	54	1.593	8.197	130	0	0	60	0	0	70	75.043	43.41	72	94.319	18.258	72	12.722	29.017	72	0	0	126	26.556	44.036	105	9.676	21.146	56	152.786	32.078	90	0	0
35 Projector	80	31.337	36.568	54	31.593	29.913	130	23.5	27.027	60	0	0	70	82.357	38.709	72	96.125	18.692	72	62.056	48.712	72	12.208	30.677	126	119.753	75.763	105	115.648	65.477	56	151.946	34.461	90	65	0
36 Projector	80	65.75	29.789	54	50.165	24.368	130	43.186	19.258	60	59.133	27.917	70	84.986	37.638	72	96.042	22.33	72	31.745	0	0	0	126	105.528	26.456	105	135.587	17.045	56	158.818	40.12	90	119.9	42.343	
		469.462	26.1	627.724	11.4	242.284	20.2	106.783	35.6				1195.274	47.8	1408.557	52.2	829.486	33.2							712.231	47.8		1285.381	51.4		4567.66	126.9		703.956	33.5	
		18	50		16	34		24	67		33	92		11	31										21	58				11	31			15	42%	