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# **Evaluating *Candida albicans* biofilm formation and novel antifungal treatment**

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Degree of Doctor of Philosophy

School of Medicine

College of Medical, Veterinary and Life Sciences

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## Abstract

*Candida* biofilms have become an increasingly important clinical problem. The widespread use of antibiotics, frequent use of indwelling medical devices, and a trend towards increased patient immuno-suppression has resulted in a creation of opportunity for clinically important yeasts to form biofilms. Whilst there is growing evidence of the importance of *Candida* biofilms in clinical medicine, not all clinical isolates are able to form biofilms. There is therefore a fundamental gap in understanding exactly what drives biofilm formation and its clinical implications. These structures have become increasingly recognised as a significant clinical problem. One of the major reasons behind this is the impact that these have upon treatment, as antifungal therapy often fails and surgical intervention is required. This places a large financial burden on health care providers. Therefore, the discovery of alternative antifungal agents to be used in the treatment of fungal biofilms is in great demand for the management of these infections.

A panel of *Candida albicans* bloodstream isolates were assessed for their biofilm forming ability by using the crystal violet assay and measuring cellular surface hydrophobicity. Scanning electron microscopy was used to visualise differences in the clinical biofilms. The impact of amphotericin B (AMB) treatment was determined next by broth microdilution method to assess differences in susceptibility profiles of the clinical isolates. The virulence of these clinical isolates was evaluated *in vivo* using a *Galleria mellonella* model and transcriptional analysis used to assess the expression of various genes associated with *C. albicans* biofilm formation within clinical isolates. Extracellular DNA (eDNA) in clinical biofilms was quantified using a microplate fluorescence assay and chitinase activity measured using a biochemical assay. Moreover, the potential of a novel antimicrobial agent Carbohydrate-derived fulvic acid (CHD-FA) was assessed against a panel of fungal and bacterial species. The mechanism of action of CHD-FA was determined using membrane assays include ATP release, and propidium iodide fluorescence, with various inhibitors used to determine whether CHD-FA activity is affected by known resistance mechanisms. Finally, the immunomodulatory properties of CHD-FA were investigated using ELISA and PCR arrays.

The results from this study have shown *C. albicans* biofilm formation is differential within clinical isolates, where those with high biofilm formation (HBF) predominately consisted of hyphal cells, were more virulent *in vivo* and had decreased susceptibility to AMB, when compared to those with low biofilm formation (LBF). Furthermore, transcriptional analysis identified a number of genes that positively correlated with *C. albicans* biofilm formation. The novel agent carbohydrate-derived fulvic acid (CHD-FA) was shown to not only be highly active against *C. albicans* biofilms, but also against a range of orally relevant bacteria through non-specific membrane activity. Furthermore, CHD-FA was shown to down-regulate a number of pro-inflammatory mediators in an oral epithelial cell line.

In conclusion, this study has characterised *C. albicans* clinical isolates based on their biological characteristics, where clear difference in virulence and antifungal treatment have been shown. It may be possible to develop a panel of genetic markers that could be used as a diagnostic tool for detecting biofilm formation in clinical isolates. CHD-FA is a microbiocidal compound that may serve as a potential novel antiseptic agent for the treatment of oral candidiasis and other candidal biofilm infections, whereby the immunomodulatory properties of CHD-FA could be exploited for controlling inflammation in a number of diseases.

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## **Author's declaration**

I declare that I have carried out the work described in this thesis unless otherwise acknowledged or cited, under the supervision of Professor Gordon Ramage. I further declare that this thesis has not, in whole or in part, been submitted for any other degree.

Leighann Sherry

July 2014

## Abbreviations

|          |   |
|----------|---|
| ABC:     | ATP-binding cassette                        |
| AIDS:    | Acquired immune deficiency syndrome         |
| Ala-Nap: | Alanine $\beta$ -naphthylamine              |
| ALT:     | Antifungal lock therapy                     |
| AmBi:    | Ambisome                                    |
| AMB:     | Amphotericin B                              |
| ANOVA:   | Analysis of variance                        |
| ATP:     | Adenosine triphosphate                      |
| AZE:     | Azetazolamide                               |
| AZL:     | Azoles                                      |
| BAL:     | Bronchopulmonary lavage                     |
| BPE:     | Bovine pituitary extract                    |
| BSA:     | Bovine serum albumin                        |
| BSI:     | Bloodstream infections                      |
| cDNA:    | Complementary deoxyribonucleic acid         |
| CF:      | Cystic fibrosis                             |
| CFU:     | Colony forming units                        |
| CFW:     | Calcofluor white                            |
| CHD-FA:  | Carbohydrate derived fulvic acid            |
| CHX:     | Chlorhexidine                               |
| CLSI:    | Clinical and laboratory standards institute |
| CLSM:    | Confocal laser scanning microscopy          |
| CSH:     | Cellular surface hydrophobicity             |

|                    |  |
|--------------------|--|
| CSP:               | Caspofungin                            |
| Ct:                | Cycle threshold                        |
| CV:                | Crystal violet                         |
| CVC:               | Central venous catheters               |
| dH <sub>2</sub> O: | Distilled water                        |
| DIS:               | Denture induced stomatitis             |
| D-KSFM:            | Defined keratinocyte serum free medium |
| DMEM:              | Dulbecco's modified eagle's media      |
| DMSO:              | Dimethyl sulfoxide                     |
| DNA:               | Deoxyribonucleic acid                  |
| DW:                | Dry weight                             |
| ECM:               | Extracellular matrix                   |
| ECN:               | Echinocandins                          |
| eDNA:              | Extracellular DNA                      |
| EDTA:              | Ethylenediaminetetraacetic acid        |
| EGF:               | Epidermal growth factor                |
| ELISA:             | Enzyme linked immunosorbent assay      |
| EPI:               | Efflux pump inhibitor                  |
| FCS:               | Foetal calf serum                      |
| FLZ:               | Fluconazole                            |
| GDN:               | Geldanamycin                           |
| GFP:               | Green fluorescent protein              |
| GTT:               | Germ tube test                         |
| HAI:               | Hospital acquired infections           |
| HBF:               | High biofilm formation                 |

|             |  |
|-------------|--|
| HIV:        | Human immunodeficiency virus                                 |
| HMDS:       | Hexamethyldisilazane   |
| HSP:        | Heat shock protein   |
| IL-8:       | Interleukin-8  |
| INF:        | Intermediate biofilm formation                               |
| ICU:        | Intensive care unit  |
| KSFM:       | Keratinocyte serum free medium                               |
| LBF:        | Low biofilm formation  |
| MAPK:       | Mitogen activated protein kinase                             |
| MDR:        | Multi drug resistance  |
| MFA:        | Microplate fluorescence assay                                |
| MFG:        | Micafungin   |
| MFS:        | Major facilitator superfamily                                |
| MIC:        | Minimum inhibitory concentration                             |
| MTT:        | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NAC:        | N-acetylcysteine   |
| NKZ:        | Nikkomycin Z   |
| NT:         | Newton's type  |
| NTC:        | Non-template control   |
| OD:         | Optical density  |
| OKF6/TERT2: | Oral keratinocyte cell line                                  |
| PBS:        | Phosphate buffered saline                                    |
| PC:         | Pseudomembranous candidiasis                                 |
| PCR:        | Polymerase chain reaction                                    |

|            |  |
|------------|--|
| PI:        | Propidium iodide   |
| POL:       | Polyenes   |
| qPCR:      | Quantitative polymerase chain reaction                                   |
| RFU:       | Relative fluorescence units  |
| RNA:       | Ribonucleic acid   |
| ROS:       | Reactive oxygen species  |
| rpm:       | revolutions per minute   |
| RPMI-1640: | Roswell Park Memorial Institute - 1640 media                             |
| RT:        | Reverse transcription  |
| SAB:       | Sabouraud dextrose agar  |
| SAP:       | Secreted aspartyl proteinases  |
| SD:        | Standard deviation   |
| SEM:       | Scanning electron microscopy   |
| SMIC:      | Sessile minimum inhibitory concentration                                 |
| SOD:       | Superoxide dismutase   |
| TBE:       | Tris Borate EDTA   |
| TLDA:      | Taqman Low Density Array   |
| TTO:       | Tea tree oil   |
| VAP:       | Ventilator associated pneumonia  |
| VRZ:       | Voriconazole   |
| WT:        | Wild type  |
| XTT:       | 2,3 bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide |
| YNB:       | Yeast nitrogen base  |
| YPD:       | Yeast peptone dextrose   |

Chapter 1:  
Clinical significance of  
fungal biofilms

## 1.1 Introduction

Fungi represent a significant burden of infection within the hospital population. There are a number of factors that predispose us to invasive fungal infections including, the use of broad-spectrum antibiotics, presence of indwelling catheters, immuno-suppression or disruption of mucosal barriers due to surgery, chemotherapy and radiotherapy (Odds, 1988). *Candida* is the third most common cause of bloodstream infections (BSI) in patients requiring intensive care in the US (Wisplinghoff et al., 2004) and the most common etiologic agent of fungal related biofilm infections. *C. albicans*, normally a commensal of human mucosal surfaces yet an opportunistic pathogen in immunocompromised patients, has the ability to form biofilms on a number of medical devices including central venous catheters (CVC) and a variety of prostheses. These surfaces are ideal for the colonisation of *Candida spp.* and allow the development of mature biofilm communities where planktonic cells can detach cause an acute candidaemia and/or disseminated infection. Furthermore, a study investigating the pathogenicity of cells dispersed from biofilms had shown a greater association with mortality than equivalent planktonic yeasts (Uppuluri et al., 2010). Infections associated with medical devices are essentially difficult to eradicate, with the only method of controlling such an infection being long-term use of antifungals and the removal of the implant. Although *C. albicans* remains the dominant species responsible for human disease, *C. glabrata*, *C. parapsilosis*, *C. dubliniensis*, *C. krusei* and *C. tropicalis* (Ramage et al., 2006, Shin et al., 2002, Tumbarello et al., 2007) are associated with biofilm formation and contribute to device-related bloodstream infections.

This chapter provides a review of the current literature around fungal biofilms, the use of current antifungal strategies use clinically and highlights the importance of identifying novel antimicrobials. Some aspects of this chapter have been published in:

Ramage G, Rajendran R, Sherry L and Williams C. 2012. Fungal biofilm resistance. Int J Microbiol:528521. Epub 2012 Feb 8.

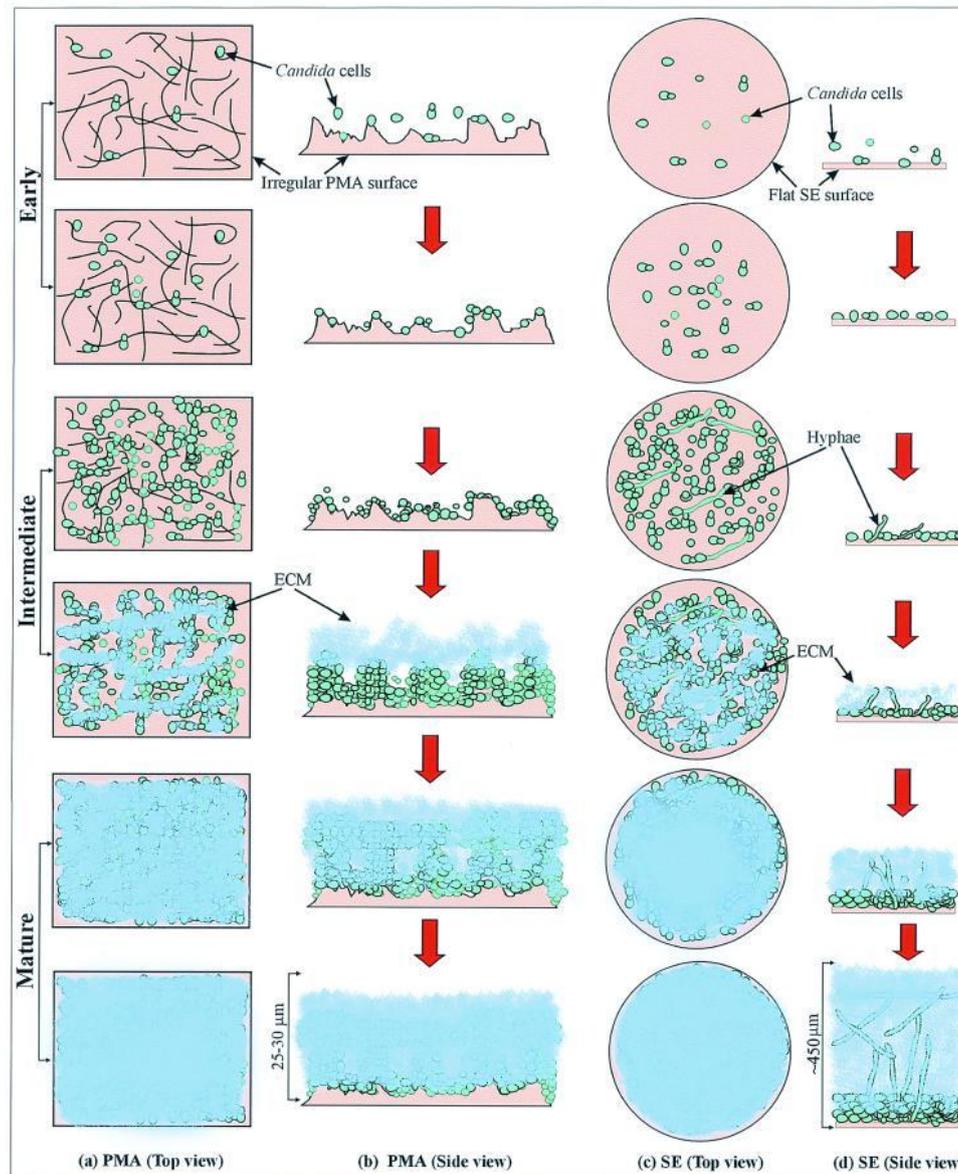
## 1.2 Biofilms

Historically, microbiologists have studied free-floating planktonic cells in pure culture. However, it is now widely accepted a link exists between sessile (surface attached and heterogeneous) cells, microbial pathogenesis and human infection (Costerton et al., 1995). It is apparent that a wide range of fungi alternate between planktonic cells and multi-cellular communities, known as biofilms. It has been estimated that up to 80% of all microorganisms in the environment exist in sessile communities (Donlan, 2002). Biofilms are defined as a group of microorganisms with the ability to attach to surfaces, and or one another, and are enclosed within a self-produced extracellular polymeric matrix (Ramage et al., 2009).

It has been estimated that 65% of all clinical infections are due to the presence of a biofilm, which considering these structures are highly associated with antimicrobial resistance makes the management of such infections difficult within the clinical setting (Donlan and Costerton, 2002). In recent years it has been appreciated that fungal species have the ability to form biofilms and have a significant impact clinically (Ramage et al., 2009, Sayed et al., 2012a, Fanning and Mitchell, 2012). The main reason to why *C. albicans* is the most studied *Candida spp.* is because it is a highly adaptable opportunistic pathogen with excellent biofilm activity on a number of substrates (Ramage et al., 2006). *C. albicans* is a dimorphic microorganism that can exist either as yeast or as hyphal cells and form the foundations of a complex multicellular biofilm.

The adhesion and colonisation of biological and innate surfaces, such as the oral mucosa or denture acrylic substrates, is commonplace for *C. albicans* (Ellepola and Samaranayake, 1998, Holmes et al., 2002, Radford et al., 1999, Williams et al., 2011). The initial attachment to the surface is controlled by a number of factors, including the flow of the surrounding medium (urine, blood, saliva, mucus), pH, temperature, osmolarity, bacteria, presence of antimicrobial agents, and host immune factors (Hawser et al., 1998a, Baillie and Douglas, 2000, Richard et al., 2005, Ramage et al., 2002d, Ramage et al., 2008, Chandra et al., 2001b). *C. albicans* biofilms have defined phases of development that have been described through the use of defined model systems (Al-Fattani and Douglas, 2004, Baillie and Douglas, 1998a, Chandra et al., 2001a, Garcia-Sanchez

et al., 2004, Nikawa et al., 2003, Ramage et al., 2001a, Ramage et al., 2008), as shown in Figure 1.1. These key phases include presence of an appropriate substratum, adhesion, colonisation, extracellular matrix (ECM) production, biofilm maturation and dispersal (Blankenship and Mitchell, 2006, Chandra et al., 2001a, Ramage et al., 2001b).



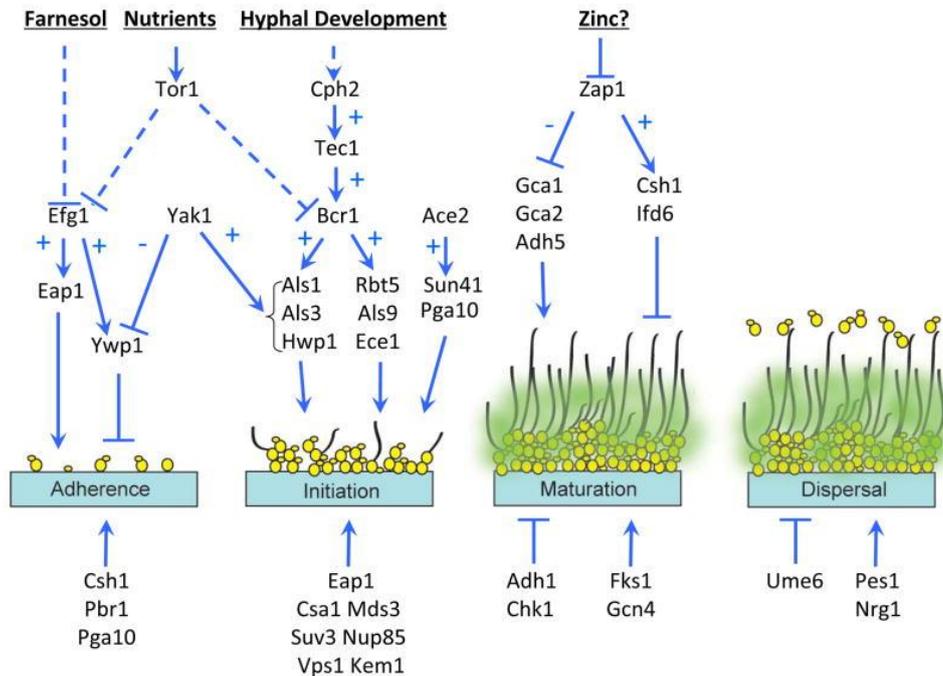
**Figure 1.1 - Stages of *C. albicans* biofilm development.**

There are three stages of biofilm formation, early - adhesion and germination (0 - 8 h), intermediate - hyphal development and ECM production (9 -18 h) and maturation (19-24 h), which is followed by dispersal of yeast cells. Circles = yeast cells, black lines = hyphae, blue = extracellular matrix (Chandra et al., 2001a).

Simplistically, yeast cells attach to a surface via adhesins including, agglutinin like sequence protein Als3p and the cell wall protein Eap1p (Zhao et al., 2006, Li et al., 2007). This leads to the formation of microcolonies and the morphology of

yeast cells switches to pseudo- and true-hyphae by the regulator Efg1p (Ramage et al., 2002d), to form a complex network of hyphal structures with budding yeast cells distributed throughout.

Next, as the biofilm matures, a glucan rich extracellular matrix (ECM) encompasses the structure (Nett et al., 2010c), providing a protective barrier from host defences, antimicrobial agents and environmental stresses. During the development of a mature biofilm, a hypoxic environment is created which induces the up-regulation of glycolytic genes that control filamentation (Bonhomme et al., 2011). Finally, planktonic yeast cells are able to disperse from the mature biofilm and colonise a new surface to begin the development of a new biofilm (Uppuluri et al., 2010). The process of biofilm formation is complex, controlled by a variety of transcription factors including Bcr1p, Ace2p, Efg1p and Zap1p, all of which are involved in sophisticated molecular pathways (Finkel and Mitchell, 2011, Nobile and Mitchell, 2006, Zhao et al., 2006, Fanning et al., 2012). Figure 1.2 illustrates our current understanding of the genes involved in *C. albicans* biofilm development and maintenance. The importance of understanding this entire process cannot be understated as it enables us to fully comprehend the mechanisms involved in the process and exploit these for the clinical management of such infections.

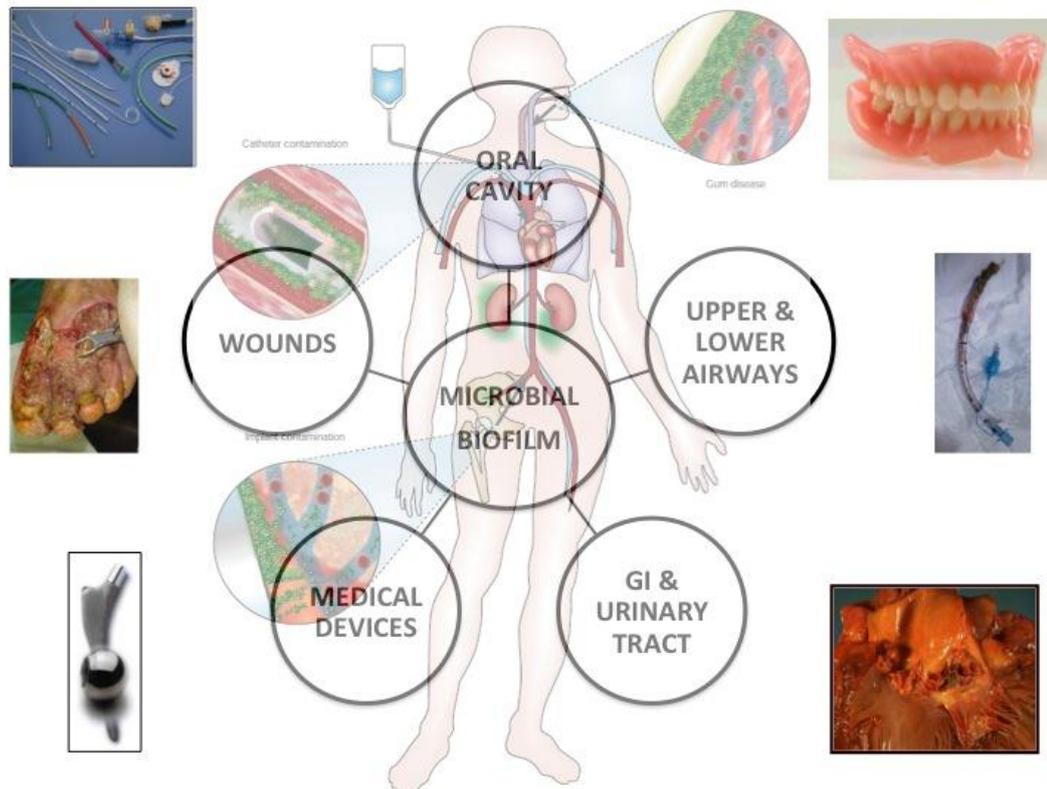


**Figure 1.2 - Genes involved for *C. albicans* biofilm formation.**

A complex molecular pathway is involved in the *C. albicans* biofilm development process, where genes are responsible for the progression from the adherence stage to biofilm dispersal through activation and inhibition of certain genes (Finkel and Mitchell, 2011).

### 1.3 Clinical relevance

*C. albicans* biofilms are able to develop in a number of body sites in the human host, including indwelling medical devices; prosthetic heart valves, breast implants and central venous catheters (Ramage et al., 2006). Figure 1.3 illustrates some of the key sites that can be impacted by *C. albicans* biofilms.



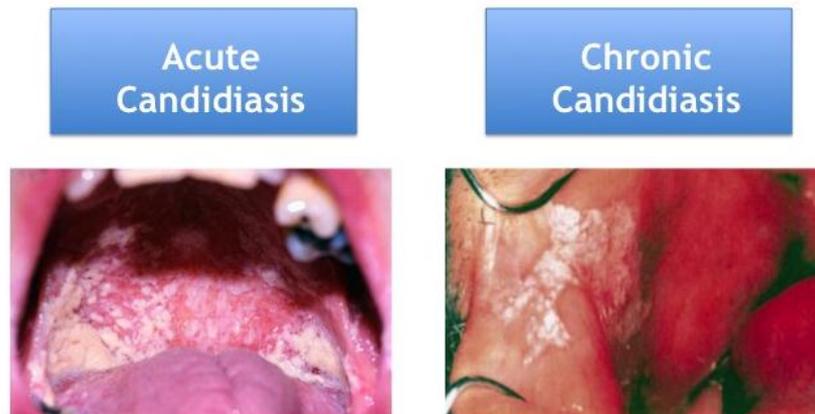
**Figure 1.3 - Key sites of the human host impacted by *C. albicans* biofilms.**

Adapted from (Ramage and Williams, 2013).

### 1.3.1 Oral Cavity

The oral cavity is one of the initial entrance sites for microorganisms, and is an area that a range of multispecies microbial biofilms can be found (Jakubovics, 2010). Biofilms are responsible for oral diseases such as caries, periodontal disease and mucosal infections (Peyyala et al., 2013, Yamakami et al., 2013). Analysis of the oral fungal microbiome revealed 74 culturable and 11 non-culturable genera from twenty healthy individuals (Ghannoum et al., 2010), of which *Candida* species were the most dominant. Oral candidosis is one of the most well studied fungal biofilm infections and affects both soft and hard tissue. The interaction between *Candida* species, bacterial species and a number of host factors allow the formation of complex biofilms (Dongari-Bagtzoglou et al., 2009, Rautemaa and Ramage, 2011). This infection is commonly observed in immunocompromised patients including those on long term corticosteroid treatments, diabetes patients and those with a human immunodeficiency virus (HIV) infection. Acute candidiasis is also referred to pseudomembranous candidiasis (PC) and has been estimated that to affect ~5% of newborns and 10% of the elderly population (Samaranayake et al., 2009a). PC is clinically

represented by white, curd-like patches found on the tongue and other mucous membranes which can be removed upon gentle scraping, leaving an area of inflammation, as shown in Figure 1.4. Oral leukoplakia is classified as chronic candidiasis and represents itself as persistent white lesions on the inside of the cheeks (Figure 1.4). These lesions have been described as potentially carcinogenic and occur with specific risk factors including tobacco and alcohol consumption (van der Waal, 2009, Marichalar-Mendia et al., 2010).



**Figure 1.4 - Classifications of oral candidiasis.**

Acute candidiasis - oral cavity contains white patches that can be removed upon gentle scraping (<http://depts.washington.edu/hiv aids/oral/case1/discussion>). Chronic candidiasis - white areas of the inner cheek are persistent and not easily removed (<http://www.exodontia.info/CandidalLeukoplakia>).

Denture induced stomatitis (DIS) is another infection of the oral cavity, which is directly caused by *Candida* species on a denture prosthesis (Pereira-Cenci et al., 2008). The disease is typically linked to an ill-fitting upper fitting denture sitting adjacent to the oral mucosa, providing an environment for *Candida* spp. to form biofilms (Ramage et al., 2004), where *C. albicans* remains the species most isolated. The severity of DIS is characterised by the degree of inflammation on the palate, defined by Newton's classification (NT) (Newton, 1962), as shown in Figure 1.5.



**Figure 1.5 - Newton's classification of DIS types.**

(I) localised erythema, (II) moderate erythema, (III) severe erythema (Rautemaa and Ramage, 2011).

A number of factors are considered when defining the severity of DIS including, colour, texture and overall appearance. Type I is characterised by small areas of pinpoint hyperaemia, type II is identified by more diffused erythema that covers most of the palate and type III is found with severe erythema and granular inflammation. Recently, NT3 was associated predominantly with *C. albicans* (Coco et al., 2008) and subsequent *in vitro* analysis of clinical isolates found that biofilm formation positively correlated with disease severity and the release of secreted aspartyl proteinases (SAP) (Ramage et al., 2012a). This finding not only highlights the importance of these enzymes in biofilm formation but also agrees with other studies where they have been shown to contribute to *Candida* virulence and pathogenicity *in vivo* (Naglik et al., 2006, Naglik et al., 2004, Naglik et al., 2003). Furthermore, in addition to tissue damage, Saps have recently been shown to be involved in filamentation via the activation of the Cek MAPK pathway (Puri et al., 2012), a defining feature of biofilm development (Ramage et al., 2002d). Altogether these studies have suggested a physical and regulatory role for proteolytic enzymes in *C. albicans* biofilms.

*C. albicans* has been shown to interact with other *Candida* species in DIS patients, particularly with *C. glabrata*, as these two species were frequently co-isolated from patients with severe inflammation (Coco et al., 2008). As *C. glabrata* is unable to form hyphae it is hypothesised that this organism gains entry to the host via its interactions with *C. albicans*. A further study has confirmed this by showing the invasion of an *in vitro* reconstituted epithelial biofilm model by *C. glabrata*, assisted by *C. albicans* (Silva et al., 2011a). Currently the mechanisms by which *C. albicans* assists *C. glabrata* invasion are

yet to be defined. However, it could be argued that specific enzymes involved in tissue damage promote increased invasion of *C. albicans* hyphae that in turn allows the entry of *C. glabrata* and contributes to host pathogenicity. Therefore, the use of *in vivo* models would be of much importance for studying the pathogenesis of DIS (Nett et al., 2010b).

*Candida* species do not exclusively adhere to dentures and mucosal surfaces, as they have also been found in periodontal pockets, orthodontic appliances and on the enamel surface (Arslan et al., 2008, de Carvalho et al., 2006, Sardi et al., 2010). A recent study has showed *C. albicans* yeast cells were highly prevalent within a mixed species subgingival biofilm in patients with moderate to severe periodontitis (Canabarro et al., 2012). However, it is yet to be established if the *C. albicans* present within these biofilms contribute to disease pathogenesis. It has been shown recently that the increased prevalence of *Candida* was linked to an alteration in the oral bacterial flora. Patients with higher *Candida* loads had a less diverse microbial population and were dominated by streptococci (Kraneveld et al., 2012). Many studies have investigated the relationship between *C. albicans* and oral streptococci and have shown an increase in colonisation of mucosal surfaces and biofilm formation by these bacteria when grown in the presence of *Candida* (Diaz et al., 2012, Silverman et al., 2010, Xu et al., 2013). Specifically, the interaction between *Streptococcus gordonii* and *C. albicans* involves the surface proteins SspB and Als3p, respectively, which are responsible for increased biofilm formation (Silverman et al., 2010). Therefore, consideration of bacterial-fungal interactions in mixed species biofilms must be considered when managing oral candidosis, particularly in terms of the antimicrobial strategy.

### 1.3.2 Upper Respiratory Tract

Facial prosthesis is an example of one of number of head and neck related devices shown to allow the adherence and growth of *Candida* biofilms, particularly with respect to polymicrobial infections (Ariani et al., 2012). Specifically, *C. albicans* and *C. glabrata* are the most frequently isolated *Candida* species found associated with voice prosthesis biofilms (Buijssen et al., 2012, Ell, 1996). These species are known to bind to salivary proteins (Holmes et al., 2006), and are often found co-habiting with bacterial species (Kania et al.,

2010). These are important in the clinical setting as they can impact the patients impede speech, swallowing and respiration (Sayed et al., 2012a) and restrict airflow (Elving et al., 2001).

Ventilator associated pneumonia (VAP) is also of particular interest when considering upper airway infections due to the close proximity of the oral cavity to the trachea and bronchioles. Previous studies with patients suspected of having VAP have shown an increased mortality rate when *Candida* species are present, compared to bacterial counts alone (Delisle et al., 2011). However, the question of whether *Candida* species is a marker of VAP or is a direct cause of disease remains unanswered. The use of broad-spectrum antibiotics may be a possible reason for the prevalence of *Candida* in VAP, where recently more than half of patients with fungi present were colonised with *C. albicans* (Serban et al., 2010). Within the intensive care unit (ICU) VAP has been significantly controlled through the use of oral rinses containing chlorhexidine (Stonecypher, 2010, Caserta et al., 2012), therefore, confirms the benefits of good oral hygiene upon other infections.

### 1.3.3 Lower Respiratory Tract

Biofilm associated infections are frequently associated with the respiratory tract where the presence of fungal biofilms in the lungs are able to contribute to the infection. Respiratory diseases including invasive pulmonary aspergillosis (IPA), allergic bronchopulmonary aspergillosis (ABPA) and aspergilloma are due to the presence of a filamentous moulds such as *Aspergillus fumigatus* (Denning, 1998). It is not surprising that *A. fumigatus* can adhere to and form multispecies biofilms in this environment as this organism is ubiquitous with thousands of conidia readily inhaled daily (Richardson, 2009). It is of no doubt that *Pseudomonas aeruginosa* is one of the major prokaryotic organisms isolated in these infections (Singh et al., 2000) and along with *A. fumigatus*, are frequently isolated from cystic fibrous (CF) patients (Mowat et al., 2010). Recent reports have identified that co-infection with these two opportunistic pathogens reduces lung function, when compared to mono-species infections (Amin et al., 2010, Gangell et al., 2011). A similar observation was made with *P. aeruginosa* and *Candida* species (Chotirmall et al., 2010). The understanding to how these organisms are able to interact with one another and cause polymicrobial

infections is still under investigation. Both organisms have been shown to impact one another through various mechanisms. *P. aeruginosa* has been shown to adhere to *Candida* hyphae, form biofilms and elicit antimicrobial activity (Hogan and Kolter, 2002) due to the release of phenazines (Gibson et al., 2009, Morales et al., 2010). In contrast, *C. albicans* releases farnesol, a quorum sensing molecule, that down-regulates cell to cell signalling in *P. aeruginosa* causing pyocyanin levels to decrease (Cugini et al., 2007), a toxin that is normally involved in killing of competing organisms. Therefore, the pathogenesis of CF lies with the interactions between these opportunistic pathogens.

### 1.3.4 Gastrointestinal tract

It has been estimated that up to 80% of healthy adults have their gastrointestinal (GI) tract colonised by *Candida* spp. (Damman et al., 2012). However, immunocompromised patients may suffer from candidiasis of the GI tract if colonisation becomes chronic (Kumamoto, 2011). The GI tract is another location prone to of polymicrobial infections, where prokaryotes and eukaryotes interact with one another and contribute to the level of disease. Unlike the competitive inhibition found in the lung, polymicrobial infections in the GI tract have been shown to be beneficial to the organisms *in vivo* (Mason et al., 2012). For example, *C. albicans* was able to influence the composition of non-pathogenic bacteria of the gut microbiota. Furthermore, co-infection with *C. albicans* and *Escherichia coli* has revealed a synergistic relationship (Klaerner et al., 1997).

### 1.3.5 Urinary tract

The urinary tract is home to a variety of bacteria and fungi that are able to prevent infections including sexually transmitted diseases (STDs), vaginosis and urinary tract infections (UTIs). The presence of *Candida* spp. in these infections is known as candiduria and has been estimated to account for 10-15% of all UTIs (Kauffman et al., 2000, Lundstrom and Sobel, 2001). Furthermore, *Candida* is responsible for other infections of the urinary tract, including prostatitis, pyelonephritis and cystitis (Kauffman et al., 2011, Sobel et al., 2011). In some cases, the implementation of urethral stents is required to help treat a UTI. However, this provides a surface for *Candida* to adhere to and allows for biofilm formation (Reid et al., 1992, Harriott et al., 2010). Within the ICU, the use of urinary catheters contributes to a number of healthcare associated fungal

infections (Yang et al., 2013) and candiduria should be recognised as a marker for invasive candidiasis in these patients (Kauffman et al., 2011).

Another infection cause by *Candida* species is vulvovaginal candidiasis. Up to 75% of woman experience an episode of vulvovaginal candidiasis at some point during their life and 5% have recurring candidiasis, showing the importance of this organism at this body site. As with oral candidiasis, many factors contribute to the pathogenesis of this infection including the use of antibiotics and hormone imbalances. Lactobacilli are abundant in this area and are thought to help control the numbers of *Candida* through the production of lactic acid (Gajer et al., 2012), however this is still unproven (McMillan et al., 2011).

### 1.3.6 Wounds

Chronic wounds that fail to heal clinically impact patient care, particularly foot ulcers and tend to be due to the presence of microbial biofilms (Seth et al., 2012). Although bacteria are the most pathogenic organisms isolated from these sites, there is evidence to suggest that fungi also play a role in these infections (Branski et al., 2009). Recent analysis of leg ulcers using a metagenomic approach has identified the presence of *Candida* and *Aspergillus* species (Wolcott et al., 2009). In addition, a recent study showed that fungi could be isolated from 23% of all chronic wounds formed at various sites (Dowd et al., 2011), with the majority of these being *Candida* species. Furthermore, when mixed polymicrobial infections were considered fungi accounted for greater than 50% of all cases. Although this study failed to determine any correlation between *Candida* and *Staphylococcus*, previous investigations have identified a positive relationship with *Staphylococcus aureus* binding to the hyphae in *C. albicans* biofilms through the adhesion Als3p. This is similar to other polymicrobial infections including the interaction between *Streptococcus gordonii* and *C. glabrata* within the oral cavity (Coco et al., 2008, Harriott and Noverr, 2009, Silverman et al., 2010).

### 1.3.7 Biomaterials

The use of temporary and fixed biomaterials provides a substrate for *Candida* species to adhere to and initiate biofilm formation and in turn can cause

systemic candidiasis. A list of implantable devices where *Candida* biofilms can develop is shown in Table 1.1 (Ramage et al., 2006).

**Table 1.1 - Implantable medical devices in which *Candida* biofilms develop (Ramage et al., 2006)**

| Device   | Usage per year   | Infection risk (%) | Main <i>Candida</i> species  |
|--|------------------|--------------------|--|
| Central and peripheral venous catheters        | 5 million        | 3–8                | <i>albicans</i><br><i>glabrata</i><br><i>parapsilosis</i>                      |
| Hemodialysis and peritoneal dialysis catheters | 240 000          | 1–20               | <i>albicans</i><br><i>parapsilosis</i>   |
| Urinary catheters                              | Tens of millions | 10–30              | <i>albicans</i><br><br><i>glabrata</i>   |
| Endotracheal tubes                             | Millions         | 10–25              | <i>albicans</i>  |
| Intracardiac prosthetic devices                | 400 000          | 1–3                | <i>albicans</i><br><i>glabrata</i><br><i>parapsilosis</i><br><i>tropicalis</i> |
| Breast implants                                | 130 000          | 1–2                | <i>albicans</i>  |
| Prosthetic joints                              | 600 000          | 1–3                | <i>parapsilosis</i><br><i>albicans</i><br><i>glabrata</i>                      |
| Neurosurgical shunts                           | 40 000           | 6–15               | <i>albicans</i>  |
| Voice prostheses                               | Thousands        | 50–100             | <i>albicans</i><br><i>tropicalis</i>   |
| Dentures                                       | > 1 million      | 5–10               | <i>albicans</i><br><i>glabrata</i>   |

Furthermore, other predisposing factors for invasive infection include; the use of broad-spectrum antibiotics, parenteral nutrition and immuno-suppression due to chemotherapy and radiotherapy, and disruption of mucosal barriers due to surgery (Odds, 1988). *Candida* species are the most dominant fungi isolated in these infections and are the third most common cause of BSI in patients within the ICU (Wisplinghoff et al., 2004). These infections are difficult to treat, as removal of the biomaterials in addition to long-term antifungal therapy, may be required to resolve such infections.

Intravascular catheters are a typical example of a medical device that can become colonised with *Candida* spp., allowing for the development of mature biofilms from which cells can disperse and cause acute candidaemia. Recently it was shown that cells dispersed from a biofilm were associated with greater cytotoxicity and had a higher mortality rate than their equivalent planktonic yeast cells (Uppuluri et al., 2010). Therefore it is no surprise that studies have

been investigating the role of different biofilm phenotypes within the clinical setting. An initial study identified biofilm formation and inadequate antifungal therapy amongst the top risk factors responsible for mortality rates in candidaemia patients (Tumbarello et al., 2007). The correlation between biofilm formation and patient mortality was not only true for *C. albicans* but was also the case for non-*albicans* species such as *C. parapsilosis*. Furthermore, a follow up study by the same group identified the presence of a *Candida* biofilm forming isolate could be predicted based on a number of patient factors including diabetes mellitus and the use of catheters (Tumbarello et al., 2012). These biofilm forming isolates also had a financial impact with increased lengths of stay in hospital and greater use of antifungals, which in turn carried a greater risk of mortality. Despite these findings, caveats to such studies include the methodologies employed to assess biofilm formation. In these studies, XTT and spectrophotometric transmittance were used to determine whether an isolate displayed biofilm growth, rather than using a technique that directly measures biofilm biomass (Taff et al., 2012a, Kuhn et al., 2003). Non-*albicans* species such as *C. glabrata* do not form true hyphae (Kuhn et al., 2002a), and as a result form scant biofilms, though the data in these studies indicate otherwise. Biofilm formation in non-*albicans* species was found to be significantly greater than *C. albicans* (Tumbarello et al., 2012), as found in a separate study elsewhere (Pannanusorn et al., 2012). Nevertheless, methodology aside, this does not take away the importance of these clinical studies in terms of highlighting biofilm formation in all *Candida* species, particularly in the presence of indwelling biomaterials.

*C. glabrata* is currently increasing in candidaemia patients, and was involved in one of the earliest cases of biofilm related disease within patients with intravenous catheters (Valdivieso et al., 1976). Despite candidaemia being the cause of death in these terminally ill patients, the removal of the devices was able to halt the disease, similar to another study around the same time (Berkowitz et al., 1979). *C. glabrata* is known to form biofilms on a range of devices including patients with, but not limited to; endocarditis (Heffner and Franklin, 1978), prosthetic joints (Goodman et al., 1983) and venous catheters (Paige et al., 1987). Therefore, it is of no surprise that catheters represented a risk factor of *C. glabrata* candidaemia (Fortun et al., 2012).

Another important species of *Candida* in biofilm related infections is *C. parapsilosis*. Similarities between this species and *C. albicans* in their ability to form biofilms has been linked to the key transcriptional biofilm regulator *BCR1* (Ding et al., 2011). Although *C. parapsilosis* can adhere to biomaterials such as prosthetic knees, hip joints and breast implants (Wada et al., 1998, Fox and Lee, 2012, Younkin et al., 1984), it is more commonly associated with being a pathogen in neonates with indwelling catheters (Pammi et al., 2013a). Furthermore, *C. parapsilosis* biofilm forming ability *in vitro* and *in vivo* has been demonstrated in bioprosthetic heart valves and catheter related disease (Mansur et al., 1996, Negri et al., 2012).

The biomaterials mentioned above do not represent the complete range of surfaces that can be potentially colonised with *Candida* species, but does however provide an insight into the clinical importance of fungal biofilms within the host. Therefore, as fungal biofilms grow and persist in a variety of locations including the environment, careful clinical management is required to overcome these infections.

## 1.4 Clinical management

The importance of studying *Candida* biofilms is to ascertain new therapeutics and techniques to manage these infections clinically as these are associated with high morbidity and mortality. In addition to controlling fungal biofilms through the use of established antifungals and novel therapies, the method by which *Candida* infections are diagnosed could also improve clinical outcomes.

### 1.4.1 Diagnostic approaches

Currently there are no defined diagnostics for *Candida* biofilm infections, as the most appropriate type of clinical specimen and the location it is retrieved from are yet to be determined as a reliable source for diagnosis. Specimens can be collected from implanted devices (dentures, catheters, implants etc) or directly from infected tissues by means of body fluids (blood, urine, sputum etc), swabs and biopsies, amongst other specimens and used for diagnostics tests including but not limited to; standard culture, MALDI-TOF, quantitative PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA) (Ramage and Williams, 2013).

A defined diagnostic that is able to detect the presence of bacterial biofilms has received some interest recently (Hall-Stoodley et al., 2012). However, such a task is difficult as these organisms are responsible for a range of complex infections therefore one defined factor may not be suitable for all. In regards to fungal biofilms, a number of potential factors could be considered when determining the presence of a biofilm: microscopic investigation to determine organism phenotype (yeast, hyphae); host status predisposing to infection (medical device, immunocompromised); failed antifungal therapy due to non biofilm active agent and clinical representation of fungal infection (Ramage and Williams, 2013) .

Another hurdle to overcome in the diagnosis of biofilm infections is the accessibility of infection for sampling. The oral cavity is a highly accessible environment where a swab or a sonicate sample from dentures is easy to retrieve (Coco et al., 2008, Samaranayake et al., 1986). If sampling can be taken directly from the biomaterial, such as the tip from a catheter (Tchekmedyan et al., 1986, Park et al., 2010) and voice prosthesis (Sayed et al., 2012b), it can be easily be determined whether the medical device is the source of infection. In contrast, CF patients presenting with a chest infection may require a bronchoalveolar lavage (BAL) to diagnose the infection using microscopic techniques (Chotirmall et al., 2010, De Vos et al., 2006), which is clinically more challenging to obtain. Furthermore, the use of culture and microscopic analysis may be less important due to recent developments in rapid molecular techniques (Dowd et al., 2011), including qPCR (Buess et al., 2012). Despite this there are still issues with using these molecular assays, one of which being that detection of DNA does not correlate with a defined biofilm phenotype. Instead, transcriptional targets that have been shown to be essential in *C. albicans* biofilm formation (Nobile et al., 2012) may provide a more reliable diagnostic for the detection of biofilms. Many fungi undergo morphological switching during biofilm formation and therefore genes involved in this process may aid in the development of a biofilm diagnostic. Such gene targets in *C. albicans* could include *EFG1*, a transcription factor required for regulating cellular morphology and virulence (Connolly et al., 2013). Strains defective in this gene fail to undergo yeast to hyphal switching (Lo et al., 1997, Sudbery, 2011) and are unable to invade reconstituted human epithelia (Dieterich et al., 2002).

Currently, antimicrobial testing is carried out using planktonic cells and therefore does not take in to consideration how therapy may be impacted if a biofilm is present. As technology continues to advance, the idea of a biofilm molecular diagnostic becomes more likely, which would in turn decrease the time taken to diagnose infections and direct more effective and timely treatment.

#### 1.4.2 Current antifungal therapies

Although prevention of fungal infections is easier than treatment, a number of fungistatic and fungicidal agents exist to combat fungal diseases which promote including physical disruption of biofilm biomass and the removal from biomaterials which may be harbouring biofilm structures (Ramage and Williams, 2013). In fact, guidelines published recently in relation to catheter-related infections have indicated in the first instance there should be the removal of such devices, if possible (Cornely et al., 2012). The impact this has clinically has differential results depending on which antifungal therapy is used. A recent study determined the removal of the CVC within 24 to 48 h of antifungal initiation was not associated with any clinical benefit, however this may be due to echinocandins (ECN) and liposomal AMB being highly active against *Candida* biofilms (Nucci et al., 2010). In contrast, a prospective randomised trial concluded the removal of a catheter was beneficial with the duration of candidaemia shortened, when comparing AMB deoxycholate to fluconazole (FLZ) (Rex et al., 1995). Furthermore, when several clinical trials were considered using meta-analysis, it was shown the removal of CVCs significantly correlated with a reduction in mortality (Andes et al., 2012).

However, it may not be possible to remove these devices in all situations whether it is due to host status or location of the biomaterial. Therefore treatment of these infections must be given using antifungals agents, although there is little evidence to suggest the most appropriate therapy for the treatment of *Candida* biomaterial infections (O'Grady et al., 2011). Saying this, one potential treatment option for device-related infections is the use of antifungal lock therapy (ALT), with several case studies highlighted in a recent review supporting its use clinically, when appropriate (Walraven and Lee, 2013). Although the number of studies using ALT is limited, polyene (POL) agents were

most frequently used and had success rates of 76.9% and 60% when AMB (Arnow and Kushner, 1991, Johnson et al., 1994, Krzywda et al., 1995, Benoit et al., 1995, Viale et al., 2001, Angel-Moreno et al., 2005, Wu and Lee, 2007) and liposomal AMB (Castagnola et al., 2005, Buckler et al., 2008) were used, respectively, with negative blood cultures apparent after 14 days therapy. Another agent used in ALT was caspofungin, however only one study showed the effectiveness of this agent against *C. lipolytica* (Ozdemir et al., 2011). Overall, these studies illustrate the potential use of ALT in fungal biofilm infections, particularly using POL and ECN formulations (Cornely et al., 2012).

The use of POL and ECN against *C. albicans* biofilm infections is based upon a number of *in vitro* studies, where they were shown to be highly active (Bachmann et al., 2002, Kuhn et al., 2002b). Also, a number of studies have set out to test the potential use of ALT against a range of *Candida* species, comparing various antifungal agents (Walraven and Lee, 2013). When investigating ECN, micafungin (MFG) and caspofungin (MFG) both had high anti-biofilm activity, yet failed to fully eradicate the biofilm (Cateau et al., 2011, Cateau et al., 2008), with liposomal AMB killing biofilms significantly quicker than those treated with ECN (Ramage et al., 2013). Surprisingly, in a separate ALT study it was shown that azoles (highly inactive biofilm agents) were more active against *C. albicans* and *C. glabrata* biofilms than AMB and CSP (Ko et al., 2010). This finding was unexpected as it is well documented that azoles (AZL) are inactive against *Candida* biofilms *in vitro* (Ramage et al., 2013) and *in vivo* (Andes et al., 2004, Kucharikova et al., 2010).

The success rate of each class of antifungal has been assessed in animal catheter models (Lazzell et al., 2009, Schinabeck et al., 2004, Mukherjee et al., 2009), where it was shown that liposomal AMB significantly reduced the *C. albicans* burden in all subjects, whereas the same concentration of FLZ was ineffective (Schinabeck et al., 2004). Furthermore, AMB deoxycholate and CSP were both used in rabbit catheter models and showed a significant reduction in *C. albicans* colony counts cultured from the catheter by 81% and 100%, respectively (Shuford et al., 2006). When considering these ALT studies altogether, it is evident the production of an effective and concise therapy for the treatment of catheter infections is possible. This is particularly desirable for biofilm infections where

the removal of the catheter is not an option due to the status of the host or the location of the device.

When considering biofilm infections of other medical devices, such as prosthetic heart valves, removal of the device is not as simple as infections originating from catheters. Infective endocarditis caused by *Candida* species remains rare and tends to be recognised after a case of candidaemia, with most patients already having a healthcare-associated infection (HAI) (Falcone et al., 2009). Prognosis is poor, with mortality rates higher than 50%, with 30% of survivors relapsing (Ellis et al., 2001). The best treatment method for this infection is surgery combined with antifungal therapy (Ellis et al., 2001, Falcone et al., 2009). However, if this is not an option then liposomal AMB and CSP should be used to treat this infection (Boland et al., 2011).

*Candida* biofilms are also responsible for infections not associated with a medical device, including those of wounds and joints. The normal treatment for wound biofilms would be the removal of infected and dead tissue. However, in a recent study the use of liposomal AMB in conjunction with either voriconazole (VRZ) and posaconazole (PSZ) was a more popular choice in more complicated wound infections (Warkentien et al., 2012). The effective use of AZL in wound infections may suggest that the structure of fungal biofilms may differ in this anatomical site to fungal biofilms found on biomaterials, as AZL have shown to be normally unresponsive to biofilm infections. However, the main finding here is that combinational antifungal therapy is a more effective treatment option than monotherapy.

Combination treatment is also used in the management of oral fungal infections including DIS and oral candidiasis, whereby antimicrobials in the form of denture cleansers or oral rinses are combined with brushing to mechanically disrupt the biofilm (Grimoud et al., 2005, Rautemaa and Ramage, 2011). However despite this, it has been shown that the removal of oral biofilms completely is actually quite rare (Ramage et al., 2011a, Ramage et al., 2012d, Jose et al., 2010). In exceptional cases antifungal treatment may be given topically and AZL tend to be the primary option. This in itself presents a problem as the use of AZL in recurring oral biofilm infections can lead to the emergence of resistant strains (Rautemaa et al., 2007). In summary, this confirms that despite the use of

various antifungal strategies, *Candida* biofilms persist due to a number of resistance mechanisms and meaning additional therapeutics are required to treat biofilm infections.

## 1.5 Studying biofilm resistance

Initial studies of biofilm resistance mechanisms were basic, investigating antifungal effects purely at the phenotypic level through descriptive analyses. The pioneering work by Julia Douglas's group working on *C. albicans* biofilms utilised some of the earliest models, from which quantitative assessment using dry weight measurements, tetrazolium salt (MTT) reduction assays and incorporation of [<sup>3</sup>H] leucine were described (Hawser and Douglas, 1994, Hawser and Douglas, 1995). These simple static models were expanded to include flow cell models, which was shown to alter antifungal susceptibility (Hawser et al., 1998a). However, typically these models were cumbersome, requiring expert handling, long processing times and the use of specialised equipment not generally available. Therefore, methods for rapid high throughput testing were preferable, and around this time the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium hydroxide (XTT) colorimetric method was described for investigating yeast adhesion and susceptibility (Hawser, 1996, Hawser et al., 1998b). This assay measures the collective metabolic activity of the cells within a biofilm and is used as the basis for developing a standardized high throughput susceptibility screen on *Candida* biofilms (Chandra et al., 2001a, Ramage et al., 2001a). The XTT assay is non-invasive and non-destructive, requiring minimal post-processing of samples compared to other methods; such as viable cell counts (Nett et al., 2011a). Using this technique multiple microtitre plates can be processed simultaneously without compromising accuracy. A caveat to its use is that it does not quantify biofilm dependant characteristics, such as biomass or morphological status, and caution must be exercised when evaluating XTT data from different isolates as there is often dramatic variability between strains (Kuhn et al., 2003). Therefore, it should only be used for direct comparison of a treated isolate to an untreated control rather than absolute quantification of biofilm formation *per se*. The next breakthrough in high throughput biofilm testing has recently been described where nano-production of *C. albicans* biofilms is achievable, creating 768 equivalent and spatially distinct nano-biofilms on a single glass microarray

(Srinivasan et al., 2011). However, it remains to be determined whether there is an assay system sensitive enough to quantify the metabolic activity of each nano-biofilm.

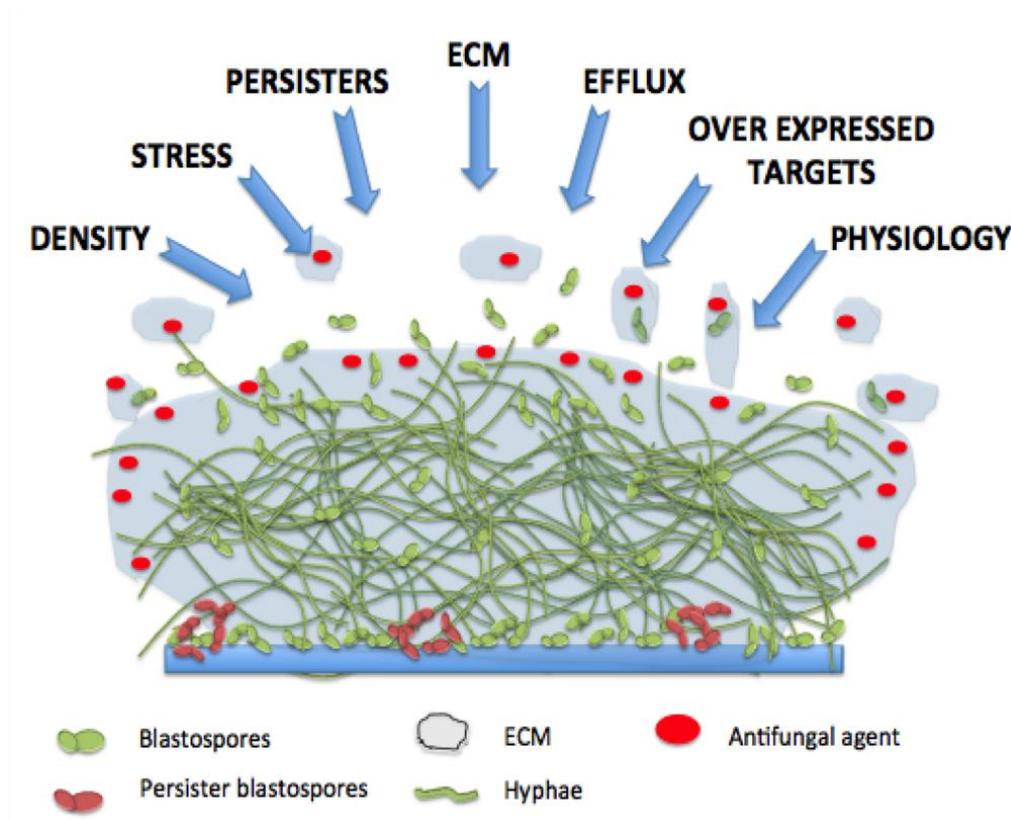
As mentioned, the presence of flowing liquid over the biofilm can alter antifungal sensitivity (Hawser et al., 1998a). There is a growing range of flow systems utilized to model biofilm development (Elving et al., 2003, Garcia-Sanchez et al., 2004, Mercier-Bonin et al., 2004, Ramage et al., 2008, Khot et al., 2006). For example, a 'seed and feed' modified Robbin's device that permits multiple biofilms to be formed under constant flow conditions, cylindrical cellulose filters, constant depth film fermenters, perfusion fermenters, flow chambers and a Robbin's device have all been described (Baillie and Douglas, 1998a, Baillie and Douglas, 1999, Chandra et al., 2001a, Chandra et al., 2001b, Lamfon et al., 2003, Ramage et al., 2008). The Lopez-Ribot group described a simple flow model based on a gravity fed flow method that enabled the group to demonstrate that biofilms were thicker and more resistant to POL and ECN by 4- and 2 -fold, respectively (Uppuluri et al., 2009a). Interestingly, perfusion of biofilms created under flow with these two antifungal agents showed time and dose dependant activity, which were potent against cells dispersed from the biofilm (Uppuluri et al., 2011). These systems will prove useful for future investigations of invasive candidiasis where biofilms are common, particularly for catheter related infections in the ICU, where there is a growing interest in catheter-lock therapy (Ghannoum et al., 2011, Mukherjee et al., 2009). In addition, there are now also a significant number of biofilm models available for *in vivo* investigations, and many of these have been utilised to elucidate biofilm resistance mechanisms (Nett and Andes, 2006), including an implanted chamber under the skin (Rajendran et al., 2011), catheter models (Andes et al., 2004, Schinabeck et al., 2004), vaginal model (Harriott et al., 2010) and a denture model (Nett et al., 2010b).

### *1.5.1 Fungal biofilm resistance mechanisms*

One of the defining characteristics of biofilms is their increased resistance to antimicrobial agents. When it comes to the treatment of fungal biofilms, up to 1000-fold higher concentrations of antifungal agents are required to effectively kill the biofilm structure than is required for their planktonic counterparts (Di

Bonaventura et al., 2006, Ramage et al., 2001a, Tre-Hardy et al., 2008). Despite some antifungal agents being efficacious against biofilms, particularly the ECN and liposomal AMB formulations, the adaptive resistance exhibited by these complex structures has promoted detailed investigation (Kuhn et al., 2002b, Ramage et al., 2002c, Bachmann et al., 2003, Bachmann et al., 2002, Mowat et al., 2008).

Antifungal resistance is both complex and multifaceted. It can be inducible in response to a compound, or an irreversible genetic change resulting from prolonged exposure. Specifically, these include alterations or over-expression of target molecules, active extrusion through efflux pumps, limited diffusion, tolerance and cell density, which are all characterised mechanisms utilised by fungi to combat the effects of antifungal treatment (Niimi et al., 2010). Planktonic cells generally rely on irreversible genetic changes to maintain a resistant phenotype, whereas biofilms are able to persist due to their physical presence and the density of the population, which provides an almost inducible resistant phenotype irrespective of defined genetic alterations. Figure 1.6 provides an overview of some of the pivotal factors associated with fungal biofilm resistance.



**Figure 1.6 - Schematic overview of fungal biofilm resistance mechanisms.**

Generic overview of key biofilm resistance mechanisms associated with *C. albicans*, but which are likely to be common to other fungi. This figure illustrates the density and complexity of the *C. albicans* biofilm, with different morphotypes present surrounded by extracellular matrix (ECM). The arrows represent the different factors that drive antifungal resistance within the biofilm, including density, stress, persisters, ECM, efflux, over-expressed targets and the general physiology of the biofilm. These have been placed according to their contribution to resistance, with those that have a greater effect closer to the middle and those with less impact at the edges (Ramage et al., 2012c).

### 1.5.1.1 Physiological state

The general physiological state of cells in sessile populations has also been implicated to influence the susceptibility profiles of biofilms. Metabolic dye assays (e.g. XTT-based assays) confirm that cells within biofilms are undergoing mitochondrial respiration during development (Chandra et al., 2001a, Hawser, 1996, Ramage et al., 2001a, Kuhn et al., 2003, Mowat et al., 2008). Other factors including the effect of growth rate on *C. albicans* biofilm resistance have also been studied, and varying growth rates were shown to play no role in resistance to AMB (Baillie and Douglas, 1998a). Similarly, biofilms of *C. albicans* grown under glucose and iron limited conditions were shown to be highly resistant to AMB (Baillie and Douglas, 1998b). Furthermore, studies of biofilms grown under anaerobic conditions demonstrated resistance to high levels of AMB

and different AZL antifungals (Dumitru et al., 2004). Nevertheless, factors including pH, temperature, oxygen availability and other environmental stresses will alter the biofilm architecture and possibly antifungal sensitivity (Kucharikova et al., 2011, Pettit et al., 2010). Therefore, whilst the physiological state of the cell may have a minor role in resistance (e.g. dormancy), it is more likely that more complex factors are involved.

### 1.5.1.2 Cell density

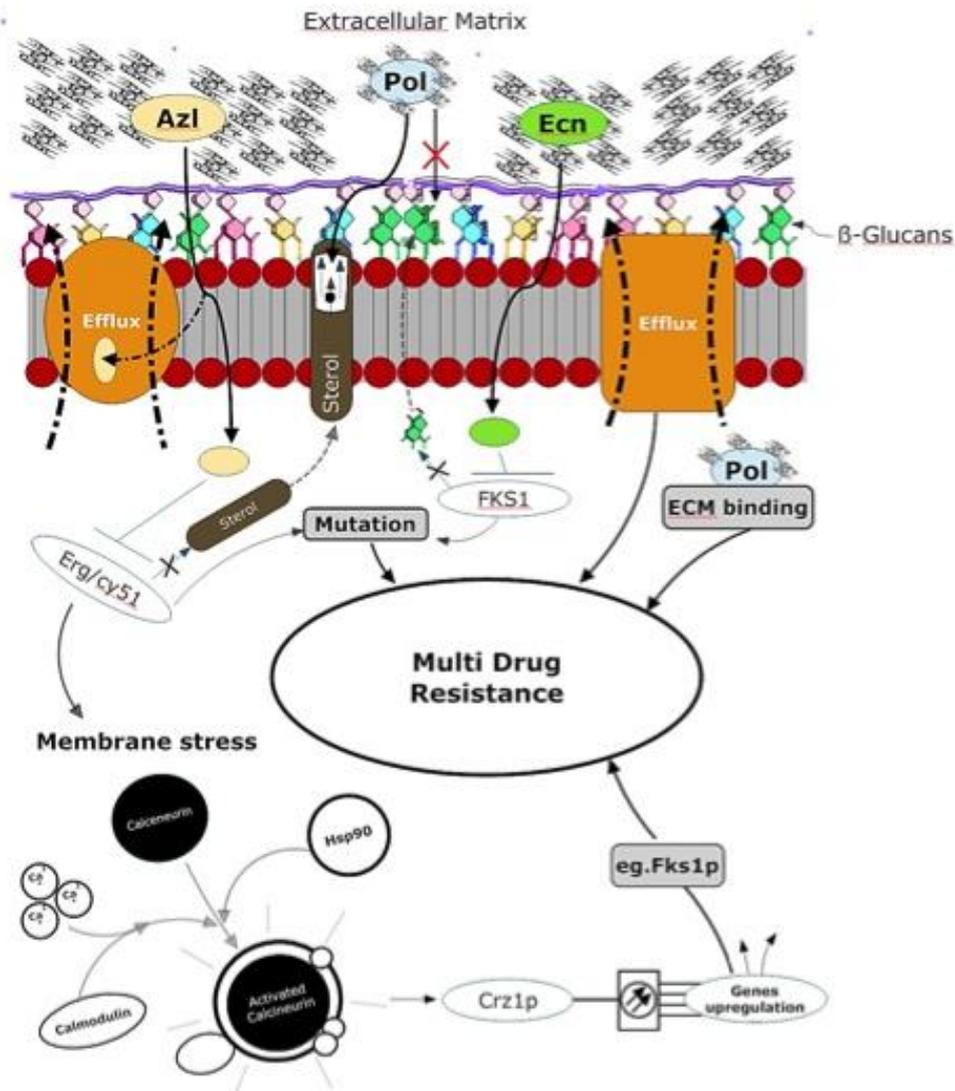
The architecture of biofilms is highly ordered to enable the perfusion of nutrients and expulsion of waste products. Mature biofilms, whilst densely populated, exhibit spatial heterogeneity with microcolonies and water channels being present (Chandra et al., 2001a, Lawrence et al., 1991, de Beer et al., 1994). Cell density is therefore an important resistance factor within complex biofilm populations of yeast and filamentous fungal biofilms, particularly towards AZL. It was demonstrated that both planktonic and resuspended *C. albicans* biofilm cells exhibited AZL sensitivity at low cell numbers ( $10^3$  cells/mL), which became increasingly resistant as the density of the cells increased (Perumal et al., 2007), a phenomenon also been demonstrated in *A. fumigatus* (Lass-Flörl et al., 2003). It has been shown in *C. albicans* that phase-dependant antifungal resistance occurs (Mowat et al., 2008, Mukherjee et al., 2003), which support the idea that the physical density of the cells within the biofilm produces recalcitrance to antifungal agents.

Within dense biofilms there is cooperation between individual cells through quorum sensing. Quorum sensing gives microorganisms the ability to communicate and coordinate their behaviour via the secretion of signalling molecules in a population dependent manner (Miller and Bassler, 2001). This was first described in *C. albicans* when Hornby and colleagues identified farnesol (3,7,11-trimethyl-2,6,10-dodecatrien-1-ol) (Hornby et al., 2001). Exposing *C. albicans* to exogenous farnesol results in genome wide responses, including activation of genes involved in drug resistance (*CaFCR1* and *CaPDR16*) (Cao et al., 2005, Enjalbert and Whiteway, 2005). It has now been shown that quorum sensing in *C. albicans* is driven by the two component regulatory system of Chk1p (Kruppa et al., 2004). However, when deleted the *chk1Δ* strain shows a similar AZL resistance profile to that of wild type (Perumal et al., 2007),

indicating that the regulatory circuit controlling biofilm resistance may be yet to be discovered, or cell density is not a defined biofilm resistance factor. However, given that ECN are highly effective against biofilms suggests cell density has a limited effect against this compound (Bachmann et al., 2002). In addition, previous work has shown that disrupted biofilms that are resuspended and tested using the Clinical and Laboratory Standards Institute (CLSI) methodology in comparison to planktonic cells retain a resistant phenotype (Rajendran et al., 2011, Ramage et al., 2002a), indicating alternative mechanisms of resistance.

### 1.5.1.3 Over expression of drug targets

The AZL are generally fungistatic against yeasts. The fungistatic nature of the AZL towards *C. albicans* induces a strong directional selection on the surviving population to evolve drug resistance (Anderson, 2005, Cowen, 2008). In fact, high levels of AZL resistance in *C. albicans* clinical isolates often accumulate through multiple mechanisms including the alteration of *ERG11* (Anderson, 2005). Figure 1.7 illustrates the molecular mechanisms contributing to fungal biofilm resistance, including those involved in AZL resistance.



**Figure 1.7 - Molecular mechanisms of fungal biofilm resistance.**

Antifungal drug resistance in fungal biofilms is both complex and multi-factorial. The diagram illustrates the mechanisms of different class of antifungal agent action AZL, POL and ECN and resistance: (a) The layer of ECM present in the biofilm shields the cells from antifungal agents by binding and reduced penetration; (b) The membrane transporter system ABC and MFS efflux pumps extrudes antifungal molecules and reduces the intracellular concentration; (c) Mutation in *ERG*, *CYP51* and *FKS1* genes alters the drug target leading to cross resistance; (d) Antifungal pressure induces stress responses, such as the calcineurin signalling pathway, which is activated and coping responses occurs through up-regulation of various signal transducers.

AZL actively target the 14  $\alpha$ -demethylase enzyme encoded by *ERG11*, blocking ergosterol biosynthesis and leading to depletion of the ergosterol content of membranes. This ultimately results in the accumulation of toxic sterol intermediates, such as 14 $\alpha$ -methylergosta-8,24(28)-dien-3 $\beta$ ,6 $\alpha$ -diol, which inhibits growth (Akins, 2005, Cannon et al., 2007). The principle drug target, ERG11p, can develop point mutations or be overexpressed (Akins, 2005, Cannon

et al., 2007, White, 1997). Common mutations in the *ERG11* that confer moderate AZL resistance are S405F, Y132H, R467K and G464S (Marichal et al., 1999, Perea et al., 2001, Sanglard et al., 1998).

Given the importance of ergosterol as a target of AZL and the high level resistance exhibited by these structures, then the sterol composition of *C. albicans* biofilms has been investigated. Sterol analyses showed that ergosterol levels were significantly decreased in intermediate (12 h) and mature phases (48 h), compared to those in early-phase biofilms (6 h) (Mukherjee et al., 2003). In contrast, in one of the first *C. albicans* biofilm studies to use microarray analysis over-expression of *CaERG25* and *CaERG11* was reported (Garcia-Sanchez et al., 2004). Alteration of ergosterols in biofilm membranes may explain their resistance to both AZL and POL derived antifungal agents. For example, *C. albicans* biofilms cultured in a flow cell for 36 h were compared to planktonic cells, where it was shown that a subpopulation of blastospores from the biofilm were 10 times more resistant to AMB than planktonic populations (Khot et al., 2006). Transcriptional analysis of this biofilm subpopulation for genes from the beta-1,6-glucan pathways indicated a possible association between the high level of resistance and up-regulation of *CaSKN1* and *CaKRE1* in the biofilm blastospore population. Therefore, changes in both the cell membrane and the cell wall may be important determinants of resistance in the biofilm. Subsequent work in *C. albicans* has shown that transcriptional responses in young and mature biofilms after exposure to high doses of FLZ or AMB induced differential antifungal drug responses (Nailis et al., 2010b). Exposure of both young and mature biofilms to FLZ induced up-regulation of genes encoding enzymes involved in ergosterol biosynthesis (*CaERG1*, *CaERG3*, *CaERG11* and *CaERG25*), particularly biofilms exposed for longer periods (22 h). Whereas, treatment of both young and mature biofilms with AMB resulted in an overexpression of *CaSKN1*, with only a modest up-regulation of *CaKRE1*. Removal of the antifungal in this study depleted further transcriptional changes, except for *CaSKN1*, which was impacted by prior FLZ exposure. It was speculated that this related to biofilm regrowth. Increased ergosterol gene expression was also reported *in vivo* in a *C. albicans* CVC biofilm model, demonstrating the importance of the molecule within the biofilm (Nett et al., 2009).

Induction of ergosterol genes has also been described in *C. dubliniensis*, where incubation with FLZ and formation of biofilm was coupled with up-regulation of the *CdERG3* and *CdERG25* (Borecka-Melkusova et al., 2009). Moreover, up-regulation of genes involved in ergosterol biosynthesis has been described in *C. parapsilosis* biofilms (Rossignol et al., 2009), which are also resistant to AZL antifungal therapy (Katragkou et al., 2008). Overall these data highlight the importance of ergosterol in biofilm resistance, particularly with respect to azoles, which indirectly inhibit their biosynthesis. Recent studies have shown that simvastatin, which impairs cholesterol metabolism in humans, is capable of inhibiting *C. albicans* biofilms, thus providing a potential novel strategy of combating these tenacious infections (Liu et al., 2009).

#### 1.5.1.4 Efflux pump mediated resistance

The primary molecular mechanism leading to high level AZL resistance in *C. albicans* is the increased efflux of drug, mediated mostly by the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporters (Albertson et al., 1996, Lopez-Ribot et al., 1999, Sanglard et al., 1997). The ABC transporters in *C. albicans* constitute a multi-gene family, which includes several *CDR* genes (*CDR1-4*) (Prasad et al., 1995, Walsh et al., 1997). The ABC transporters include a membrane pore composed of transmembrane segments and two ABCs on the cytosolic side of the membrane, which provide the energy source for the pump (Higgins, 1992, Balzi and Goffeau, 1995). Importantly, multiple antifungal agents can be substrates for these transporters and thus their over-expression can lead to cross-resistance between different drugs, particularly AZL. Among members of the MFS, which are secondary transporters and use proton-motive force across the plasma membrane, the *MDR1* gene encodes a major facilitator that has been implicated in *C. albicans* AZL resistance, and its over-expression leads to exclusive FLZ resistance (White, 1997, Williams et al., 2011). ECN sensitivity is unaffected by efflux pumps (Niimi et al., 2006).

Genes encoding for drug efflux pumps have been reported in biofilms to be differentially regulated during development and upon exposure to antimicrobial agents include *CaCDR1*, *CaCDR2* and *CaMDR1* (Lepak et al., 2006, Mateus et al., 2004, Mukherjee et al., 2003, Ramage et al., 2002a). In the first study to

investigate the role of efflux pumps it was demonstrated that expression of genes encoding both types of efflux pumps were up-regulated during the course of biofilm formation and development. Both *CaCDR1* and *CaCDR2* were up-regulated in 24 and 48 h biofilms, whereas *CaMDR1* was transiently up-regulated at 24 h (Ramage et al., 2002a). However, their contribution to resistance in the biofilm phenotype was placed in doubt when a set of *C. albicans* isogenic strains deficient in efflux pumps, carrying single and double deletion mutations (*cdr1Δ*, *cdr2Δ*, *mdr1Δ*, *cdr1/cdr2Δ* and *mdr/cdr1Δ*), rendered planktonic cells hypersusceptible to FLZ, retained their resistant phenotype during biofilm growth. In a subsequent investigation *C. albicans* biofilms were formed through three distinct developmental phases that were associated with high FLZ resistance. Again the same set of isogenic *C. albicans* strains were utilised and it was shown that 6 h old biofilms formed by double and triple mutants were >4 to 16-fold more susceptible to FLZ than the wild-type strain (Mukherjee et al., 2003). At 12 and 48 h all the strains became highly resistant to this AZL, indicating lack of involvement of efflux pumps in resistance at late stages of biofilm formation. In cell density studies, the efflux pump isogenic strains remain hypersensitive at low cell concentrations yet resistant at high cell concentrations and in biofilm, indicating a contributory resistance role of cell density (Perumal et al., 2007). Nevertheless, similar to the study by Ramage and coworkers (Ramage et al., 2002a), *C. albicans* biofilms were shown to express *CaCDR* and *CaMDR1* genes in all three phases (6, 12 and 48 h), whilst planktonic cells expressed these genes transiently. In fact, GFP promoter studies have shown induction of efflux pumps after 15 min adherence to provide a tolerant biofilm phenotype (Mateus et al., 2004). Animal studies have also shown that biofilms formed on implanted catheters expressed efflux pumps (Andes et al., 2004, Nett et al., 2009). Transcript up-regulation of *CaCDR2* at 12 h (1.5-fold) and *CaMDR1* at both 12 h (2.1-fold) and 24 h (1.9-fold) was demonstrated (Nett et al., 2009). In *C. glabrata* similar results are reported, where expression of *CgCDR1* and *CgCDR2* was investigated during the early (6 h), intermediate (15 h), and mature (48 h) phases of biofilm development. At 6 h and 15 h the biofilms exhibited approximately 1.5- and 3.3-fold upregulation of *CgCDR1* and 0.5- and 3.1-fold upregulation of *CgCDR2*, respectively, in comparison to planktonic cells (Won Song et al., 2008). Expression of *CtMDR* in *C. tropicalis* biofilms has also been reported (Bizerra et al., 2008).

Collectively, the available literature supports the hypothesis that efflux pumps are an important, but not an exclusive determinant of *C. albicans* biofilm resistance to AZL (Morschhauser, 2010, Cannon et al., 2009). Their primary role may be for homeostasis within complex environments to protect themselves from acute toxicity (Piddock, 2006). However, within clinical environments exposure to AZL drugs may enhance the levels of efflux pump expression, therefore either contributing towards or inducing clinical resistance (Bueid et al., 2010). However, it is likely that they play a greater protective (resistance) role in the early phases of biofilm growth until the production of ECM, one of the primary mechanisms of biofilm resistance.

#### 1.5.1.5 Extracellular matrix

The ECM is a defining characteristic of fungal biofilms, providing the cells protection from hostile factors such as host immunity and antifungal agents (Ramage et al., 2009). *C. albicans* ECM was shown to increase when biofilms are grown under dynamic flow conditions (Al-Fattani and Douglas, 2004, Baillie and Douglas, 2000, Hawser et al., 1998a). However, subsequent work has shown that while diffusion is hampered by the ECM, penetration of antifungal drugs is not thought to play a key role in biofilm resistance (Al-Fattani and Douglas, 2004). Recent studies have provided new insights that suggest the chemical composition of the ECM and its regulation may play a central role in resistance.

The composition of the ECM in *C. albicans* and *C. tropicalis* biofilms is complex, comprising of protein, hexosamine, phosphorus, uronic acid and carbohydrates (Al-Fattani and Douglas, 2006). It has been well established that extracellular DNA (eDNA) has been found within the ECM of bacterial biofilms (Allesen-Holm et al., 2006, Vilain et al., 2009). However it was not until recently the importance of eDNA in *C. albicans* biofilms was established (Martins et al., 2010). Here it was shown that eDNA contributes to the structural integrity and maintenance of *C. albicans* biofilms and the addition of DNase decreases biofilm biomass. Furthermore, DNase treatment improved the efficacy of POL and ECN, but this was not the case with AZL (Martins et al., 2011).

One of the principle carbohydrate components of the ECM is beta-1,3 glucans. Treatment of *C. albicans* biofilms with beta-1,3 glucanase helps detach biofilms

from a substrate (Al-Fattani and Douglas, 2006). Its contribution is confirmed in a series of investigations by the Andes group where it was shown to increase in *C. albicans* biofilm cell walls compared to planktonic organisms, and was also detected in the surrounding biofilm milieu and as part of the ECM (Nett et al., 2007). Beta-1,3 glucans have also been shown to increase in investigations of three specific phases of biofilm development grown on both denture acrylic and catheter substrates (Yeater et al., 2007). Its contribution to resistance was realised when it was also shown that biofilm cell walls bound 4 to 5-fold more AZL than equivalent planktonic cells, and culture supernatant bound a quantifiable amount of this antifungal agent. Moreover, beta-1,3 glucanase markedly improved the activity of both FLZ and AMB. Addition of exogenous biofilm ECM and commercial beta-1,3 glucan also reduced the activity of FLZ against planktonic *C. albicans in vitro* (Nett et al., 2007). The group have recently shown that the ECM  $\beta$ -1,3 glucan is synthesised from *FKS1* using a defined knockout and over-expressing strain (Nett et al., 2010c). This study also demonstrated that beta-1,3, glucan is responsible for sequestering AZL, acting as a 'drug sponge' and conferring resistance on *C. albicans* biofilms (Nett et al., 2010c). Further studies have shown that they are also responsible for sequestering ECN, pyrimidines, and POL (Nett et al., 2010c). This has been confirmed independently where AMB was shown to physically bind *C. albicans* biofilms and beta-glucans (Vediyappan et al., 2010). Subsequent studies have identified a role for *CaSMI1*, a gene involved in cell wall glucans, in biofilm ECM production and development of a drug resistant phenotype, which appears to act through transcription factor *CaRLMP* and glucan synthase *FKS1p* (Nett et al., 2011b).

In addition to *CaFKS1*, a zinc-response transcription factor *CaZAP1* has been shown to be a negative regulator of ECM soluble beta-1,3 glucan in both *in vitro* and *in vivo* (Nobile et al., 2009). Conversely, two glucoamylases, *CaGCA1* and *CaGCA2*, are thought to have positive roles in ECM production. A group of alcohol dehydrogenases *CaADH5*, *CaCSH1*, and *CaLFD6* also have roles in matrix production, with *CaADH5* acting positively, and *CaCSH1* and *CaLFD6* acting negatively (Nobile et al., 2009). It is thought that these alcohol dehydrogenases generate quorum-sensing aryl and acyl alcohols, which co-ordinate biofilm maturation. Collectively, it appears that *C. albicans* ECM production is highly

regulated and is a key resistance factor. It is also present in a number of other *Candida spp.*, including *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. dubliniensis* (Silva et al., 2009, Silva et al., 2011b).

Therefore, ECM clearly plays a critical role in biofilm resistance, particularly for *C. albicans*, about which we currently understand the greatest. It is one of the most significant and regulated resistance mechanisms utilised in the biofilm phenotype.

#### 1.5.1.6 Persisters

Persister cells are an important mechanism of resistance in chronic infections (Fauvart et al., 2011) and a mechanism of resistance that has gathered some attention recently in fungal biofilms (Bink et al., 2011, LaFleur et al., 2006, Lewis, 2008). Persister cells are “dormant variants of regular cells that form stochastically in microbial populations and are highly tolerant to antibiotics” (Lewis, 2010). In *C. albicans* biofilms a small subset of yeast cells have been described that are highly resistant to AMB following adhesion, which is independent to up-regulation of efflux pumps and cell membrane composition (Khot et al., 2006, LaFleur et al., 2006). *C. albicans* persisters have been detected only in biofilms and not in planktonic populations (LaFleur et al., 2006). Re-inoculation of cells that survived killing by AMB produced a new biofilm with a new subpopulation of persisters, suggesting that these were not mutants but phenotypic variants of the wild type, and that attachment to a substratum initiated dormancy. The presence of persisters in *C. krusei* and *C. parapsilosis* biofilms treated with AMB has also been described (Al-Dhaheri and Douglas, 2008). It was further hypothesized that the periodic application of antimicrobial agents may select for strains with increased levels of persister cells, so 150 isolates of *C. albicans* and *C. glabrata* were obtained from cancer patients who were at high risk for the development of oral candidiasis and who had been treated with topical chlorhexidine once a day. It was shown that the persister levels of the isolates varied from 0.2 to 9%, and strains isolated from patients with long-term carriage had high levels of persisters, whereas those from transient carriage did not (LaFleur et al., 2010). Therefore, in this clinically relevant scenario prolonged and ineffectual antifungal treatment may be

beneficial to the biofilm population, which may be responsible for antimicrobial drug failure and relapsing infections.

The role of reactive oxygen species (ROS) in sessile *C. albicans* cells was investigated as they are known to be induced by high concentrations of miconazole, allowing 1-2% of miconazole-tolerant cells to persist (Vandenbosch et al., 2010). Superoxide dismutases (SOD) were found to be differentially expressed by miconazole treated sessile *C. albicans* cells compared to untreated cells. Inhibition of superoxide dismutase resulted in a 18-fold reduction of the miconazole-tolerant persister cells and increased endogenous ROS levels in these cells (Bink et al., 2011). In biofilms from strains lacking *sod4/sod5Δ* at least 3-fold less miconazole-tolerant persisters were observed and ROS levels were increased compared to the isogenic wild type. Therefore, miconazole-tolerant persisters are linked to the ROS-detoxifying activity of SOD. Whether this is the definitive molecular basis for *C. albicans* persister cells or a tolerance mechanism still remains to be determined, but these subpopulations are clearly another important fungal biofilm resistance mechanism.

#### 1.5.1.7 Tolerance

Stress responses have become more fully recognised as defined mechanisms of antifungal resistance. Pathogenic fungi encounter a range of physiological stresses from different environments, including temperature changes, ionic stress, changes in osmolarity, and oxidative stress, such as that experienced in the phagosomes of neutrophils (Cannon et al., 2007). These stresses are sensed through various receptors, which elicit responses through conserved signalling pathways. One of the most important is the mitogen-activated protein kinase (MAPK) signal transduction network (Cannon et al., 2007). It was first shown that the MAPK Mck1p, which is activated by contact stress, is involved in biofilm development. Moreover, the null mutant (*mck1Δ*) biofilms were AZL sensitive, in contrast to the sessile wild type and both planktonic strains. This indicates that Mck1p is involved in biofilm resistance through a stress pathway (Kumamoto, 2005).

Calcineurin is a  $\text{Ca}^{2+}$ -calmodulin-activated serine/threonine-specific protein phosphatase that plays many critical stress roles in the fungal cell, including

amongst other things antifungal drug responses (Steinbach et al., 2007). In planktonic cells, calcineurin is critical for *C. albicans* survival during AZL treatment (Sanglard et al., 2003). Inhibiting calcineurin pharmacologically or impairing calcineurin function genetically, has synergistic activity with FLZ and renders the AZL fungicidal against *C. albicans* (Onyewu et al., 2003). Calcineurin has also been implicated in mediating resistance to the AZL in both *in vitro* and *in vivo* models of biofilm formation (Uppuluri et al., 2008). *C. albicans* cells in biofilms are up to 1,000-fold more resistant to FLZ than planktonic cells, indicating that inhibitors could be used in combinations as novel therapeutic interventions to treat or prevent biofilms, whereas *C. dubliniensis* calcineurin inhibitors were unable to form biofilms (Chen et al., 2011). Similar studies have evaluated the efficacy of a VRZ-MFG combination against *C. albicans* biofilms. VRZ significantly antagonized the fungicidal effect of MFG against biofilms. To investigate the mechanism of antagonism, an inhibitor of calcineurin was evaluated, which reversed the VRZ-induced resistance to MFG (Kaneko et al., 2010a). This study also suggested that heat shock protein 90 (Hsp90p) molecular chaperone played a role in this antagonism. *HSP90* regulates complex cellular circuitry in eukaryotes and potentiates the emergence and maintenance of resistance to AZL and ECN in *C. albicans*, at least in part via calcineurin (Cowen and Lindquist, 2005). It physically interacts with the catalytic subunit of calcineurin, keeping it stable and poised for activation (Singh et al., 2009). A recent study demonstrated led by the Cowen group demonstrated that genetic depletion of *HSP90* reduced *C. albicans* biofilm growth and maturation *in vitro*, and interestingly impaired dispersal of biofilm cells (Robbins et al., 2011). It also abrogated resistance of *C. albicans* biofilms to the AZL, which was also shown *in vivo*. Furthermore, depletion of *HSP90* led to reduction of calcineurin and *MKC1* in planktonic but not biofilm conditions, suggesting that *HSP90* regulates drug resistance through different mechanisms. A marked decrease in matrix glucan levels were also observed, providing a mechanism through which HSP90 might regulate biofilm AZL resistance. In *A. fumigatus*, pharmacological depletion of *HSP90* led to reduced resistance to the ECN (Robbins et al., 2011). Moreover, a recent investigation of the *C. glabrata* biofilm proteome demonstrated up-regulation of a heat shock protein (Hsp12p) and other stress proteins (Trx1p, Pep4p) (Seneviratne et al., 2010). Therefore, targeting HSP90 may provide a novel strategy for treating fungal biofilm infections.

## 1.6 Alternative therapies

### 1.6.1 Physical interference

One of the main challenges faced by antifungal effectiveness is the ECM, which acts as a physical barrier preventing the penetration of drugs. Therefore, novel therapies that are able to disrupt the ECM are highly sought after. This is particularly true for use as denture cleansers due to current agents being ineffective against fungal biofilms, unless used on a daily basis (Jose et al., 2010, Ramage et al., 2012d). One way of overcoming the ECM would be to directly target components of the ECM such as eDNA as this has been shown to improve antifungal treatment (Martins et al., 2011, Taff et al., 2012b). With the recent finding of eDNA being present in *C. albicans* biofilms (Martins et al., 2010), it was shown that combinational therapy of AMB and CSP with DNase significantly improved antifungal sensitivity (Martins et al., 2011). Therefore, the penetration of such antifungals could be enhanced by combining therapy with a biofilm disruptive agent such as Dornase Alfa Pulmozyme<sup>®</sup>, currently used for the breakdown of DNA present in mucus of CF patients. Furthermore, *C. parapsilosis* biofilms have been shown to be more sensitive to AZL treatment when used in conjunction with ambroxol, a mucoactive agent used in respiratory infections (Pulcrano et al., 2012). Other anti-biofilm agents shown to have disruptive properties against fungal biofilms include N-acetylcysteine (NAC) (Aslam and Darouiche, 2011), ester lauroyl glucose (Dusane et al., 2008) and alginate lyase (Bugli et al., 2012, Papi et al., 2012). Overall, these studies illustrate the potential of biofilm disruption agents have as an alternative or complementary therapy for treating fungal biofilms.

Silver has been known to have broad-spectrum antimicrobial activity for some time now. Silver interferes with DNA replication and in turn denatures proteins, leading to the inhibition of oxidative enzymes (Kim et al., 2007). The production of silver nanoparticles has been shown to impact *C. albicans* and *C. glabrata* biofilms at various stages of development (Monteiro et al., 2011, Monteiro et al., 2012b) and have been utilised in various experimental models including the release from hydrogels used in chronic wounds and denture acrylic (Humphreys et al., 2011, Monteiro et al., 2012a).

Another strategy that has been shown to effectively prevent fungal biofilm formation on biomaterials is coating the surface with chitosan, a naturally-occurring polysaccharide from shellfish (Carlson et al., 2008). This agent has activity against both bacterial and fungal biofilms, including *Candida* species (Martinez et al., 2010) and is thought to inhibit biofilms through the interference of cell membranes.

### 1.6.2 Microbial interference

From the infections detailed earlier, it is evident that polymicrobial interactions contribute to fungal biofilm formation. However, it is possible to utilise these interactions to help control the formation of *Candida* biofilms. One possibility is by the use of probiotics, whereby one organism can control or inhibit the growth of another (Meurman, 2005). An example of this has been shown whereby the release of biosurfactants from *Streptococcus thermophilus B* prevented the adhesion of *Candida* species on silicone (Busscher et al., 1997). Many probiotics are found in dairy products and therefore easily consumed, including *Lactobacillus lactis* that has been shown to play a role in controlling *Candida* biofilms (Rodrigues et al., 2006, Rodrigues et al., 2004). However, conflicting evidence exists around the use of *Saccharomyces boulardii* as a probiotic. Despite this organism being able to control *C. albicans* biofilm formation by the inhibition of filamentation (Murzyn et al., 2010), it has also been found to possibly cause fungaemia within immunocompromised patients (Santino et al., 2014).

A number of secondary metabolites isolated from various organisms have been shown to have potential in the treatment of fungal infections. An environmental strain of *A. fumigatus* produced a prenylated indole alkaloid called waikialoid A, identified to having inhibitory properties towards *C. albicans* biofilms (Wang et al., 2012b). In addition, the release of mutanobactin by *S. mutans*, a hybrid peptide metabolite, has been shown to inhibit *C. albicans* biofilm formation by preventing morphological switching (Wang et al., 2012a, Joyner et al., 2010). The shared aims of these studies are the utilisation of molecules isolated from organisms and using these to inhibit hyphal development and biofilm formation within *C. albicans*.

The relationships between bacteria and fungi have been shown previously to have serious implications on health, particularly within the lung of CF patients. However, the interactions between fungi and *P. aeruginosa* have found *C. albicans* and *A. fumigatus* hyphae and biofilm development can be inhibited through the secretion of quorum sensing molecules such as decanol, dodecanol and 3-oxo-C12 homoserine lactone (Hogan et al., 2004, Mowat et al., 2010). Another quorum sensing molecule shown to have anti-biofilm activity against a range of *Candida* and *Aspergillus* species is farnesol, whereby the molecule can cause apoptosis and behave as a cell wall stressor impacting morphogenesis and cellular development (Albuquerque and Casadevall, 2012, Ramage et al., 2002b). Despite these findings, contradicting *in vivo* studies have shown an increase in mortality in mice when farnesol was administered, suggesting that this molecule could contribute to disease pathogenesis (Navarathna et al., 2007). However, with antifungal resistance currently increasing, any molecules that have activity against fungal biofilms warrant further investigation (Guo et al., 2013).

### 1.6.3 Natural antifungals

Alternative therapies including the use of natural compounds are of great interest clinically as they are thought to be less toxic to the host, fairly cheap and the generation of resistant phenotypes appears to be less common compared to current antifungals used. One of the most documented naturals is tea tree oil (TTO), an essential oil obtained from the leaves of *Melaleuca alternifolia*. TTO is made up of a various components including sesquiterpenes, monoterpenes and alcohols, with the active ingredient being terpinene-4-ol (Carson et al., 2006). Studies have shown the antifungal activity of these agents against *C. albicans* through the inhibition of biofilm formation (De Prijck et al., 2010, Ramage et al., 2012b). Furthermore, *in vivo* studies have shown terpinen-4-ol is able to prevent the development of oral candidiasis within a murine model (Ninomiya et al., 2012).

In addition to the antimicrobial properties natural compounds have to offer, agents that are also anti-inflammatory are of increased value for the treatment of fungal infections. TTO is an example of a compound with dual action as it is able to down-regulate key pro-inflammatory mediators including IL-8 (Ramage et al., 2012b), a similar finding was also found when using proanthocyanidins

isolated from cranberries (Feldman et al., 2012). A number of other naturally occurring molecules identified for having anti-biofilm activity include a number of plants and spices including turmeric, cinnamon oil, garlic, usnic acid and linalool (Hsu et al., 2012, Rukayadi and Hwang, 2012, Sardi et al., 2013). Further studies have investigated fulvic acids and showed in addition to a reduction of inflammation *in vivo* (Gandy et al., 2011, Sabi et al., 2012), these compounds also have broad-spectrum antimicrobial activity (van Rensburg et al., 2000).

Ethanol is another natural antimicrobial agent that can either be used alone or in combinational therapy to control *C. albicans* biofilm formation (Blackwood et al., 2011, Pieroni et al., 2012). The broad-spectrum activity of ethanol has also allowed its use in catheter locks to inhibit bacterial biofilm development (Oliveira et al., 2012), with biofilms found within polymicrobial infections also being treated effectively with ethanol (Peters et al., 2013). Other mixed species biofilms including *C. parapsilosis* and MRSA have shown to be eradicated when ethanol and ethylenediaminetetraacetic acid (EDTA) are used in combination (Raad et al., 2007), however EDTA treatment alone can also be used in endodontic infections (Siqueira and Sen, 2004).

Overall, these studies have shown the potential that natural antimicrobials possess when it comes to the treatment of biofilm infections. The main advantage of using these is their broad-spectrum antimicrobial activity as many of these infections consist of mixed bacterial-fungal biofilms.

## 1.7 Aims and hypothesis

It is clear by reviewing the current literature that *C. albicans* biofilms have a great impact on the clinical management of candidiasis. At present there are no definitive diagnostic tools to aid in identification of fungal biofilm infections, and antifungal therapies are increasingly failing.

Given that these biofilm infections caused by *C. albicans* do not have consistent responses to clinical management, the overall hypothesis of this study was that standard antifungal therapies are ineffective and that these biofilms vary physically and transcriptionally, which therefore impacts their clinical management.

The aims of this study were therefore to, 1) investigate the variation of biofilm development within clinical isolates, 2) identify a potential mechanism responsible for differential biofilm formation and to 3) explore the potential of a natural broad-spectrum antimicrobial agent against *C. albicans* and polymicrobial biofilms.

Chapter 2:  
*Candida albicans* biofilm  
heterogeneity is associated with  
resistance and pathogenicity

## 2.1 Introduction

*Candida* species are frequently isolated commensals of the human skin and mucosal membranes, but if these barriers become compromised in any way then there can be an increase in *Candida* colonisation leading to invasive forms of candidiasis (Eggimann et al., 2011). *Candida* is the most common fungal pathogen of bloodstream infections (BSI) where it is the fourth most prevalent causative organism of these infections and the third most commonly isolated species within the ICU in the USA (Wisplinghoff et al., 2004). Candidaemia is often associated with the ability of *Candida* to adhere to and form biofilms on indwelling medical devices, such as CVC and prostheses (Kojic and Darouiche, 2004, Lynch and Robertson, 2008).

Biofilms are a population of microorganisms attached to one another and/or a surface, surrounded by an ECM (Ramage et al., 2012c). A defining feature of these is their resistance to antimicrobial therapy, with higher drug concentrations required to kill biofilms and their dispersed cells when compared to equivalent free-floating planktonic cells (Ramage et al., 2012c, Taff et al., 2012b, Uppuluri et al., 2010). Another feature of *C. albicans* biofilms is their enhanced pathogenicity. For example, cells detaching from biofilms have been shown to be more cytotoxic *in vivo* than their planktonic counterparts and significantly increase mortality within a murine model of infection (Uppuluri et al., 2010). These observations have been demonstrated clinically, where a significant association was observed between *C. albicans* biofilm formation and mortality rates in candidaemia patients (Tumbarello et al., 2007).

Whilst there is growing evidence of the importance of *Candida* biofilms in clinical medicine, not all clinical isolates are able to form biofilms (Tumbarello et al., 2012). There is, therefore, a fundamental gap in understanding exactly what drives biofilm formation and its clinical implications. Establishing methods to differentiate these isolates is challenging, as many studies rely on either metabolic assays or biomass, and these frequently use a variety of different substrates and media (Daniels et al., 2013, Kucharikova et al., 2011, Pierce et al., 2008, Samaranayake et al., 2009b). Therefore, comparison between these studies is not possible, and further interpretation of the data to improve clinical management both for diagnostics and antifungal therapy is limited.

## 2.2 Aims

The aim of this chapter was to investigate and characterise biofilm formation by clinical isolates of *C. albicans* using standard methodologies and subsequently analyse biofilm subsets at a phenotypic level. Next, the antifungal susceptibility profiles of these clinical isolates were established using amphotericin B, with the impact of *C. albicans* strain variation assessed *in vivo* using a *Galleria mellonella* model. Finally, a number of *C. albicans* biological markers were used to assess biofilm variation at a transcriptional level and to determine if these could potentially be used as a diagnostic tool for identifying *C. albicans* biofilm formation.

The data represented in this chapter has been accepted for publication in:

Sherry L, Rajendran R, Lappin D, Borghi E, Perdoni F, Falleni M, Tosi D, Williams C, Jones, Nile C, Smith K and Ramage G. Biofilms formed by *Candida albicans* bloodstream isolates display phenotypic and transcriptional heterogeneity that are associated with resistance and pathogenicity. *BMC Micro*, epub ahead of print.

Work from this chapter has been presented at the following conferences:

Sherry L, Rai M, Murray C, Nile C, Jones B, Williams C and Ramage G. 'Evaluating the incidence of biofilm formation from candidaemia isolates and the effects of Ambisome®.' *ASM Candida and Candidiasis*. March 2012.

Sherry L, Rajendran R, Jones B, Williams C, Nile C and Ramage G. 'Biofilm phenotypic and transcriptional heterogeneity amongst *Candida albicans* bloodstream isolates differentially affect amphotericin B tolerance.' *ECCMID*, Barcelona, Spain. May 2014.

## 2.3 Materials and Methods

To investigate the biofilm forming ability of clinical *C. albicans* isolates, an established method used for evaluating and quantifying biofilm biomass was employed. Based on this initial screening, three distinct groups of isolates were identified based upon their ability to form a biofilm; low biofilm formation (LBF), intermediate biofilm formation (IBF) and high biofilm formation (HBF). Minimum inhibitory concentrations (MIC) of these selected isolates were determined and the clinical significance of biofilm formation by *C. albicans* in relation to antifungal therapy and pathogenicity was assessed.

### 2.3.1 Culture conditions and standardisation

#### 2.3.1.1 *C. albicans* strain collection

A collection of *C. albicans* clinical bloodstream isolates (n=42) was obtained from the Royal Hospital of Sick Children (Yorkhill Division, Glasgow, UK) between January 2009 to July 2011 (Appendix 1). The laboratory strains SC5314 and 3153A were also included as appropriate control isolates. All *Candida* isolates obtained during this period of time were independently identified using Colorex *Candida* chromogenic plates (E&O Laboratories Ltd, Bonnybridge, UK) and purity plates made for each isolate. All isolates were stored in Microbank<sup>®</sup> vials (Pro-Lab Diagnostics, Cheshire, UK) at -80°C until further use.

#### 2.3.1.2 Optimisation of *C. albicans* biofilm formation

*C. albicans* SC5314 and 3153A biofilm formation was optimised as described previously (Ramage et al., 2001a). Initially, isolates were retrieved from Microbank<sup>®</sup> vials and sub-cultured on to Sabouraud's dextrose agar (SAB [Sigma-Aldrich, Dorset, UK]). Plates were incubated at 30°C for 48 h and maintained at 4°C. A loopful of each isolate was inoculated into 10 mL of yeast peptone dextrose (YPD) medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose [Sigma-Aldrich, Dorset, UK]) contained in a 25 mL universal (Sterilin<sup>®</sup> Limited, Cambridge, UK) and placed in the orbital shaking incubator at 150 rpm and 30°C for 18 h. Cells were centrifuged at 3,000 rpm for 5 min and the resultant supernatant containing cellular debris was discarded. The remaining pellet was washed with 10 mL of phosphate buffered saline (PBS [Sigma-Aldrich, Dorset,

UK]) and centrifuged for 5 min at 3,000 rpm. The supernatant was discarded once more, the pellet was resuspended in PBS, and cells were counted using a Naeuber haemocytometer. *C. albicans* cells were standardised to the desired cellular density of  $1 \times 10^6$  cells/mL in either RPMI-1640 (Sigma-Aldrich), YPD + 10% foetal calf serum (FCS), yeast nitrogen base (YNB [Sigma-Aldrich]) + 100 mM glucose or Spider media (2% nutrient broth [Oxoid], 2% mannitol, 0.4%  $K_2HPO_4$  and 2.7% agar, pH adjusted to 7.2). The selection of media was based upon a range of growth conditions currently used in various laboratories (Ferreira et al., 2013, Ramage et al., 2001a, Midkiff et al., 2011). Standardised cells (200  $\mu$ L) within each medium were dispensed into defined wells of a pre-sterilised, polystyrene, 96 well flat-bottom microtitre plate (Corning Incorporated, NY, USA), which were incubated for 24, 48 and 72 h at 37°C. Following incubation, biofilms were carefully washed twice with PBS to remove planktonic cells and biofilm biomass was determined using the crystal violet (CV) assay (Jose et al., 2010), described in detail in 2.3.2.1. Negative controls containing no organisms were also included. Testing was carried out in triplicate for each isolate, on two separate occasions.

### **2.3.2 Characterisation of *Candida albicans* biofilm formation by clinical isolates**

Based on this initial optimisation experiment above, all other clinical *C. albicans* isolates (n=42) were assessed for their biofilm forming ability in RPMI (Roswell Park Memorial Institute) 1640 media (Sigma-Aldrich, Dorset, UK). *C. albicans* cells were standardised to  $1 \times 10^6$  cells/mL in RPMI and 200  $\mu$ L dispensed into each well of pre-sterilised, polystyrene, 96 well flat-bottom microtitre plate (Corning Incorporated, NY, USA). Plates were incubated for 24 h at 37°C. Following incubation, the culture media was discarded and biofilms were carefully washed twice with PBS to remove planktonic cells. Biofilm biomass was quantified using the CV assay.

#### **2.3.2.1 Biomass quantification by crystal violet**

Biofilms were grown as previously described in 2.3.1. Biofilms were carefully washed with PBS and allowed to air dry. One hundred microlitres of 0.05% w/v CV solution was added to each biofilm and incubated at room temperature for 20 min to allow uptake of the dye. Following incubation, the CV solution was

discarded and the biofilms were washed with running tap water to remove any unbound dye, and 100  $\mu$ L of 100% ethanol applied to destain each biofilm. The contents of the wells were mixed thoroughly by pipetting and transferred to a new 96 well flat-bottom microtitre plate for measurement. The biomass was quantified spectrophotometrically by reading absorbance at 570 nm in a microtitre plate reader (FluoStar Omega, BMG Labtech). All absorbance values were blank corrected based upon the negative control where no biofilms were formed. Isolates were grouped based on their level of biomass distribution ( $OD_{570nm}$  values). Isolates that fell below the 1st quartile (Q1) were classed as having LBF, strains with a biomass greater than the 3rd quartile (Q3) were deemed isolates with HBF, and those that lay in between were classified as IBF (Q2). Each isolate had eight replicates and biomass measured on two separate occasions. Biofilms were also formed in 12 well flat-bottom microtitre plate (Corning Incorporated, NY, USA) for 24 h before carefully washing with PBS and stained with 0.05% CV solution. Macroscopic images were digitally taken (Canon IXUS 220 HS) to show biomass after 24 h of biofilm development.

### **2.3.2.2 Biomass quantification by dry weight**

*C. albicans* biomass was further assessed using dry weight measurements. Selected isolates with LBF (n=3) and HBF (n=3) were grown as biofilms in 12 well plates for 24 h, as previously described, and the resulting biomass collected in 1 mL of PBS using a cell scraper (STARLAB, Milton Keynes, UK). This was then passed through a 0.22  $\mu$ m filter disc (Satorius Stedim, Surrey, UK) using a vacuum, and the filters subsequently dried at 40°C overnight before measuring each isolates dry weight. Un-inoculated controls were used for background correction.

### **2.3.3 Scanning electron microscopy**

For biofilm visualisation, sessile cells of isolates with proven high (n=2 [28158, 34106]) and low (n=2 [27915, 74174]) biofilm forming ability were grown directly on Thermanox™ coverslips (Nunc, Roskilde, Denmark) for 4 and 24 h at 37°C. Following incubation, biofilms were carefully washed with PBS and then fixed with a fixative solution containing 2% para-formaldehyde, 2% glutaraldehyde and 0.15 M sodium cacodylate, and 0.15% w/v alcian blue (pH 7.4) and left in solution for 18 h. The fixative was carefully discarded and replaced with 0.15 M

sodium cacodylate buffer and stored at 4°C until processing. Samples were then prepared for SEM as previously described (Erlandsen et al., 2004). Samples were washed 3 × 5 min with 0.15 M cacodylate to ensure all gluteraldehyde had been removed. Samples were then treated with 1% osmium tetroxide solution containing 0.15 M sodium cacodylate (1:1) and incubated in the fume hood for 1 h. Samples were rinsed 3 × 10 min with distilled water and then treated with 0.5% uranyl acetate and incubated in the dark for 1 h. Uranyl acetate was removed from the samples and quickly rinsed with water before a series of dehydration steps were carried out. Two 5 min rinses of 30, 50, 70 and 90% alcohol were followed by 4 × 10 min rinses of absolute and dried absolute alcohol. Hexamethyldisilazane (HMDS) was used to dry the specimens by soaking the samples for 5 min before transferring to a plate containing fresh HMDS. All samples were then placed in a dessicator overnight to allow evaporation of any residue and drying. The specimens were then mounted and sputter-coated with gold in an argon filled chamber, and then viewed under a JEOL JSM-6400 scanning electron microscope. Images were assembled using Photoshop software (Adobe, San Jose, CA, USA).

### 2.3.4 Growth kinetics

The growth kinetics of *C. albicans* clinical isolates with LBF (n=10) and HBF (n=10) were assessed to determine if growth rate played a role in their ability to form biofilms. Each isolate was standardised to  $1 \times 10^4$  cells/mL in YPD, with 200 µL added to each well of a 96 well round-bottomed plate. The plate was incubated at 37°C for 24 h with absorbance measured at 530 nm every 1 h, following shaking at 100 rpm for 30 s. Each isolate was tested in duplicate and this experiment was repeated on three independent occasions. Negative controls containing no *C. albicans* were included for background correction.

### 2.3.5 Cellular surface hydrophobicity assay

Cellular surface hydrophobicity (CSH) was determined for selected *C. albicans* clinical isolates with LBF (n=10) and HBF (n=10). CSH was assessed using the microbial adhesion to hydrocarbon test, with a few modifications (Yoshijima et al., 2010, Rosenberg et al., 1980). Isolates were standardised to  $1 \times 10^6$  cells/mL in RPMI-1640 and grown as biofilms for 4 and 24 h in 75 cm<sup>2</sup> flasks (Nunc, Rochester, NY) at 37°C. These were then carefully washed with PBS and

the resultant biomass scraped off using a cell scraper and homogenised in YPD. Cells were standardised ( $OD_{590nm}$  1.0) and cells transferred into a glass tube and overlaid with  $1/5^{th}$  volume of xylene. Contents were vortexed for 1 min and phases allowed to separate over 30 min. The aqueous phase was carefully removed and  $OD_{590nm}$  measured again. The percentage of hydrophobicity was calculated as  $([OD_{590nm}$  before xylene overlay -  $OD_{590nm}$  after xylene overlay]/  $OD_{590nm}$  before xylene overlay)  $\times$  100%. Controls containing no *C. albicans* were used for background correction. Each isolate was tested on three separate occasions.

### 2.3.6 Antifungal susceptibility testing

The following antifungal agents were used in the course of this study: AmBisome<sup>®</sup> (AmBi, Gilead, UK), amphotericin b (AMB, Sigma-Aldrich, Gillingham UK), caspofungin (CSP, Merck Sharp & Dohme, Hertfordshire, UK) and micafungin (MFG, Astellas, UK). All antifungals were prepared at a stock concentration of 2 mg/mL in sterile water and stored at  $-20^{\circ}C$  until required.

#### 2.3.6.1 Planktonic and sessile inhibitory testing

Antifungal testing to determine minimum inhibitory concentrations (MICs) of planktonic cells was performed using the CLSI M-27A broth microdilution method (CLSI, 2008). Two hundred microlitres of each drug was added to the appropriate wells of a 96 well round-bottom plate (Corning Incorporated, NY, USA) and serial 2-fold dilutions were made in RPMI-1640. Next, 100  $\mu$ L of standardised *C. albicans* cells ( $1 \times 10^4$  cells/mL) was added to each well containing the drug, giving a final volume of 200  $\mu$ L, before incubation at  $37^{\circ}C$  for 24 h. Appropriate positive and negative controls were also included. Biofilms were grown for 24 h at  $37^{\circ}C$ , washed with PBS and sessile susceptibility testing was performed, as previously described, in commercially available pre-sterilised, polystyrene, flat-bottomed, 96 well microtitre plates (Corning Incorporated, NY, USA) (Ramage et al., 2001a). Two-fold serial dilutions were prepared as described for planktonic susceptibility testing with a final 100  $\mu$ L of RPMI-1640 added to each well to take the final volume to 200  $\mu$ L in each well. Biofilms were incubated at  $37^{\circ}C$  for 24 h. Sessile minimum inhibitory concentrations (SMIC) were determined at 90% inhibition using an XTT metabolic reduction assay, as described in 2.3.6.2

Ch.2 *C. albicans* biofilm heterogeneity is associated with resistance and pathogenicity (Pierce et al., 2008). Testing of all planktonic and sessile isolates was performed in triplicate.

Further sessile susceptibility testing was carried out using AMB against selected *C. albicans* isolates with LBF (n=10) and HBF (n=10) to determine if biofilm phenotype impacts antifungal treatment, see appendix 1 for highlighted strains. Testing of each isolate was carried out in duplicate on three separate occasions.

### **2.3.6.2 Quantification of biofilm metabolic activity**

A semi-quantitative measure of each biofilm was calculated using a formazan salt-based XTT reduction assay (Pierce et al., 2008). Briefly, XTT (Sigma-Aldrich, Dorset, UK) was prepared as a solution of 0.25 g/L in ddH<sub>2</sub>O. The solution was then filtered through a 0.22 µm filter, aliquoted and stored at -80 °C. Prior to use, XTT was thawed and menadione ([Sigma-Aldrich, Dorset, UK], 10 mM prepared in acetone) added to a final concentration of 1 µM. A 100 µL aliquot of the XTT/menadione solution was subsequently added to each pre-washed biofilm, and to control wells (for measurement of background XTT-reduction levels). Plates were then incubated in the dark for 2 h at 37 °C. A colorimetric change in the XTT reduction assay, representing a direct correlation of metabolic activity of the biofilm, was then measured at 492 nm in a microtitre plate reader (FluoStar Omega, BMG Labtech, Aylesbury, Buckinghamshire, UK). Negative controls containing no biofilms were used for background correction. Testing of all planktonic and sessile isolates (n=42) was performed in duplicate.

### **2.3.7 Investigating pathogenicity of two biofilm sub-sets**

#### **2.3.7.1 *Galleria mellonella* pathogenicity assay**

The pathogenicity of *C. albicans* isolates pre-defined as LBF (n=3), HBF (n=3) and SC5314 were assessed using the *G. mellonella* killing assay, as described previously (Cotter et al., 2000). This biological model has been shown previously to be useful in the study of fungal virulence (Gago et al., 2014, Cirasola et al., 2013, Fallon et al., 2012). Sixth-instar *G. mellonella* larvae (Livefoods Direct Ltd, UK) were stored in the dark and used within 7 days of shipment. Ten random larvae with a bodyweight of between 200 to 300 mg were used for each group. Overnight YPD cultures of each isolate were washed and standardised to 1

Ch.2 *C. albicans* biofilm heterogeneity is associated with resistance and pathogenicity

$\times 10^7$  cells/mL in PBS. Larvae were inoculated using a 50  $\mu$ L Hamilton syringe with 26 g needle by injecting 10  $\mu$ L aliquots ( $1 \times 10^5$  cells/larva) into the haemocoel, through the hindmost proleg. In addition, mock inoculated larvae pierced on the proleg with a sterile needle and a PBS inoculated control group were also included in each experiment. Each group contained ten replicates for each condition. The infected larvae were placed in sterile petri dishes, incubated at 37°C and the number of dead larvae were scored daily. Larva was considered dead when it displayed no movement in response to touch together with a dark discolouration of the cuticle. Pathogenicity of *C. albicans* isolates with LBF and HBF was assessed using a Kaplan-Meier plot with percentage survival monitored over 7 days.

### **2.3.7.2 Histology analysis of infected *G. mellonella***

These experiments were performed with the help and assistance from Elisa Bourghi from the University of Milan.

The morphology of the larvae infected with two isolates of *C. albicans* LBF and HBF was examined. Larvae were infected with the respective strains as described previously, and after post-infection (24, 48 and 72 h) larvae were fixed by a direct injection of formalin into the haemocoel and by formalin immersion at room temperature for 24 h. Paraffin embedded samples were then transversally sectioned into four-micron thickness using a microtome (microm HM 3335H, ThermoScientific). Sections were then stained with Periodic Acid-Schiff (PAS) to evaluate *C. albicans* infected cells. Whole larvae sections were examined for characterisation and localisation of nodules by light optical microscope visualisation (Leica microscope, model 020-519.502). Two larvae were processed for each isolate, carried out on three separate occasions.

### **2.3.8 Optimisation of PCR conditions for transcriptional analysis**

Before the transcriptional profiles of *C. albicans* could be determined, a series of primer and PCR optimisation stages were required. All future PCR studies were carried out in a laminar flow hood (Bioquell UK Ltd, Hampshire, UK) with UV-radiated equipment and filter tips to ensure no contamination occurred within the samples. Specific genes related to adhesion, cell wall structure,

Ch.2 *C. albicans* biofilm heterogeneity is associated with resistance and pathogenicity morphology, pathogenicity and antimicrobial resistance were selected and used for analysis in gene expression studies.

### **2.3.8.1 DNA extraction for PCR optimisation**

*C. albicans* SC5314 was used as a template for all subsequent PCR optimisation studies. An overnight culture of *C. albicans* SC5314 in 10 mL of YPD broth, incubated at 30°C at 150 rpm, was used from the DNA extraction. The MasterPure™ Yeast DNA purification kit from Epicentre® Biotechnologies (Cambio, Cambridge UK) was used, following the manufacturer's instructions. One and a half millilitres of the overnight culture was transferred into a microfuge tube and centrifuged at 10,000 rpm for 5 min to pellet the cells. The supernatant was discarded and the remaining pellet resuspended in 300 µL of yeast cell lysis solution. The cells were incubated at 65°C for 15 min and then cooled on ice for 5 min. Following incubation, 150 µL of MPC protein precipitation reagent was added to the solution and mixed by vortexing. Samples were centrifuged for 10 min at 13,000 rpm to ensure all cellular debris had been pelleted before transferring the supernatant to a fresh microfuge tube. Five hundred microlitres of isopropanol was mixed with the solution by inversion and centrifuged for a final 10 min at 10,000 rpm. The supernatant was discarded and the remaining genomic DNA was washed with 500 µL of 70% ethanol before being resuspended in 35 µL of TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). DNA was quantified using the NanoDrop™ ND-1000 spectrophotometer (Labtech) as described in 2.3.8.2.

### **2.3.8.2 Nucleic acid quantification**

Nucleic acid was quantified using the NanoDrop™ ND-1000 spectrophotometer (Labtech International, Ringmer, East Sussex, UK). TE buffer was used as a reference before the DNA was quantified and concentrations were recorded in ng/µL. Samples with a 260/280 nm ratio of 1.8 to 2.2 were deemed to be of high quality and were used for subsequent PCR reactions. DNA was stored at -20°C until required.

### 2.3.8.3 Primer designing

All primers used for qPCR throughout this study were either taken from peer-reviewed papers or designed based upon their sequence, obtained from the *Candida* Genome Database (CGD) web site (<http://www.Candidagenome.org>). For primer sets being designed in house, the web-based primer design software program, Primer3 (<http://frodo.wi.mit.edu/cgi-bin/primer3/>) was used. Primers were designed with a PCR product size of 70-120 base pairs. The National Institute of Health's Basic Local Alignment Search Tool (NIH-BLAST) was used to check the specificity of designed primers to *C. albicans* (<http://www.nlm.nih.gov/BLAST>). Oligonucleotides matching the resulting primer sequences were synthesised (Invitrogen, Paisley, UK) for the *C. albicans* genes listed in Table 2.1.

**Table 2.1 *C. albicans* primers used for real time qPCR**

| Gene        | Sequence (5' - 3')  | Reference                |
|-------------|---|--------------------------|
| <b>ALS1</b> | F - TTCTCATGAATCAGCATCCACAA<br>R - CAGAATTTTCACCCATACTTGGTTTC     | (Nailis et al., 2010a)   |
| <b>ALS3</b> | F - CAACTTGGGTTATTGAAACAAAAACA<br>R - AGAAACAGAAACCCAAGAACAACCT   | (Nailis et al., 2006)    |
| <b>ALS5</b> | F - CTGCCGTTATCGTCCATTTA<br>R - ATTGATACTGGTTATTATCTGAGGGAGAAA    | (Green et al., 2005)     |
| <b>EAP1</b> | F - ACCACCACCGGTATACAAA<br>R - GCCATCACATTTGGTGACAG               | In house                 |
| <b>HWP1</b> | F - GCTCAACTTATTGCTATCGCTTATTACA<br>R - GACCGTCTACCTGTGGGACAGT    | (Nailis et al., 2010a)   |
| <b>BCR1</b> | F - ATTGCCACCAATACCTGCTC<br>R - GGCTGTCCATGTTGTTGTTG              | In house                 |
| <b>CPH1</b> | F - ACGCAGCCACAAGCTCTACT<br>R - GTTGTGTGTGGAGGTTGCAC              | In house                 |
| <b>EFG1</b> | F - CCAGTGGTGGCAGTAATGTG<br>R - CAGTGGCAGCCTTGGTATTT              | In house                 |
| <b>TUP1</b> | F - GCTTCAGGTAACCCATTGTTGAT<br>R - CTTCGGTTCCTTTGAGTTTAGG         | (Uppuluri et al., 2009a) |
| <b>OCH1</b> | F - TCATCCAATGTTGCGTGAAT<br>R - TCATGATATCGCCACCTTCA              | In house                 |
| <b>PMR1</b> | F - GAATCCCCGCAGACATTAGA<br>R - GGGCCTGTTTTACCAGTTA               | In house                 |
| <b>MNN4</b> | F - TGAGCAATCGTCAAACCAG<br>R - GGCGGTTGTCATTTGTTGAT               | In house                 |
| <b>MNT2</b> | F - CGTCAAGGTGCCTGAAGAAT<br>R - GAGGAGGAGGAGGATTTTGG              | In house                 |
| <b>SAP1</b> | F - ACAAGCCCTCCCAGTTACTTTAAA<br>R - AACCAATAGTGATGTCAGCAGCAT      | (Nailis et al., 2010a)   |
| <b>SAP2</b> | F-GAATTAAGAATTAGTTTGGGTTTCAGTTGA<br>R - CCACAAGAACATCGACATTATCAGT | (Nailis et al., 2010a)   |
| <b>SAP3</b> | F - CAGCTTCTGAATTTACTGCTCCATT<br>R - TCCAAAAAGAAGTTGACATTGATCA    | (Nailis et al., 2010a)   |
| <b>SAP4</b> | F -CAAAACTTAGCGTTATTGTTGACACT<br>R - AAACGGCATTGGAATCTGGAA        | (Nailis et al., 2010a)   |
| <b>SAP5</b> | F - CCAGCATCTTCCCGCACTT<br>R - GCGTAAGAACCCTCACCATATTTAA          | (Nailis et al., 2010a)   |

|             |  |                         |
|-------------|--|-------------------------|
| <b>SAP6</b> | F - GCTAACGTTTGGTCTACTAGTGCTCATA<br>R - TGGTAGCTTCGTTGGCTTGA | (Nailis et al., 2010a)  |
| <b>PLB1</b> | F - GGTGGAGAAGATGGCCAAA<br>R - AGCACTTACGTTACGATGCAACA       | (Nailis et al., 2010a)  |
| <b>CDR1</b> | F - GTACTATCCATCAACCATCAGCACTT<br>R - GCCGTTCTTCCACCTTTTTGTA | (Watamoto et al., 2011) |
| <b>MDR1</b> | F - TCAGTCCGATGTCAGAAAATGC<br>R - GCAGTGGGAATTTGTAGTATGACAA  | (Watamoto et al., 2011) |
| <b>ZAP1</b> | F - CGACTACAAACCACCAGCTTCATC<br>R - CCCCTGTTGCTCATGTTTTGTT   | (Nobile et al., 2009)   |
| <b>ACT1</b> | F - AAGAATTGATTTGGCTGGTAGAGA<br>R - TGGCAGAAGATTGAGAAGAAGTTT | (Ricardo et al., 2009)  |

#### 2.3.8.4 Primer optimisation by gradient PCR

Gradient PCR was used to optimise the primers designed in-house, as illustrated in section 2.3.8.3, to obtain the most appropriate annealing temperature. All other primer sets used in the gene expression studies had already being optimised by other groups.

Primers were diluted to a working concentration of 100  $\mu\text{M}$  in sterile water. A mastermix was prepared consisting of 22.5  $\mu\text{L}$  of ReddyMix (Abgene, ThermoFisher, Epsom, Surrey, UK), 1  $\mu\text{L}$  of forward and reverse primers and 0.5  $\mu\text{L}$  of DNA at 200 ng/ $\mu\text{L}$ , previously extracted from *C. albicans* SC5314. Eight replicates for each gene of interest were carried out in 200  $\mu\text{L}$  PCR tubes. A negative control containing no DNA template was also included. The tubes were placed in a temperature gradient heat block with PCR cycling capability (MJ Research, Waltham, MA, USA), where annealing temperatures ranged from 56.0°C to 63.0°C. The thermal profile included a 2 min initial denaturation step at 95°C, followed by an amplification cycle of 40 cycles of 95°C for 25 s, the appropriate annealing temperature (56.0°C to 63.0°C) for 35 s, and a 65 s extension at 72°C. A final 5 min extension step at 72°C was followed by a 4°C holding stage. Amplified samples were then visualised using gel electrophoresis to assess to most appropriate annealing temperature.

### 2.3.8.5 Gel electrophoresis

A 2% (w/v) agarose (Life Technologies, Paisley, UK) gel was prepared in 100 mL of 0.5× TBE buffer and melted in a microwave, with frequent stirring until the agarose had dissolved. This solution was allowed to cool before 5 µL of 10 mg/mL ethidium bromide was added. The gel apparatus was assembled according to the manufacturer's instructions (Advance Mupid EX, Japan) and a 13-lane comb and was inserted into the tray. The agarose solution was initially used to seal the tray at both ends to ensure a tight fit was achieved before the remainder was poured into the tray, ensuring any air bubbles were removed. The agarose was allowed to set for 20 min before the tray was placed inside the electrophoresis tank and covered with 0.5 × TBE buffer. The comb was carefully removed and 10 µL of each PCR product was added to an individual well. In addition, 10 µL of a 1000 base pair hyperladder (1 in 10 dilution) and a water control were also included on the gel. The gel tank was connected to the power supply and run for 2 h at 60 v. Upon completion, the gel was observed under UV light and photographed (BioRad Gel Doc 2000, Hemel Hempstead, Hertfordshire UK).

### 2.3.8.6 Optimisation of quantitative PCR

The efficiencies of each primer set designed in 2.3.8.3 were assessed using a standard curve to confirm linear PCR kinetics independent of the genomic DNA concentration. The simplest method to determine the amplification efficiencies was to use the DNA extracted from *C. albicans* SC5314 in 2.3.8.1 as a template and to serially dilute the DNA using 1 in 10 dilutions, from 400 ng to 0.04 ng/µL. For each dilution, 1 µL of each concentration of genomic DNA was added to a mastermix containing 12.5 µL of SYBR<sup>®</sup> GreenER<sup>™</sup> (Life Technologies, Paisley, UK), 0.5 µL of forward and reverse primers and 10.5 µL of sterile distilled water. All reactions were carried out in 96 well plates and sealed using caps (Agilent Technologies, Cambridge, UK). The thermal profile cycle was carried out on a Strategene MX5300x Pro (Agilent Technologies) and consisted of a UDG incubation of 2 min at 50°C, an UDG inactivation and DNA polymerase activation of 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. A melt curve was also carried out after the end of the PCR thermal profile to confirm

Ch.2 *C. albicans* biofilm heterogeneity is associated with resistance and pathogenicity only one product had been amplified. Reactions were carried out in duplicate, on one occasion and included no template controls (NTC).

The cycle threshold (Ct) values for each sample were plotted against the  $\log_{10}$  of the DNA concentration and the efficiency of the PCR reaction was calculated from the formulas;

$$\text{Efficiency} = -1 + 10^{(-1/\text{slope})}; \text{Slope} = (y_2 - y_1) / (x_2 - x_1)$$

Efficiencies that fell between 90-110% are deemed acceptable for subsequent transcriptional analysis of *C. albicans* clinical biofilms by qPCR.

### 2.3.9 Transcriptional analysis of biofilm related genes

Based upon the initial screening of the *C. albicans* bloodstream isolates, those categorised as LBF (n=10) and HBF (n=10) were used in the transcriptional analysis studies.

#### 2.3.9.1 RNA extraction from *in vitro* biofilms

Biofilms were grown as described in 2.3.2 in 24 well flat-bottom plates for 4 h (early phase) and 24 h (mature phase) at 37°C. Following incubation, the supernatant was discarded and biofilms carefully washed with PBS. One millilitre of TRIzol™ solution (Invitrogen, Paisley, UK) was added to the wells containing pre-formed biofilms and a CytoOne™ cell scraper (STARLAB, Milton Keynes, UK) was used to remove the adherent biofilm from the plate. The resulting biomass was transferred to an O-ring screw-cap microcentrifuge tube (Stratech, Newmarket, UK) and approximately 250 µL of 0.5 mm diameter sterile glass microbeads (BioSpec, Bartlesville, Oklahoma, USA) were added to each tube and placed on ice. Cells within the TRIzol™ solution were mechanically disrupted in a Mini-Beadbeater (BioSpec, Bartlesville, Oklahoma, USA) for 3 cycles of 30 s. Samples were placed kept on ice in between each cycle and 100 µL of 1-bromo-3-chloropropane (Sigma-Aldrich, Dorset, UK) was added to each sample before they were vortexed for 30 s. Samples were left at room temperature for 5 min, with occasional inversion, before centrifuged at 13,000 rpm for 15 min at 4°C (Heraeus,) and then placed on ice. The upper aqueous layer of each sample was transferred to a sterile microfuge tube and 500 µL of ice-cold isopropanol was

added in order to precipitate the RNA. The solution was placed at  $-20^{\circ}\text{C}$  overnight to maximise the precipitation of RNA.

Following overnight precipitation, all samples were centrifuged for 10 min at 10,000 rpm at  $4^{\circ}\text{C}$ . The supernatant was discarded and the remaining pellet washed with 500  $\mu\text{L}$  of ice cold 70% ethanol. Samples were inverted 40 times then centrifuged at 10,000 rpm for 5 min at  $4^{\circ}\text{C}$ . The ethanol was carefully removed and samples were air dried for 5 min. The RNA was finally resuspended in 25  $\mu\text{L}$  of RNase free distilled water and incubated at  $65^{\circ}\text{C}$  in a heat block (Techne, Staffordshire, UK) for 5 min to aid the recovery of RNA.

### **2.3.9.2 DNase digestion of total RNA**

To ensure the extracted RNA was free from DNA contamination, a DNase digestion kit was used, as per manufacturer's instructions (Qiagen Ltd, Crawley, West Sussex, UK). Briefly, 10  $\mu\text{L}$  of RDD buffer was mixed with 2.5  $\mu\text{L}$  of DNase before being added to the extracted RNA. Finally 62.5  $\mu\text{L}$  of RNase-free distilled water was added to the reaction to make a final volume of 100  $\mu\text{L}$  and samples were incubated at room temperature for 10 min.

### **2.3.9.3 RNA cleanup**

After the DNA digestion stage, RNA was then purified using the RNeasy MinElute CleanUp kit as per manufacturer's instructions (Qiagen Ltd., Crawley, West Sussex, UK). A solution containing 350  $\mu\text{L}$  of RLT buffer and 250  $\mu\text{L}$  of 100% ethanol were added to the RNA extract and mixed thoroughly by pipetting. The 700  $\mu\text{L}$  sample was then transferred to the RNeasy MinElute spin column within a 2 mL collection tube and centrifuged for 15 s at 10,000 rpm. The flow-through was discarded and 500  $\mu\text{L}$  of RPE buffer was added directly on to the column. Samples were centrifuged at 10,000 rpm for 15 s and the flow-through discarded. To ensure there was no carry over, the column containing the RNA was transferred to a new 2 mL collection tube and centrifuged for 1 min at 10,000 rpm. Finally, the column was then placed into a 1.5 mL microfuge tube and 50  $\mu\text{L}$  of RNase free water was added directly onto the membrane before being centrifuged at 10,000 rpm for 1 min in order to release the RNA. To ensure the maximum RNA concentration was achieved, the eluted RNA was then passed

Ch.2 *C. albicans* biofilm heterogeneity is associated with resistance and pathogenicity through the membrane once more at 10,000 rpm for a further minute. The purified RNA was now ready for quantification, as described in 2.3.8.2.

#### **2.3.9.4 cDNA synthesis from total RNA**

Two hundred nanograms of purified RNA was added to 4  $\mu$ L of High-Capacity RNA-to-cDNA™ reverse transcription (RT) mastermix (Applied Biosystems, UK) within 0.2 mL dome-capped PCR microtubes (Abgene, ThermoFisher, Epsom, Surrey, UK), with a final volume of 20  $\mu$ L made up using RNase free water. No RT controls were also included using a mastermix which did not contain the reverse transcriptase enzyme. Samples were briefly centrifuged to remove any air bubbles and loaded on to the thermal cycler (Bio-Rad, Hertfordshire, UK). The cycle conditions consisted of 5 min at 25°C, 30 min at 42°C, 5 min at 85°C and a final hold stage at 4°C. cDNA was then stored at -20°C until used in subsequent PCR.

#### **2.3.9.5 Analysis of real time PCR gene expression**

The Ct concept is the basis of quantification of real-time PCR using fluorescence-based indicators, such as SYBR® GreenER™ (Invitrogen, Paisley, UK). The total amount of product amplified gives a fluorescence value that is recordable throughout the cycled reaction. An inverse relationship exists between the amount of DNA template and the cycle number. A higher amount of template present at the beginning of the reaction, correlates to a lower number of cycles required to reach the threshold where the fluorescence measured is statistically greater than background readings (Gibson et al., 1996). This is defined as the cycle threshold (Ct) and is found during the exponential phase of amplification (Higuchi et al., 1993).

#### **2.3.9.6 Assessing gene expression using real time PCR**

The selected *C. albicans* clinical isolates with LBF and HBF were used in gene expression studies to determine any transcriptional differences between the biofilm subsets. Regulation of the genes of interest within the clinical samples, were all relative to the standard housekeeping gene *ACT1*. The same SYBR® GreenER™ mastermix and thermal profile described in 2.3.8.6 was used for all gene expression studies, with the addition of 1  $\mu$ L of 200 ng/ $\mu$ L of cDNA. No RT

Ch.2 *C. albicans* biofilm heterogeneity is associated with resistance and pathogenicity and no template controls (NTC) were also included. Each parameter (LBF n=10, HBF n=10 at 4 and 24 h) was analysed in duplicate. Expression of the genes of interest were normalised to the housekeeping *ACT1* and relative expression quantified using the  $2^{-\Delta Ct}$  method (Livak and Schmittgen, 2001).

### **2.3.9.7 Clustering and heat map analysis**

Differential expression of the selected genes from isolates with LBF and HBF were assessed by clustering and heat map analysis using GenEx software (Exiqon, Vedbaek, Denmark). The Ct values from qPCR experiments were pre-processed to obtain the percentage gene expression relative to the housekeeping gene *ACT1* by  $\Delta Ct$  method. Percentage expression data was then log transformed and the mean values were used for heat map production. Each coloured cell in the heat map represents the variable expression of genes in LBF and HBF at 4 and 24 h time points. An increase in gene expression is represented by red and a down-regulation by green. Clustering techniques were used to show genes with similar expression patterns (co-regulated genes) in each set of isolates. The clustering was performed independently by average linkage and Euclidean distances used as a distance measure for both dimensions in the data.

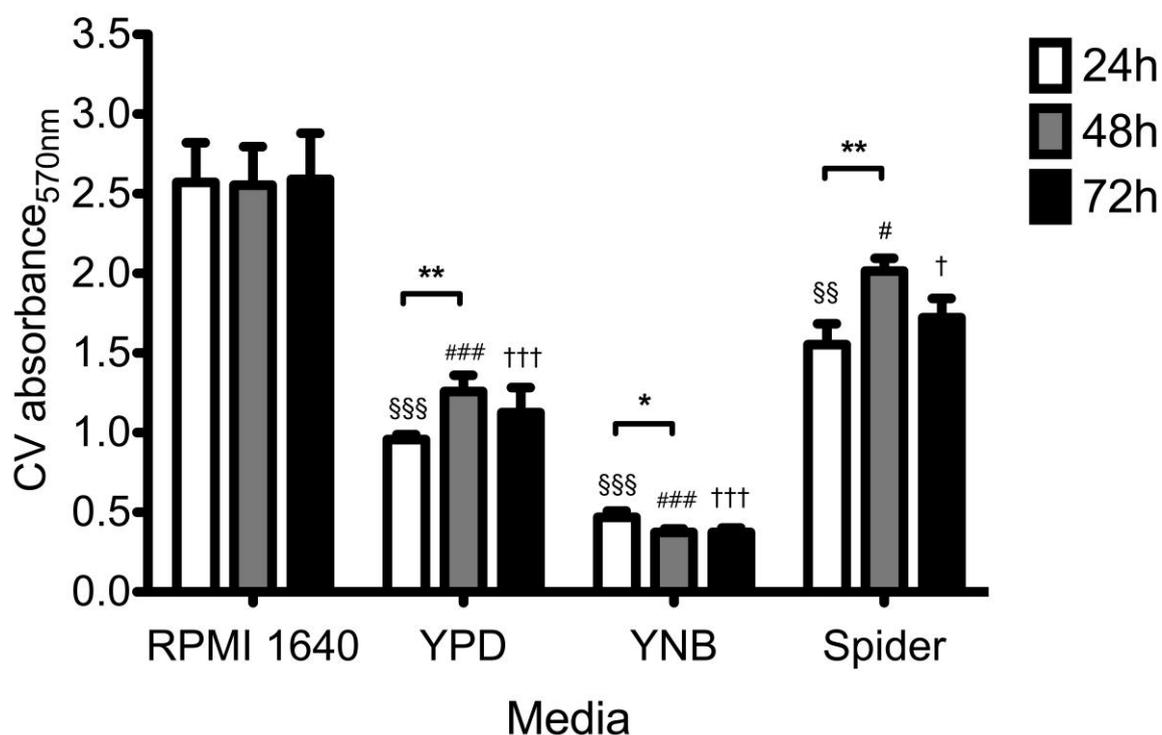
### **2.3.10 Statistical analysis**

Graph production, data distribution and statistical analysis were performed using GraphPad Prism (version 4; La Jolla, CA, USA). After assessing whether data conformed to a normal distribution data were transformed where necessary and One Way Analysis of Variance (ANOVA) was used to investigate significant differences between independent groups. A Bonferroni post-test was used to determine statistically significant differences between groups. The *G. mellonella* survival curve was analysed using log rank test. Student t-tests were used to measure statistical differences between the two independent groups assessed in gene expression studies. Statistical significance was achieved if  $p < 0.05$ . IBM SPSS<sup>®</sup> (version 20) statistical analysis software was used for correlation analysis. Two-tailed Spearman rho correlation coefficient was determined separately for all 4 and 24 h selected genes expression versus 24 h biomass data. Genes that had a significant correlation with biomass were tested for correlations with the other genes as described above.

## 2.4 Results

### 2.4.1 Growth conditions impact *C. albicans* biofilm formation

First, to determine the most appropriate medium for biofilm evaluation, an initial optimisation study was undertaken using biomass quantification using two laboratory strains, SC5314 and 3153A (Figure 2.1).



**Figure 2.1 - Optimisation of *C. albicans* biofilms.**

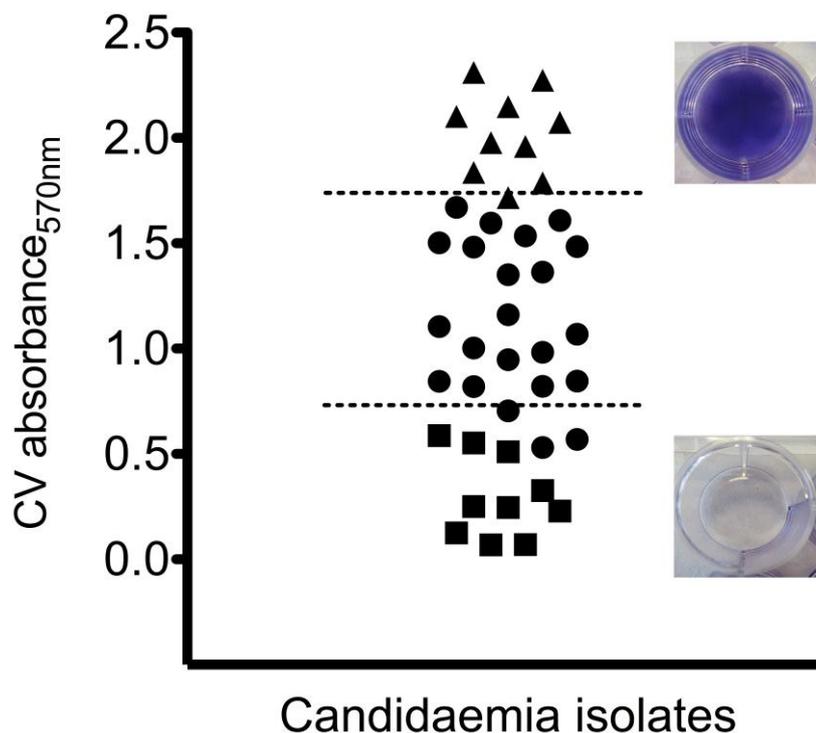
*C. albicans* SC5314 and 3153A biofilms were grown for 24, 48 and 72 h in RPMI-1640, YPD + 10% FCS, YNB + 100mM glucose and Spider media. Three replicates for each isolate were used and carried out on two separate occasions. Data represents mean  $\pm$  SEM. Significant differences were observed when comparing RPMI-1640 to all other growth media at 24 h (<sup>SS</sup> $P < 0.005$ , <sup>SSS</sup> $P < 0.0001$ ), 48 h (<sup>#</sup> $P < 0.005$ , <sup>###</sup> $P < 0.0001$ ) and 72 h (<sup>†</sup> $P < 0.05$ , <sup>†††</sup> $P < 0.0001$ ). Significant differences were also found between periods of biofilm development within each growth media, except RPMI-1640 (<sup>\*</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$ ).

*C. albicans* biofilms were shown to differ greatly in their biomass depending on the media used. YNB + 100mM glucose was the least effective medium for biofilm growth, as biomass was 5.5 - 7-fold reduced at all time points when compared to biofilms grown in RPMI-1640 ( $p < 0.0001$ ). YPD + 10% FCS was the second poorest medium for developing biofilms as biomass was 2.7, 2 and 2.3-fold less at 24, 48 and 72 h, respectively, than those developed in RPMI-1640

( $p < 0.0001$ ). Finally, despite Spider medium being able to develop moderate biofilms, it was the only growth media where the age of biofilm development played a significant role in biomass. Biofilms grown in Spider medium for 48 h had 30% greater biomass than those grown for 24 h ( $p < 0.05$ ). Based on this data, RPMI-1640 was identified as the most appropriate growth medium for the development of *C. albicans* biofilms and was therefore used throughout the remainder of this study.

#### 2.4.2 *C. albicans* clinical isolates exhibit heterogeneous biofilm formation

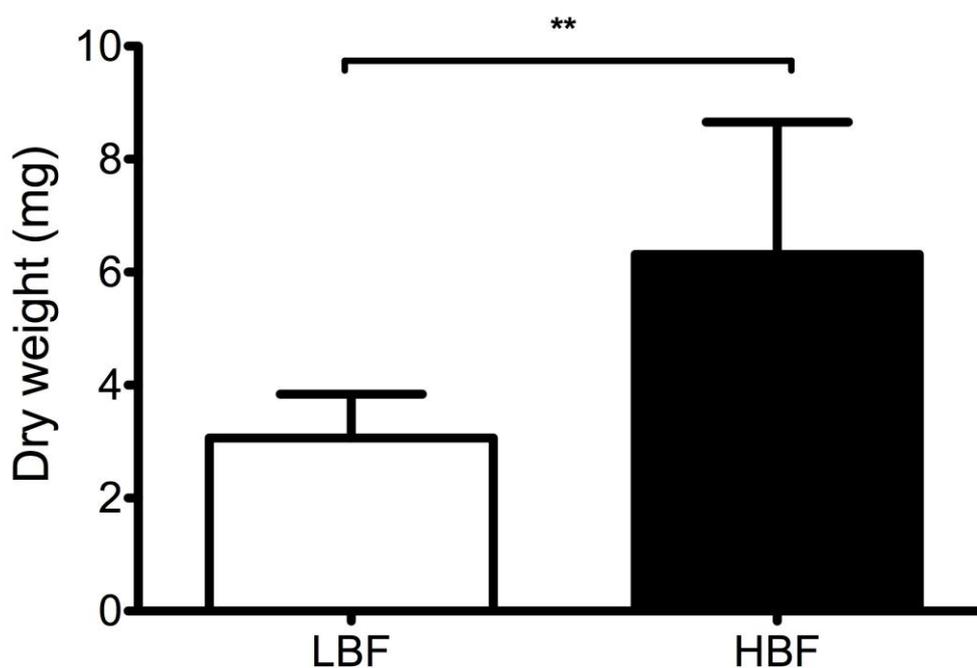
*C. albicans* bloodstream isolates displayed heterogeneity with respect to their biofilm biomass when grown in RPMI-1640 (Figure 2.2), the medium shown to support the optimal growth of *C. albicans* over various stages of biofilm development and allowed for hyphae development.



**Figure 2.2 - *C. albicans* clinical isolates form differential biofilms.**

Forty-two *C. albicans* bloodstream isolates were used to evaluate biofilm formation of strains derived from a clinical setting. Eight replicates were used for each isolate, which was carried out on two separate occasions, with the mean of each represented. *C. albicans* isolates with LBF (square), HBF (triangle) and IBF (circle) were defined by the upper and lower quartiles, as shown by CV stained biofilms.

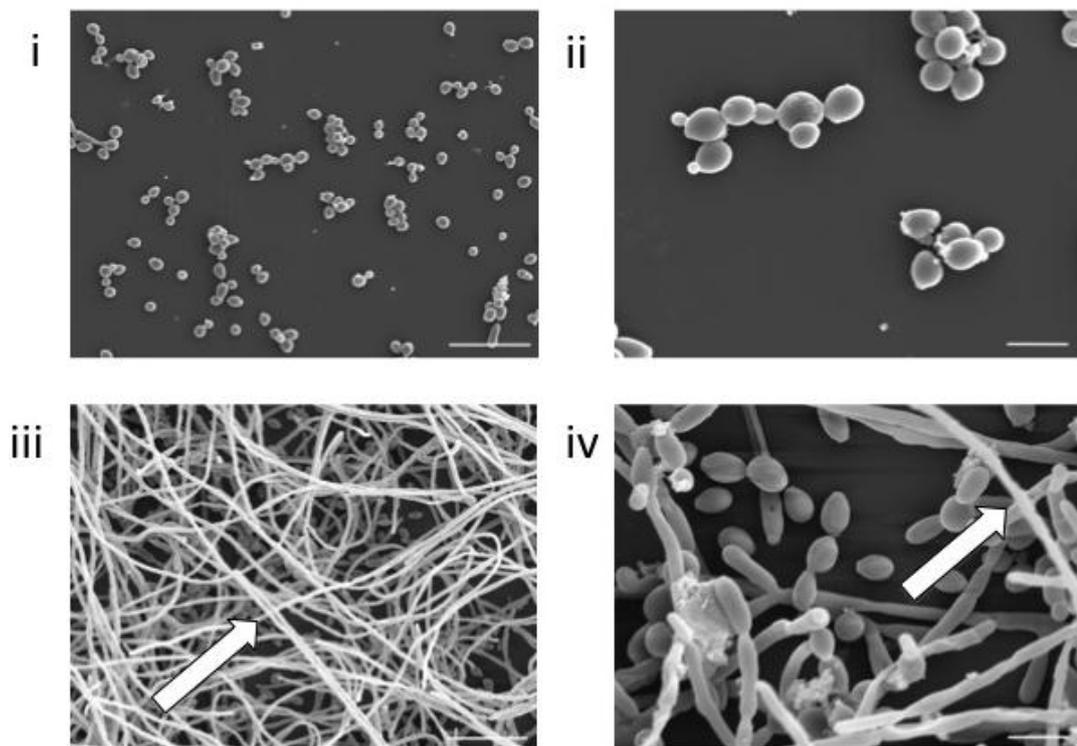
Isolates were categorised with LBF or HBF if their biomass absorbance were less than the first quartile ( $Q_1$   $OD_{570} = 0.565$ ) or greater than the third quartile ( $Q_3$   $OD_{570} = 1.682$ ), respectively. Those isolates in between the first and third quartile ( $Q_1$ - $Q_3$ ) were defined with intermediate biofilm formation (IBF). When HBF were stained with CV, the extent of the biofilm formation was observed macroscopically, where the bottom of the well was clearly covered with cellular biomass (Figure 2.2). In contrast, minimal staining was retained on isolates classed as LBF, as demonstrated by the well remaining almost colourless. Further confirmation of differences in biofilm biomass was then confirmed using dry weight measurements (Figure 2.3).



**Figure 2.3 - Dry weight is greater in *C. albicans* isolates with HBF.**

Biofilms were grown for 24 h and dry weight was measured for three *C. albicans* isolates with LBF and HBF, in triplicate on three separate occasions. Data represents mean  $\pm$  SD with significance  $**p < 0.005$ .

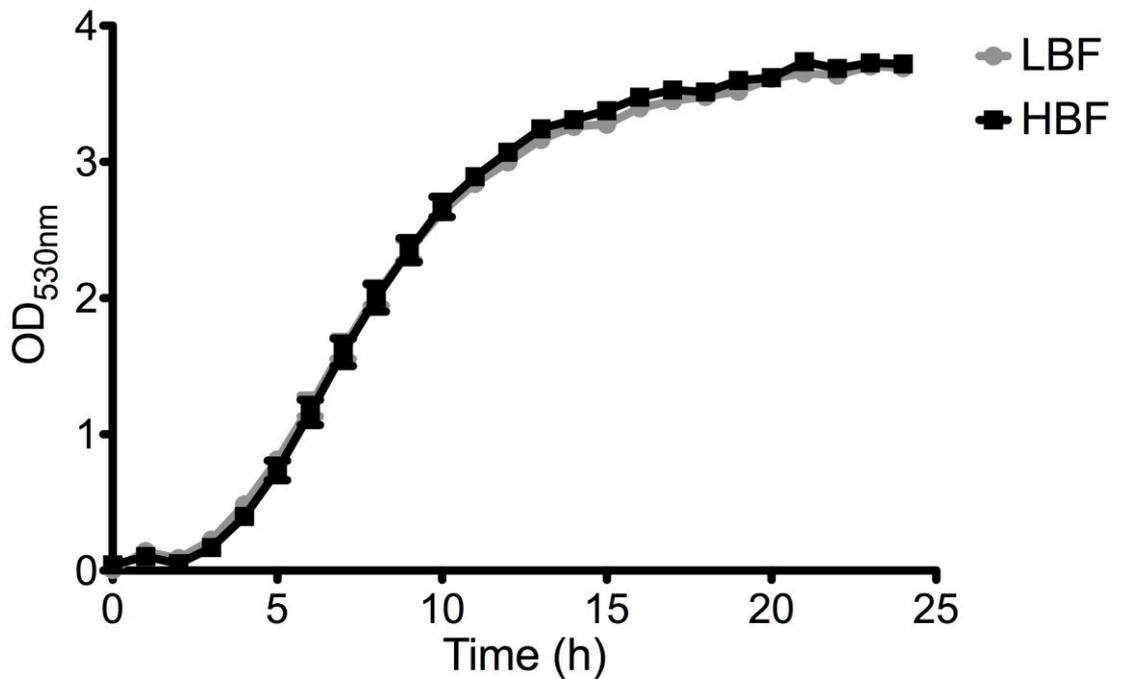
The dry weight of the *C. albicans* subsets were shown to differ significantly, where isolates with HBF had 2-fold greater compared to strains with LBF ( $p = 0.0023$ ), confirming our previous biomass observations. These differences are clearly evident when viewed under SEM (Figure 2.4).



**Figure 2.4 - *C. albicans* clinical isolates have differences in morphology.**

A panel of *C. albicans* LBF (i, ii) and HBF (iii, iv) were grown on Thermanox™ coverslips for 24 h at 37°C. Note the abundance of hyphae in HBF as denoted by arrows, which is lacking in LBF. Scale bars represent 20 µm and 5 µm for 1000 × (i, iii) and 3000 × (ii, iv) magnifications, respectively. Representative images were taken from 10 independent fields and of three isolates with LBF and HBF.

One obvious observation under microscopic viewing was the lack of hyphal cells in isolates with LBF (Figure 2.4i and ii), with biofilms mainly consisting of yeast cells. In contrast, *C. albicans* isolates with HBF were highly filamentous with a multi-dimensional structure with very few yeast cells (Figure 2.4iii and iv), illustrating a definite difference microscopically between these two biofilm subsets. These images confirmed our initial studies that identified differential biofilm formation existed between *C. albicans* clinical isolates at a phenotypic level. The next stage was to determine whether biofilm heterogeneity was simply related to defective growth (Figure 2.5).



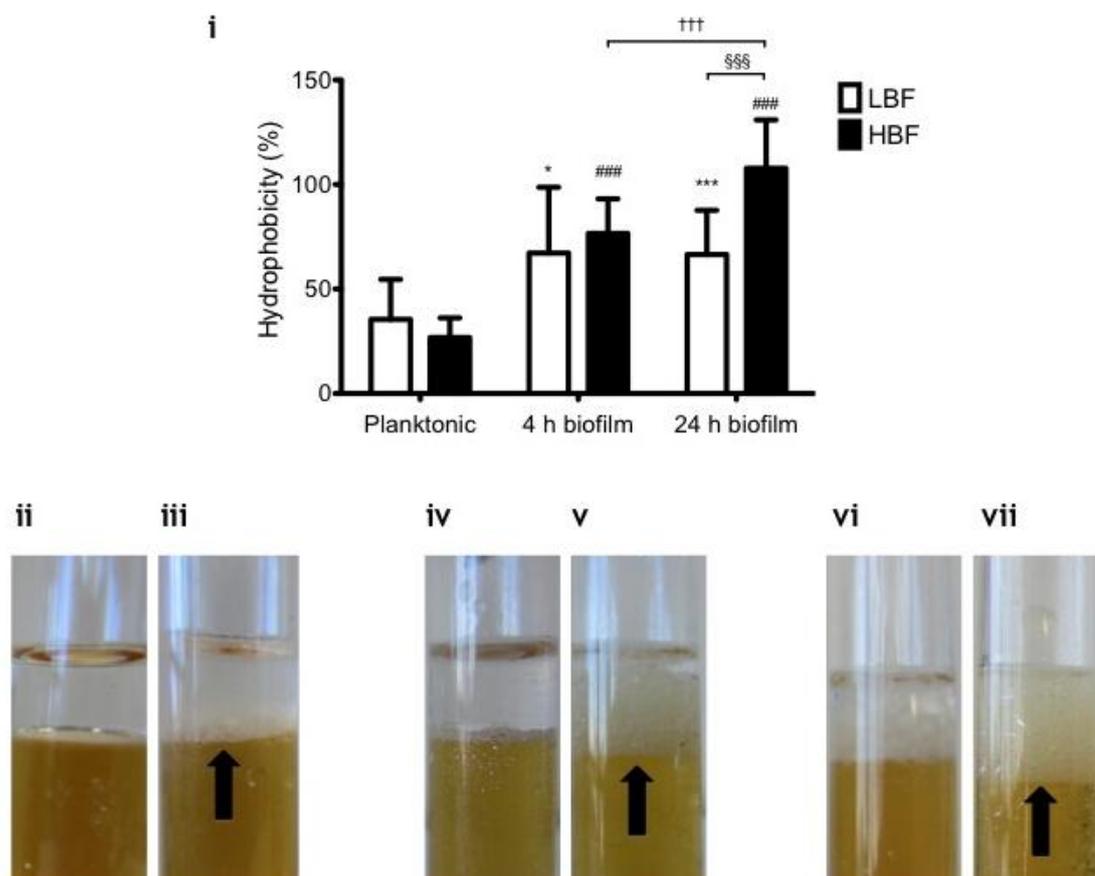
**Figure 2.5 - Variation in *C. albicans* biofilm formation is independent of growth kinetics.**

The growth kinetics of *C. albicans* isolates with LBF (grey circle) and HBF (black square) was assessed over 24 h, with absorbance read at 530 nm every hour. Isolates with LBF (n=10) and HBF (n=10) were grown in duplicate, on three separate occasions. Data represents mean  $\pm$  SEM.

Figure 2.5 shows no significant differences exist between *C. albicans* isolates with LBF and HBF at each time point ( $p > 0.05$ ). Therefore, variation in biofilm biomass in clinical strains is independent of their growth kinetics.

### 2.4.3 Biofilm phenotype is affected by cell surface hydrophobicity

The CSH of LBF and HBF isolates was quantified to determine whether it played a role in biofilm forming ability (Galan-Ladero et al., 2013). Figure 2.6 illustrates that hydrophobicity of an isolate significantly alters its ability to form a biofilm.



**Figure 2.6 - CSH influences *C. albicans* biofilm formation.**

Ten *C. albicans* isolates with LBF and HBF were assessed for their CSH at various stages of biofilm development (i). A visual representation CSH was shown for planktonic LBF (ii) and HBF (iii), 4 h LBF (iv) and HBF (v) and 24 h LBF (vi) and HBF (vii). Note the cloudy upper layer denoted by arrows showing hydrophobic cells. Each isolate was measured on two independent occasions. Data represented mean  $\pm$  SD. Significant differences between LBF and HBF were observed when 4 and 24 h biofilms were compared to their planktonic counterparts (\* $P < 0.05$ , \*\*\* $P < 0.0001$ , ### $P < 0.0001$ ). Furthermore, significant differences were found between 4 and 24 h in HBF (††† $P < 0.0001$ ) and between LBF and HBF at 24 h (<sup>§§§</sup> $P < 0.0001$ ).

Figure 2.6i shows the cellular state of *C. albicans* significantly alters the hydrophobicity, as CSH increased in isolates with LBF by ~30% in 4 ( $p < 0.05$ ) and 24 h ( $p < 0.0005$ ) biofilms, when compared to planktonic counterparts. This trend was also found in isolates with HBF where CSH increased by 50% in 4 h ( $p < 0.0001$ ) biofilms and 81% in 24 h ( $p < 0.0001$ ) biofilms, when compared to planktonic equivalents. Furthermore, CSH was significantly increased by 31% in isolates with HBF, when comparing 4 and 24 h biofilms ( $P < 0.001$ ). Of greater importance, differences in CSH between isolates with LBF and HBF were compared to determine if hydrophobicity influenced biofilm formation. Here it was shown that CSH increased significantly by 41% in isolates with HBF compared to those with LBF at 24 h ( $p < 0.0001$ ). However, no differences were observed at

Ch.2 *C. albicans* biofilm heterogeneity is associated with resistance and pathogenicity planktonic ( $p=0.2162$ ) or early biofilm stages ( $p=0.683$ ) between isolates with LBF and HBF.

Figure 2.6 ii-vii shows a visual representation of CSH in *C. albicans* isolates with LBF and HBF. Planktonic cells of LBF (ii) and HBF (iii) both had a relatively transparent upper layer, similar to 4 h biofilms in the LBF group (iv). However, it was shown at 4 h that isolates with HBF (v) had an opaque layer on top of the mixture, as indicated by arrows. Furthermore, 24 h biofilms of LBF (vi) and HBF (vii) both had a turbid opaque upper layer, which was more apparent in the HBF and corresponded to the level of CSH previously measured in Figure 2.6i.

#### ***2.4.4 Antifungal activity is impacted by biofilm phenotype***

Planktonic and sessile antifungal susceptibility testing was undertaken on all *C. albicans* clinical isolates ( $n=42$ ) using two polyenes (AmBi, AMB) and two echinocandins (CSP, MFG) to initially understand how active these agents were against clinical strains. Table 2.2 summarises these findings.

Table 2.2- Susceptibility of *C. albicans* clinical isolates to four antifungal agents

| Minimum Inhibitory Concentrations (MICs) |              |             |               |              |               |                |                     |             |
|--|--------------|-------------|---------------|--------------|---------------|----------------|---------------------|-------------|
| n = 42                                   | AmBi (mg/L)  |             | AMB (mg/L)    |              | CSP (mg/L)    |                | MFG (mg/L)          |             |
|  | PMIC*        | SMIC<br>80% | PMIC          | SMIC<br>80%  | PMIC          | SMIC<br>80%    | PMIC                | SMIC<br>80% |
| <b>Range**</b>                           | 0.125 –<br>1 | 0.5 –<br>32 | 0.25 –<br>0.5 | 0.25 –<br>32 | 0.25 –<br>0.5 | 0.125 –<br>>16 | <0.0156 –<br>0.0625 | 1 –<br>>16  |
| <b>MIC<sub>50</sub></b>                  | 0.5          | 8           | 0.5           | 4            | 0.25          | >16            | 0.0313              | >16         |
| <b>MIC<sub>90</sub></b>                  | 1            | 32          | 0.5           | 16           | 0.5           | >16            | 0.0313              | >16         |

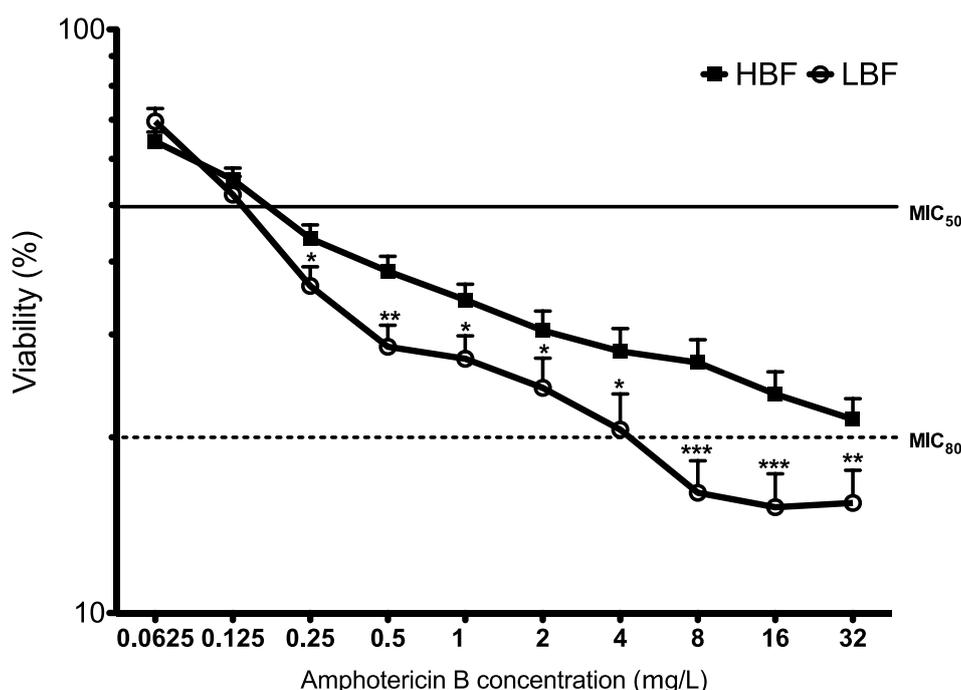
\*PMIC = Planktonic minimum inhibitory concentration, SMIC = Sessile minimum inhibitory concentration

\*\*MIC range of all isolates

SMIC<sub>80</sub> = 80% inhibition of isolate

MIC<sub>50/90</sub> = % of isolates susceptible to the antifungal concentration

All planktonic *C. albicans* isolates tested were susceptible to all four antifungal agents where MFG was the most sensitive compound with MIC<sub>90</sub> of 0.0313 mg/L for MFG, followed by CSP and AMB at 0.5 mg/L and AmBi at 1 mg/L (Table 2.2). When the sessile activity of each compound was compared only AmBi and AMB were effective at killing all *C. albicans* biofilms giving a MIC<sub>90</sub> of 32 and 16 mg/L, respectively. In contrast, the echinocandin agents showed inferior activity against biofilms as the MIC<sub>50</sub> for both compounds was >16 mg/L, a concentration substantially greater than what has been documented in the literature previously. Next, time kill analysis of sessile cells was performed on *C. albicans* isolates with LBF and HBF to determine if one group were more susceptible to treatment, using AMB which was the most effective antifungal overall (Figure 2.7).



**Figure 2.7 - Amphotericin B sensitivity is significantly impacted by biofilm formation.**

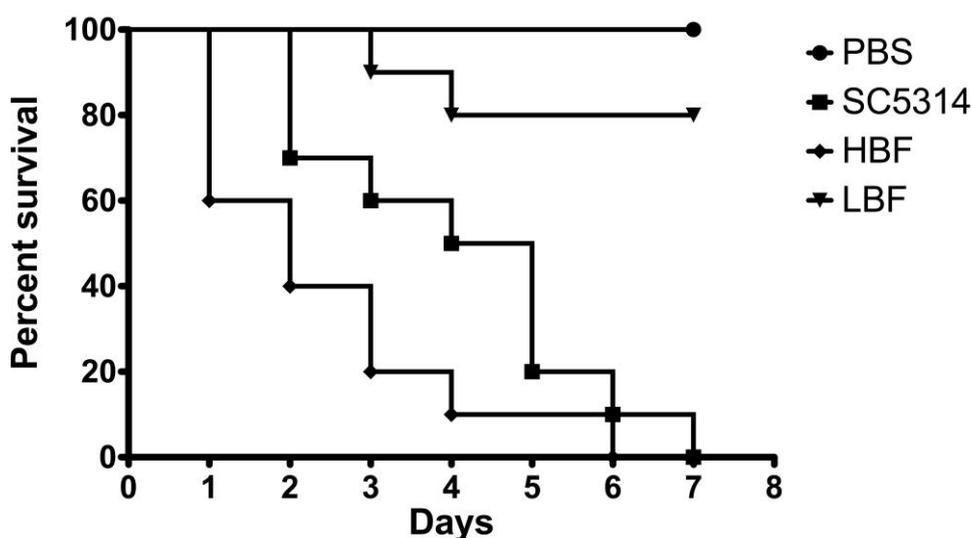
Biofilms of ten isolates with LBF (circle) and HBF (square) were grown for 24 h and tested against AMB. Each isolate was tested in duplicate, on three separate occasions with data represented by mean  $\pm$  SEM. Viability as determined using the XTT assay. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

A dose-dependent effect was evident in all isolates tested with AMB (Figure 2.7). Moreover, a significant difference was observed between LBF and HBF treated with 0.25 - 32 mg/L AMB ( $p < 0.05$ ). LBF and HBF isolates both had a MIC<sub>50</sub> of 0.25

mg/L AMB, yet isolates with LBF were significantly less viable than those with HBF at this concentration ( $p=0.0307$ ). In addition, LBF isolates suffered an ~80% kill at a concentration of 4 mg/L, whereas HBF required 32 mg/L to reach the same kill.

#### 2.4.5 *In vivo* pathogenicity is affected by biofilm phenotype

Next, the impact of the isolates ability to form biofilms based upon the severity of infection was analysed using a previously described *G. mellonella* model (Figure 2.8). Here isolates with LBF ( $n=3$ ) and HBF ( $n=3$ ) were compared to the reference strain SC5314.

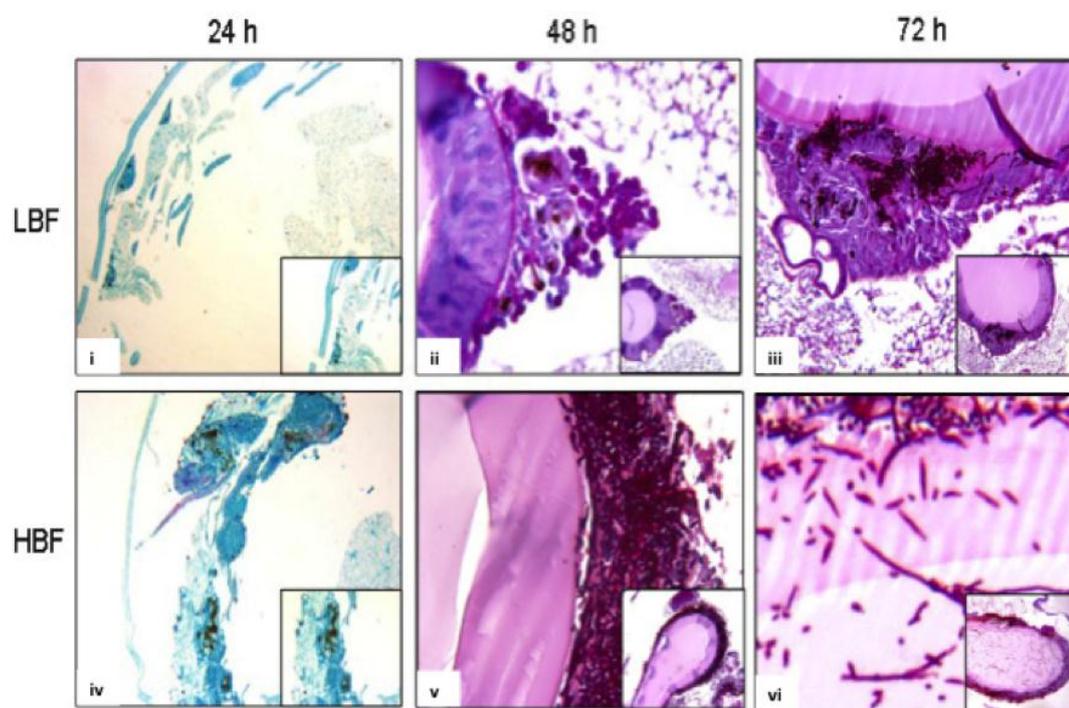


**Figure 2.8- *C. albicans* with HBF significantly impacts mortality.**

Larvae of *G. mellonella* were infected with *C. albicans* isolates with LBF ( $n=3$ ), HBF ( $n=3$ ) or SC5314 and pathogenicity assessed using a Kaplan-Meier plot, with percentage survival monitored over 7 days. Three strains with LBF and HBF contained 10 larvae and was repeated on two independent occasions, with the mean of each group represented in the survival plot.

The average rate of killing by HBF ( $n=3$ ), LBF ( $n=3$ ) and a reference strain (SC5314) of *C. albicans* were calculated to plot a survival curve (Figure 2.8). Survival data showed a significant difference in the killing of larvae between HBF and LBF ( $p<0.0001$ ). After 2 and 6 days, respectively, >50% and 100% larval death was recorded for HBF isolates, whereas larvae infected with LBF only achieved 20% killing after 7 days challenge. The reference strain SC5314 achieved 50% and 100% larval death by day 4 and 7, respectively. Similar kill rates to that of HBF were observed in SC5314, however, when compared to LBF there was a significant difference in larval mortality ( $p=0.0005$ ). Next, host-pathogen

interactions in this model were investigated by microscopically observing the morphology of the infected larvae at 24, 48 and 72 h post-infection with *C. albicans* HBF and LBF (Figure 2.9).



**Figure 2.9- *C. albicans* phenotype differentially impacts morbidity *in vivo*.**

Infected larvae were formalin fixed and sectioned for histology analysis. At 24 h, LBF (Feulgen staining, 20× original magnification (o.m.); inset: 4× o.m.) (i) and HBF (20× o.m.; inset: 10× o.m) (iv) were stained using Feulgen, whereas 48 h LBF (20× o.m.; inset: 10× o.m.) (ii) and HBF (40× o.m.; inset: 10× o.m.) (v) and 72 h LBF (20× o.m.; inset: 10× o.m.) (iii) and HBF (40× o.m.; inset: 10× o.m.) (vi) were stained using PAS. Images are representative of two isolates with LBF and HBF, repeated on two independent occasions.

At 24 h in both the LBF (Figure 2.9i) and HBF (Figure 2.9iv) groups, the nodule formation and melanin deposition were mainly observed under the cuticle and in the fat body, with mild to strong melanisation observed in the centre of the nodules, together with the presence of yeast cells and/or hyphae. The LBF nodules were smaller in dimension and dispersed mainly in the sub-cuticle area (Figure 2.9i), whereas the HBF nodules had a stronger melanisation with the tendency to converge in large aggregates, and were localised more deeply within the fat body (Figure 2.9iv). At 48 h, the LBF were confined to the external part of the visceral organs, with a spot-like distribution (Figure 2.9ii), whereas the HBF were found to display a pronounced filamentation all around the intestinal wall, with a PAS positive matrix visible surrounding the hyphae (Figure 2.9v). Furthermore at 72 h, there was a substantial invasion of both the

Ch.2 *C. albicans* biofilm heterogeneity is associated with resistance and pathogenicity gastrointestinal tract and the tracheal system with damaged gut epithelium, where yeast and hyphal cells both observed in the HBF infection (Figure 2.9vi). In contrast, a segmental invasion of the intestinal wall (Figure 2.9iii) was observed with LBF infection and the progression of the infection was to a lesser extent than that by the HBF. Moreover, Table 2.3 summarises the localisation and characterisation of the nodules with LBF and HBF infected larvae. Changes in the fat body morphology and composition including vacuolisation and haemocyte recruitment were detected during the course of the infection and were more evident in the HBF group.

**Table 2.3- Characteristics and localisation of nodules found in infected *G. mellonella* larvae**

|            |       | Nodules      |               |            |                   |        |              |    |         |    |
|------------|-------|--------------|---------------|------------|-------------------|--------|--------------|----|---------|----|
|            | Size  | Melanisation | Encapsulation | Confluence | Fungal morphology |        | Localisation |    |         |    |
|            |       |              |               |            | Yeast             | Hyphae | SC           | FB | PI      | PT |
| <b>LBF</b> | Small | +            | +             | -          | +++               | +      | +            | +  | ++      | -  |
| <b>HBF</b> | Large | ++, +++      | +++           | +          | +                 | +++    | ++           | ++ | ++<br>+ | +  |

SC: subcuticle, FB: fat body, PI: paraintestinal, PT: paratracheal

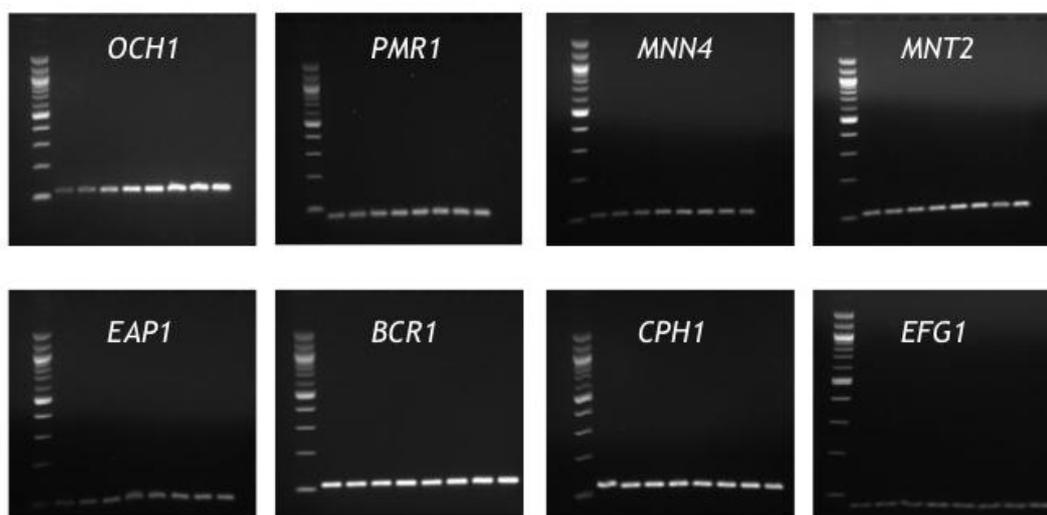
+ low, ++ medium, +++ high

### 2.4.6 *Transcriptional heterogeneity is associated with biofilm phenotype*

Before *C. albicans* clinical isolates could be assessed at a transcriptional level for their expression differences of genes associated with biofilm formation, a range of primer sets designed in house had to be validated.

#### 2.4.6.1 *C. albicans* qPCR primer optimisation

The primer sets designed in this study were shown to amplify the genes of interest at similar annealing temperatures, with a range of annealing temperatures for each primer set shown in Figure 2.10.



**Figure 2.10- Optimisation of *C. albicans* qPCR primers using gradient PCR.**

DNA extracted from *C. albicans* SC5314 was used to identify the most appropriate annealing temperature for each set of designed primers. Lane 1 contains 1000 bp hyperladder. Lanes 2-9 show the PCR products for annealing temperatures 63.0, 62.3, 61.2, 59.4, 57.3, 55.7, 54.6 and 54 °C, respectively.

The aim was to identify an annealing temperature that was able to amplify all genes of interest equally. All PCR products were amplified at 60 °C and therefore this was the annealing temperature selected for used in the remainder of the transcriptional studies. In addition to optimising the annealing temperature for the genes of interest, amplification efficiencies for each primer set were calculated for qPCR (Table 2.4).

**Table 2.4- Amplification efficiencies of primers designed for qPCR**

| <i>MNN4</i>       | Average Ct | Log (dilution) | <i>PMR1</i>       | Average Ct | Log (dilution) |
|-------------------|------------|----------------|-------------------|------------|----------------|
| 400 ng/ $\mu$ L   | 19.96      | 2.6            | 400 ng/ $\mu$ L   | 18.18      | 2.6            |
| 40 ng/ $\mu$ L    | 22.79      | 1.6            | 40 ng/ $\mu$ L    | 21.63      | 1.6            |
| 4 ng/ $\mu$ L     | 26.19      | 0.6            | 4 ng/ $\mu$ L     | 25.46      | 0.6            |
| 0.4 ng/ $\mu$ L   | 30.11      | -0.4           | 0.4 ng/ $\mu$ L   | 29.18      | -0.4           |
| 0.04 ng/ $\mu$ L  | 33.58      | -1.4           | 0.04 ng/ $\mu$ L  | 32.33      | -1.4           |
| <b>Slope</b>      |            | <b>-3.457</b>  | <b>Slope</b>      |            | <b>-3.585</b>  |
| <b>%</b>          |            | <b>95%</b>     | <b>%</b>          |            | <b>90%</b>     |
| <b>Efficiency</b> |            |                | <b>Efficiency</b> |            |                |
| <i>OCH1</i>       | Average Ct | Log (dilution) | <i>EFG1</i>       | Average Ct | Log (dilution) |
|                   |            |                | 400 ng/ $\mu$ L   | 18.91      | 2.6            |
| 40 ng/ $\mu$ L    | 24.56      | 1.6            | 40 ng/ $\mu$ L    | 22.08      | 1.6            |
| 4 ng/ $\mu$ L     | 27.84      | 0.6            | 4 ng/ $\mu$ L     | 26.35      | 0.6            |
| 0.4 ng/ $\mu$ L   | 34.12      | -0.4           | 0.4 ng/ $\mu$ L   | 28.80      | -0.4           |
| <b>Slope</b>      |            | <b>-3.211</b>  | <b>Slope</b>      |            | <b>-3.394</b>  |
| <b>%</b>          |            | <b>105%</b>    | <b>%</b>          |            | <b>97%</b>     |
| <b>Efficiency</b> |            |                | <b>Efficiency</b> |            |                |
| <i>MNT2</i>       | Average Ct | Log (dilution) | <i>CPH1</i>       | Average Ct | Log (dilution) |
| 400 ng/ $\mu$ L   | 18.16      | 2.6            | 400 ng/ $\mu$ L   | 18.57      | 2.6            |
| 40 ng/ $\mu$ L    | 21.27      | 1.6            | 40 ng/ $\mu$ L    | 21.71      | 1.6            |
| 4 ng/ $\mu$ L     | 25.50      | 0.6            | 4 ng/ $\mu$ L     | 25.49      | 0.6            |
| 0.4 ng/ $\mu$ L   | 28.20      | -0.4           | 0.4 ng/ $\mu$ L   | 28.47      | -0.4           |
| <b>Slope</b>      |            | <b>-3.465</b>  | <b>Slope</b>      |            | <b>-3.348</b>  |
| <b>%</b>          |            | <b>94%</b>     | <b>%</b>          |            | <b>99%</b>     |
| <b>Efficiency</b> |            |                | <b>Efficiency</b> |            |                |
| <i>EAP1</i>       | Average Ct | Log (dilution) | <i>BCR1</i>       | Average Ct | Log (dilution) |
| 400 ng/ $\mu$ L   | 17.94      | 2.6            | 400 ng/ $\mu$ L   | 20.91      | 2.6            |
| 40 ng/ $\mu$ L    | 20.95      | 1.6            | 40 ng/ $\mu$ L    | 23.56      | 1.6            |
| 4 ng/ $\mu$ L     | 24.78      | 0.6            | 4 ng/ $\mu$ L     | 26.62      | 0.6            |
| 0.4 ng/ $\mu$ L   | 27.54      | -0.4           | 0.4 ng/ $\mu$ L   | 29.51      | -0.4           |
| <b>Slope</b>      |            | <b>-3.262</b>  | <b>Slope</b>      |            | <b>-2.886</b>  |
| <b>%</b>          |            | <b>103%</b>    | <b>%</b>          |            | <b>122%</b>    |
| <b>Efficiency</b> |            |                | <b>Efficiency</b> |            |                |

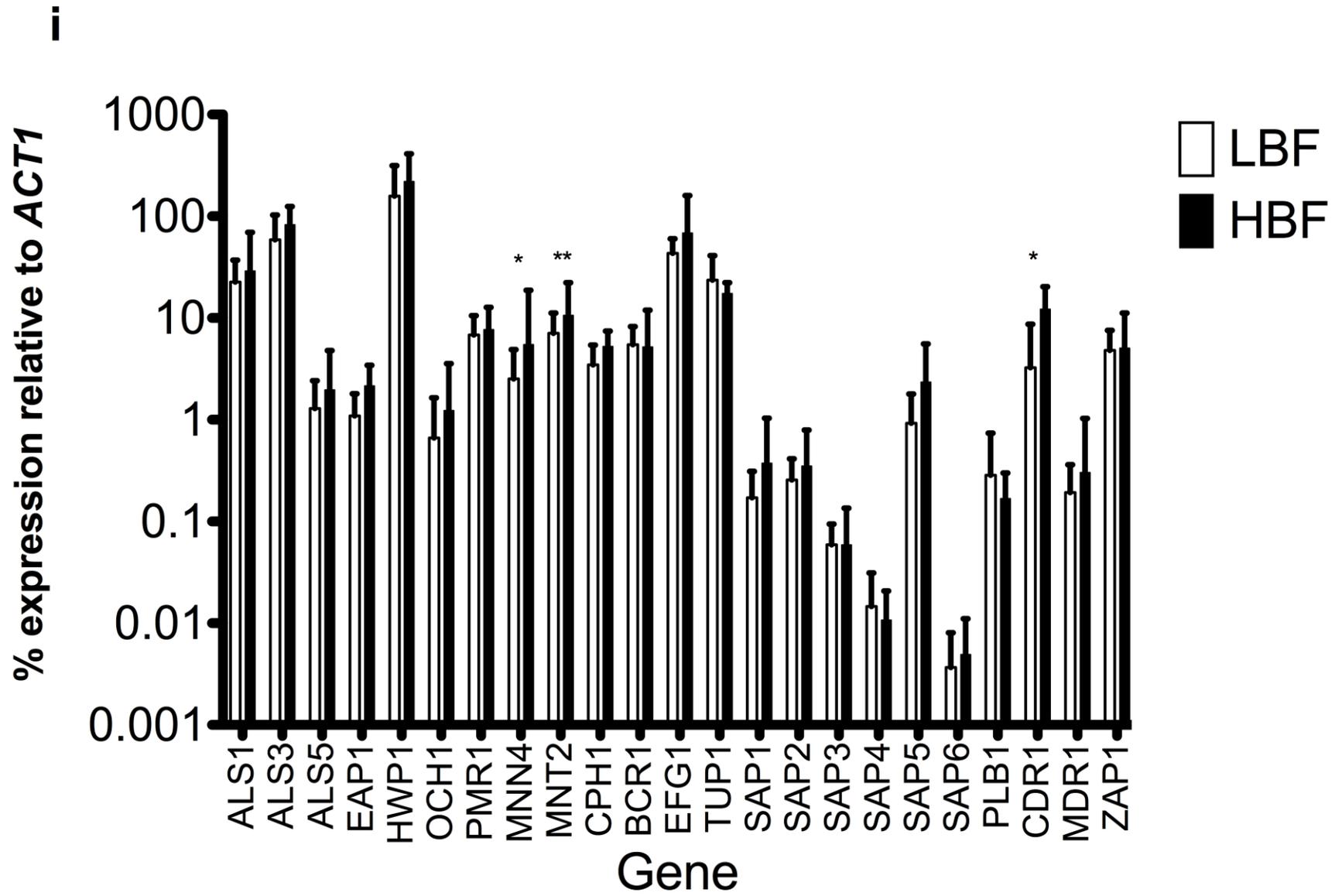
Each DNA template was then amplified to determine the efficiency of the primers. Table 2.4 shows the average Ct and log Ct of each DNA template, which was used to calculate the slope of the line, and in turn the efficiency of each primer set. The lowest concentration of 0.04 ng/μL was not amplified for most of the primer sets including *OCH1*, *EFG1*, *CPH1*, *EAP1*, *BCR1* and *MNT2*. In addition, the template did not amplify for *OCH1* at the highest concentration (400 ng/μL). Therefore these samples were excluded from the efficiency analysis.

#### 2.4.6.2 Differential expression of genes associated with *C. albicans* biofilm formation

*C. albicans* clinical isolates defined with LBF and HBF were further assessed at a transcriptional level and the expression of genes related to biofilm formation was investigated using 4 and 24 h biofilms. Percentage expression of all genes of interest normalised to *ACT1* are shown in Figure 2.11 and Table 2.5. *ACT1* was used as the housekeeping gene and was shown to be stably expressed throughout all biofilm conditions.

#### Figure 2.11- Genes associated with *C. albicans* biofilm development are up-regulated in HBF.

Ten *C. albicans* isolates with LBF and HBF were grown as biofilms for 4 (i) and 24 h (ii). Percentage of gene expression is shown as log<sub>10</sub> mean ± SD, relative to housekeeping gene *ACT1*. All strains were assessed in duplicate and included appropriate no RT and non-template controls. \* $p < 0.05$ , \*\* $p < 0.005$ .



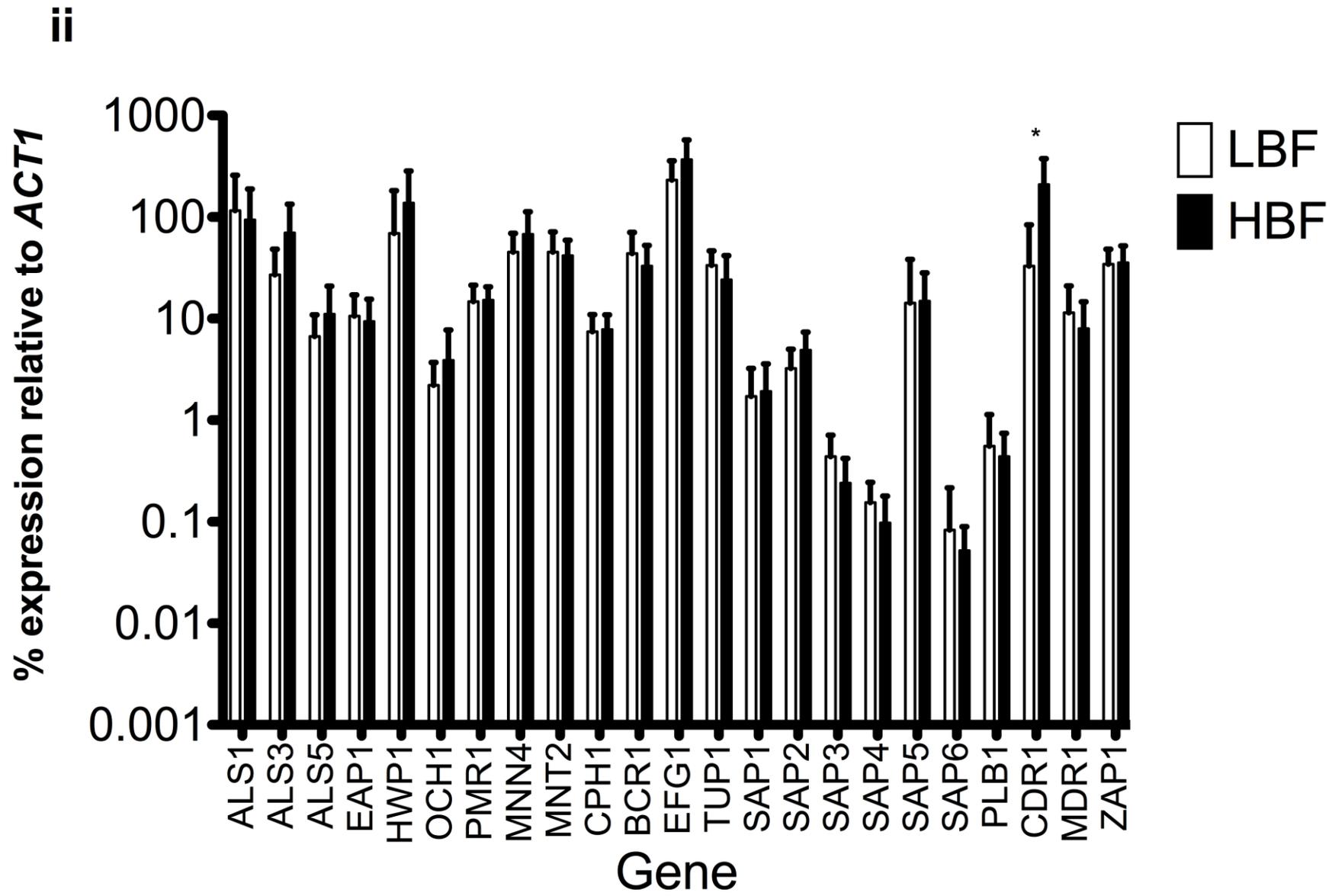
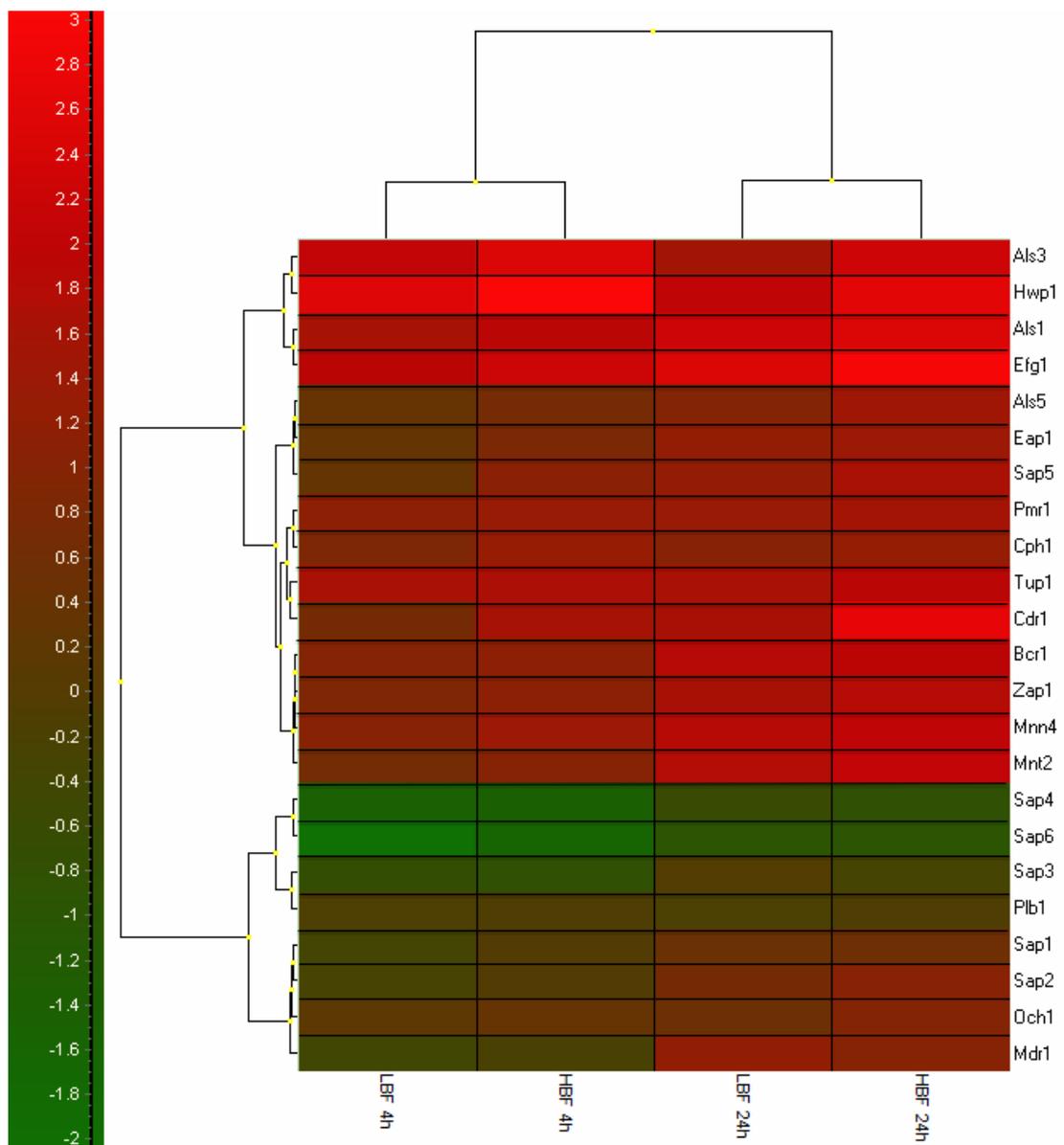


Figure 2.11 illustrates the levels of gene expression of LBF versus HBF at both (i) 4 and (ii) 24 h. Overall, the majority of the genes tested followed a trend of up-regulation in HBF compared to LBF. However, statistically significant differences were only observed in the glycosylated mannoproteins *MNN4* ( $p=0.0313$ ) and *MNT2* ( $p=0.0044$ ) at 4 h, where expression was increased by ~2 fold. Furthermore, the resistance gene *CDR1* was significantly increased in HBF by 4- and 6-fold at 4 h ( $p=0.0113$ ) and 24 h ( $p=0.0239$ ), respectively. Clustering the expression of 23 selected genes from 5 different functional groups in a heat map showed their relationship with one another and their variable expression in LBF and HBF over time (Figure 2.12).



**Figure 2.12- Clustering analysis identified the transcriptional relationship of biofilm specific genes.**

Percentage expression of each gene was also assessed by clustering and heat map analysis using GenEx software. Data was log transformed and mean values were used for heat map construction. Increased expression of genes is shown by red and a decrease is represented by green.

Here, it was found that the expression of the adhesion genes *ALS3* and *HWP1* were closely related, particularly in HBF isolates at 4 and 24 h. Furthermore, genes from different functional groups were closely related to one another irrespective of whether LBF or HBF, such as the proteinase *SAP5* and the adhesion genes *ALS5* and *EAP1*. The remaining *SAP* genes were all closely related to one another, and interestingly the resistance gene *MDR1* and the cell wall mannoprotein *OCH1*. Further analysis of *SAP3* showed an increase in

Ch. 2 *C. albicans* biofilm heterogeneity is associated with resistance and pathogenicity transcription within LBF at 24 h, despite no differences being observed at 4 h. In contrast, *SAP5* expression was consistently high at 4 and 24 h within HBF.

Analysis of Spearman rho coefficients identified that out of 23 selected genes, 7 genes, including those related to adhesion (*ALS3*, *EAP1*), filamentation (*CPH1*), hydrolytic enzymes (*SAP5*, *PLB1*) and resistance (*CDR1*, *ZAP1*) showed a significant positive correlation ( $p < 0.05$ ) with biomass data at 4 h (Table 2.6).

Table 2.5- *C. albicans* biomass correlates with biofilm gene expression at 4 h

| 4h Correlations <sup>a</sup> |      | <i>ALS3</i> | <i>EAP1</i> | <i>CPH1</i> | <i>SAP5</i> | <i>PLB1</i> | <i>CDR1</i> | <i>ZAP1</i> |
|------------------------------|------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| CV                           | Rho= | .529*       | .608**      | .534*       | .539*       | .483*       | .647**      | .515*       |
|                              | p =  | .029        | .010        | .027        | .026        | .050        | .005        | .035        |
| <i>ALS1</i>                  | Rho= | .240        | .529*       | .542*       | .336        | .608**      | .382        | .250        |
|                              | p =  | .353        | .029        | .025        | .188        | .010        | .130        | .333        |
| <i>ALS3</i>                  | Rho= | 1.000       | .385        | .708**      | .544*       | .593*       | .779**      | .365        |
|                              | p =  |             | .127        | .001        | .024        | .012        | .000        | .149        |
| <i>ALS5</i>                  | Rho= | .229        | .538*       | .644**      | .607**      | .681**      | .145        | .214        |
|                              | p =  | .378        | .026        | .005        | .010        | .003        | .579        | .410        |
| <i>EAP1</i>                  | Rho= | .385        | 1.000       | .544*       | .490*       | .860**      | .537*       | .279        |
|                              | p =  | .127        |             | .024        | .046        | .000        | .026        | .277        |
| <i>HWP1</i>                  | Rho= | .868**      | .338        | .821**      | .571*       | .532*       | .615**      | .201        |
|                              | p =  | .000        | .184        | .000        | .017        | .028        | .009        | .439        |
| <i>MNN4</i>                  | Rho= | .350        | .554*       | .645**      | .537*       | .672**      | .493*       | .309        |
|                              | p =  | .168        | .021        | .005        | .026        | .003        | .045        | .228        |
| <i>PMR1</i>                  | Rho= | .333        | .635**      | .527*       | .534*       | .784**      | .233        | .377        |
|                              | p =  | .191        | .006        | .030        | .027        | .000        | .368        | .135        |
| <i>BCR1</i>                  | Rho= | .262        | .250        | .539*       | .566*       | .485*       | .306        | .522*       |
|                              | p =  | .309        | .333        | .026        | .018        | .048        | .232        | .032        |
| <i>CPH1</i>                  | Rho= | .708**      | .544*       | 1.000       | .833**      | .748**      | .547*       | .321        |
|                              | p =  | .001        | .024        |             | .000        | .001        | .023        | .209        |
| <i>EFG1</i>                  | Rho= | .257        | .520*       | .576*       | .645**      | .605*       | .409        | .392        |
|                              | p =  | .319        | .033        | .016        | .005        | .010        | .103        | .119        |
| <i>TUP1</i>                  | Rho= | .713**      | .397        | .397        | .463        | .544*       | .684**      | .444        |
|                              | p =  | .001        | .115        | .115        | .061        | .024        | .002        | .074        |
| <i>SAP1</i>                  | Rho= | .356        | .523*       | .794**      | .744**      | .730**      | .251        | .413        |
|                              | p =  | .160        | .031        | .000        | .001        | .001        | .332        | .100        |
| <i>SAP2</i>                  | Rho= | .091        | .554*       | .478        | .417        | .654**      | .172        | .292        |
|                              | p =  | .729        | .021        | .052        | .096        | .004        | .510        | .256        |
| <i>SAP5</i>                  | Rho= | .544*       | .490        | .833**      | 1.000       | .676**      | .395        | .539*       |
|                              | p =  | .024        | .046        | .000        |             | .003        | .117        | .026        |
| <i>SAP6</i>                  | Rho= | .371        | .772**      | .637**      | .627**      | .869**      | .320        | .284        |
|                              | p =  | .143        | .000        | .006        | .007        | .000        | .211        | .269        |
| <i>PLB1</i>                  | Rho= | .593*       | .860**      | .748**      | .676**      | 1.000       | .532*       | .434        |
|                              | p =  | .012        | .000        | .001        | .003        |             | .028        | .082        |
| <i>CDR1</i>                  | Rho= | .779**      | .537*       | .547*       | .395        | .532*       | 1.000       | .262        |
|                              | p =  | .000        | .026        | .023        | .117        | .028        |             | .309        |
| <i>ZAP1</i>                  | Rho= | .365        | .279        | .321        | .539*       | .434        | .262        | 1.000       |
|                              | p =  | .149        | .277        | .209        | .026        | .082        | .309        |             |

\*Correlation is significant at the 0.05 level (2-tailed),

\*\* Correlation is significant at the 0.01 level (2-tailed).

a. List wise N = 17

Further analysis of the relationship between these seven genes and all the other genes tested presented various correlations (marked bold in Table 2.6). For example, *PLB1* was significantly correlated with all other genes tested (94.11%) except *ZAP1*, followed by *CPH1* (76.47%), *SAP5* (76.47%), *EAP1* (64.71%), *CDR1* (41.17%), *ALS3* (35.29%) and *ZAP1* (11.76%). Correlation of individual genes with one another at the 4 h time point showed that *PLB1*, *CPH1*, *MNN4* and *HWP1* all correlated with 5 of the 7 key genes defined above. Notably, 24 h gene expression revealed very few significant correlations other than CV (Table 2.7). In fact, a significant negative correlation was found between *SAP3* and the biomass data (Rho = -0.465, p=0.045). Furthermore, *SAP3* was positively correlated with *MNT2* (Rho = 0.468, p=0.043) and *Sap4* (Rho = 0.460, p=0.048).

**Table 2.6- *C. albicans* biomass correlates with biofilm gene expression at 24 h**

| 24 h Correlations <sup>b</sup> |      | <i>SAP3</i>  |
|--------------------------------|------|--------------|
| CV                             | Rho= | <b>.465*</b> |
|                                | p =  | <b>0.045</b> |
| <i>MNT2</i>                    | Rho= | <b>.468*</b> |
|                                | p =  | <b>0.043</b> |
| <i>SAP4</i>                    | Rho= | <b>.460*</b> |
|                                | p =  | <b>0.048</b> |

\* Correlation is significant at the 0.05 level (2-tailed),

\*\* Correlation is significant at the 0.01 level (2-tailed).

b. List wise N = 19

## 2.5 Discussion

The presence of medical devices, such as central venous catheters (CVCs), are known to be important risk factors for developing candidaemia (Gamaletsou et al., 2014), suggesting that biofilm formation plays a critical role. The past decade has seen a significant leap in our knowledge and understanding of the biology of *C. albicans* biofilms, particularly with respect to the molecular basis of their development and homeostasis (Blankenship and Mitchell, 2006). However, in the clinical setting it is generally assumed that all *C. albicans* isolates have the capacity to form biofilms, but often with little regard to individual differences within the species when managing the infection. Here it was demonstrated that *C. albicans* display heterogeneous biofilm characteristics, and these strain differences have important implications with respect to treatment and pathogenicity.

Previous studies have reported very defined categories in their analysis of association with clinical outcomes, i.e. biofilm formers and non-biofilm formers (Tumbarello et al., 2012, Tumbarello et al., 2007). However, these important studies fail to take into account the heterogeneous nature of individual clinical isolates forming biofilms, which based on their metabolic XTT values can range from 0.125 to 1.358 (Tumbarello et al., 2012). When looking for clinical correlations with these phenotypes important information can be missed, as the isolates at either end of the biofilm forming spectrum may lead to different clinical outcomes. For example, use of the XTT metabolic assay as quantitative measure of biomass has been shown to have pitfalls (Alnuaimi et al., 2013, Kuhn et al., 2003, Kuhn et al., 2002a). Kuhn and colleagues explored the limitations of this assay and demonstrated that although it could be used for analysing and comparing parameters of an individual strain, *Candida* spp. clinical isolates can differentially metabolise XTT and therefore and it cannot be assumed that the colorimetric signal produced is correlated with cell number (Kuhn et al., 2003). Moreover, the authors previously demonstrated non-invasive *Candida* strains had increased XTT activity when compared to invasive isolates and that significant differences were observed between XTT activity and dry weight (DW) measurements, concluding techniques that measure direct biomass instead of metabolic activity are more appropriate for comparing strains to one another (Kuhn et al., 2002a). It was hypothesised that isolates could shift their

metabolism away from routine functions and those with LBF could in fact have increased metabolic activity. Therefore, careful consideration must be taken with the conclusions drawn from studies using reference strains, as these may not relate directly to the observations found *in vivo* with regards to biofilm formation.

Another technique that could potentially be used to determine biofilm biomass is SYTO<sup>®</sup> 9, a nucleic acid stain shown previously to quantify both bacterial and fungal cells (Honraet et al., 2005, Peeters et al., 2008, Jin et al., 2005), however, due to cost limitations this assay is not suitable for large scale screening of isolates. In this current study, CV was selected as the method to determine *C. albicans* biofilm formation, as this stain was shown to have high reproducibility and is relatively inexpensive and straightforward to use, as has been described elsewhere (Peeters et al., 2008). Although this assay was able to distinguish groups with LBF and HBF, its sensitivity between strains of similar biofilm forming ability is limited.

Initially, biofilms were categorised grown in RPMI using a biomass stain (Jose et al., 2010) and confirmed by dry weight analysis, which differentiated clinical isolates into defined groupings. This approach was used in preference to metabolic assays due to the highly variable nature of XTT from strain to strain (Tumbarello et al., 2012). Moreover, the usefulness of the XTT assay is limited to antifungal drug testing of biofilms (Nett et al., 2011a, Pierce et al., 2008). This classification made in this study, based initially on biomass, was supported by observations on a macro- and microscopic level where it was clear that numerous cells consisting of hyphae and yeasts were visible in HBF, whereas scant layers of yeast cells were observed for LBF. Growth kinetics was performed and excluded isolate growth rate from being responsible for variation in biofilm formation. CSH was also investigated as an additional biofilm promoting feature, as previous studies have also shown a link between biofilm biomass and CSH (Blanco et al., 2010, Galan-Ladero et al., 2013). This study confirms that CSH impacts different phases of biofilm development, which is in agreement with previous work where it was shown that cells dispersed from mature biofilms were more hydrophobic than those dispersed from earlier stages of biofilm development (Bujdaková et al., 2013). Furthermore, it has been shown hydrophobic cells are more adherent (Perez et al., 2011), and therefore it is

unsurprising that CSH was increased in HBF isolates. Based on this overall approach to biofilm categorisation there is confidence in the phenotypes selected for further detailed analysis. It is however conceded that there are caveats to defining levels of biofilm, and this requires further work and collaboration between groups to establish a standardised method.

One of the key defining features of *C. albicans* biofilms is their insensitivity to sterol active antifungal agents (Ramage et al., 2012c). Notably, AMB was less effective against HBF biofilms than LBF, which was hypothesised due to the inability of the compound to permeate easily throughout the dense physical structure of the cells encased within ECM (Nett et al., 2010a). AZL were not used in the study due to their resistance towards *C. albicans* biofilms. Moreover, ECN were purposely excluded from this study as these have been shown to be an effective anti-biofilm antifungals, therefore quantifying differences in activity against the two populations would be difficult (Bachmann et al., 2002). Moreover, due to their paradoxical effect, then these would not be appropriate to identify subtle differences in antifungal activity (Stevens et al., 2005, Walraven et al., 2014). The observations from this study may have implications to whether a patient responds to antifungal therapy, as Tumbarello and colleagues (2012) demonstrated that inadequate antifungal therapy (azoles) and the presence of an indwelling venous catheter were key predictors of patient mortality and hospital length of stay in patients infected with biofilm forming isolates (Tumbarello et al., 2007). Guidelines have also suggested that removal of the catheter is an important factor in improving clinical outcomes, again supporting the notion that biofilm formation has a crucial role in clinical outcomes (Andes et al., 2012, Cornely et al., 2012). Given the importance of these infections, efficient and appropriate treatment in candidaemia cases has been highlighted (Almirante et al., 2005, Garey et al., 2006), as failure to treat quickly and effectively has profound consequences on mortality statistics (Kollef et al., 2012).

To this end the pathogenicity of clinical isolates capable of forming robust biofilms was investigated in comparison to non-biofilm forming isolates, which was hypothesised as a potential reason for their apparent role in infections with increased mortality (Tumbarello et al., 2012). Previous experimental work has shown that cells dispersed from biofilms are more cytotoxic and more rapidly

lethal in murine models than the equivalent planktonic cells (Uppuluri et al., 2010). Using a *G. mellonella* model it was shown that HBF isolates caused significantly greater mortality rates than LBF isolates, a finding supported elsewhere (Cirasola et al., 2013). In addition, it was previously reported that the virulence of *C. albicans* isolates was shown to vary with the levels of biofilm formation, and that mice infected with a LBF had increased survival rates compared to isolates that were infected with HBF (Hasan et al., 2009). Furthermore, strains known to be more virulent have been shown to contribute to disease severity and induce greater inflammatory responses *in vivo*, thought to be more damaging towards the host than protective (MacCallum et al., 2009a). Histological analysis of the infected larvae displayed similar cell morphology of yeast and filamentous hyphae as observed in SEM images of LBF and HBF, respectively. This is in agreement with a previous study that showed filamentation plays a role in killing *G. mellonella* larvae (Fuchs et al., 2010). A number of animal models are currently used to assess the virulence of *Candida*, with mouse and rat models being the most popular choice for both mucosal and invasive infections (Maccallum, 2012, Nett et al., 2010b, Rahman et al., 2007). Therefore, it would be ideal to utilise such models for assessing the impact of isolates with LBF and HBF *in vivo*.

Filamentous growth is a characteristic feature of *C. albicans* biofilm formation. Defective hyphal formation through deletion of *EFG1* has been shown to lead to low levels of biofilm growth (Ramage et al., 2002d). Given the growing knowledge of key biofilm related genes it was decided to investigate transcriptional changes to determine whether these are truly represented amongst clinical isolates, and therefore could be used as a more robust way to categorise biofilm formation and as potential diagnostic targets of HBF isolates. *ACT1*, the stably expressed housekeeping gene, as reported elsewhere (Lepak et al., 2006, Nailis et al., 2006, Nett et al., 2009), enabled these relative comparisons. Regulation of biofilm related genes were shown to influence an isolates biomass within clinical isolates, echoing work carried out by other groups (Nailis et al., 2010a, Uppuluri et al., 2009b). Cluster analysis of the selected biofilm related genes showed a good association with functional classes of genes, such as adhesins and proteinases, suggesting that both LBF and HBF had conserved pathways in the basic developmental phases of biofilm growth.

However, individual gene expression profiles were inconclusive, showing very few clear independent significant differences, though gene expression proved interesting at 4 h. The overall biomass at 24 h was investigated and examined how 4 h gene expression related to this. Overall *HWP1* was the most highly regulated at this time point in both LBF and HBF, as has been shown elsewhere (Nailis et al., 2010a), though no significant differences between the populations were observed. Seven other genes were however shown to have significant positive correlations with biomass. The most significant was *CDR1*, which was unsurprising as it has been shown to be transiently expressed in different biofilm studies, though does not correspond directly to antifungal resistance (Mukherjee et al., 2003, Ramage et al., 2002a). *PLB1* was shown to be significantly correlated with another 16 genes including biomass, though expression appeared constitutively low within the biofilm, which is agreement with previous studies (Nailis et al., 2010a), and may have an accessory role in the degradation of host tissue alongside *SAPs*. Of these, *SAP5* was shown to be highly expressed in mature biofilms, and correlated with biomass and 13 other genes. It was previously reported that *SAP5* was associated with higher levels of expression in *in vitro* biofilms formed from denture stomatitis *C. albicans* isolates (Ramage et al., 2012a). In addition, Nailis and colleagues (2010) demonstrated its crucial role both in a reconstituted human epithelial model and within *in vivo* biofilms (Nailis et al., 2010a). Furthermore, the role of *SAP5* in biofilm formation has recently been demonstrated in BSI, where its expression was significantly increased when compared to planktonic counterparts (Joo et al., 2013). Adhesins, such as *ALS3*, were also up-regulated, which has previously been identified to be involved in biofilm formation, particularly at early stages of biofilm development (0-6 h) (Nailis et al., 2009, Zhao et al., 2006), where *C. albicans* mutants lacking this gene produce sparse biofilms on catheter material *in vitro* (Liu and Filler, 2011). *EAP1*, though showing no clear independent association with biofilm formation *per se*, did show a clear correlation with biomass and 11 other genes. Its importance in biofilm formation has been reported previously (Li et al., 2007). Of interest was the positive correlation with *ZAP1* expression at 4 h, which is a negative regulator of matrix production (Nobile et al., 2009). It did positively correlate with *BCR1*, the global regulator of biofilm formation, suggesting that the early interaction between their proteins may be important for downstream construction of the biofilm.

Collectively the data highlighted the importance of looking at multiple genes simultaneously opposed to single gene targets.

Overall, this study has categorised isolates based on biological properties relating to biofilm characteristics, and evaluated these in models of infection and treatment, where clear differences in virulence have been shown. In an attempt to create a molecular basis of categorising these strains, gene expression studies have shown that individual gene expression analysis of the biofilm related genes to differentiate and categorise biofilm-forming isolates might be futile. Instead, it has been shown here that taking a defined panel of genes during early biofilm growth may be more informative. In particular, the panel of genes such as *SAP5*, *HWP1*, *EAP1*, *PLB1* and *CDR1* investigated in tandem could constitute an important step towards diagnostics of *C. albicans* biofilm formation, though the use of transcriptomics, such as RNA-Seq, may prove useful in identifying novel diagnostic targets. Further work is required to determine why some patients succumb to *C. albicans* biofilms whereas others do not, as the HBF isolates do have an increased pathogenic potential and are more difficult to manage with antifungal agents. Understanding the mechanism underlying these differences would be an important step to improving the clinical management of these patients.

## CHAPTER FINDINGS

The phenotype of *C. albicans* isolates varies greatly within the clinical setting.

Isolates with HBF have increased pathogenicity and are less susceptible to antifungal therapy.

Transcriptional analysis of individual biofilm-specific genes was inconclusive at grouping these organisms, though strong correlation with several of these was observed with respect to biomass.

Chapter 3:  
Investigating the role of  
extracellular DNA and chitinases  
in the formation of *C. albicans*  
biofilms?

### 3.1 Introduction

The mechanism of biofilm formation by *C. albicans* is complex and multifactorial, yet is not fully understood. Previous studies have tended to focus primarily on molecular mechanisms through characterised laboratory strains. This, however, bears no resemblance to the clinical heterogeneity that exists in patient cohorts. One of the pivotal characteristics of fungal biofilms is the extracellular matrix (ECM), which provides a structural scaffold and protection for biofilm cells from hostile factors, including antifungal agents (Flemming and Wingender, 2010, Ramage et al., 2012c). Typically the ECM consists of exopolysaccharides, proteins, surfactants, lipids and water (Al-Fattani and Douglas, 2006, Martins et al., 2010). Recent studies showed another important component of a fungal biofilm matrix is extracellular DNA (eDNA) (Martins, et al., 2010, Rajendran et al., 2013). These studies demonstrated the role of eDNA in fungal biofilm formation and antifungal susceptibility. In both *C. albicans* and *A. fumigatus* it was shown that addition of eDNA significantly improved biofilm formation, and depletion of biofilm eDNA adversely affects the overall biomass. Moreover, bacterial biofilm studies have suggested that eDNA has a multifactorial purpose, as a nutrient source (Mulcahy et al., 2010), facilitator of genetic information exchange (Molin and Tolker-Nielsen, 2003), contributor to biofilm stability and dispersal (Whitchurch et al., 2002, Izano et al., 2008, Allesen-Holm et al., 2006, Berne et al., 2010), and as an antimicrobial resistance factor (Mulcahy et al., 2008, Tetz et al., 2009).

The mechanism of eDNA release from biofilms is not yet fully understood. Work in bacterial cells has suggested various mechanisms potentially responsible for eDNA release, including cell lysis (Allesen-Holm et al., 2006, Qin et al., 2007). In a recent study by our group it was demonstrated that the chitinase regulated autolytic pathway was associated with eDNA release in *A. fumigatus* (Rajendran et al., 2013). To date chitinase activity with respect to eDNA release in *C. albicans* biofilms has yet to be demonstrated.

## 3.2 Aims

It is hypothesised that variation in *C. albicans* biofilm formation is due to the release of eDNA through chitinase activity. Therefore the aim of this study was to evaluate and quantify eDNA within *C. albicans* isolates with variable biofilm formation. Chitinase activity was assessed to determine if these contribute to the presence of eDNA within *C. albicans* biofilms. Moreover, inhibitors of eDNA and chitinases were used to determine if these have an impact on antifungal therapy.

Data presented in this chapter has been submitted and is under review for publication in:

Rajendran R, Sherry L, Williams C, Munro C and Ramage G. 2014. Extracellular DNA confers variation to *C. albicans* biofilm formation. *BMC Microbiology*.

Work from this chapter has been presented orally at the following conference:

Rajendran R, Sherry L, Williams C, Munro C and Ramage G. 'Strain dependent extracellular DNA release affects *Candida albicans* biofilm formation and antifungal sensitivity.' OMIG, Gregynog, UK. April 2014.

### 3.3 Materials and Methods

#### 3.3.1 Culture conditions and standardisation

A number of clinical BSI previously identified and characterised as LBF (n=3 [27915, 50348, 74174]) and HBF (n=3 [9629, 28158, 34106]) were processed for biofilm assessment as described in section 2.3.2.1. These representative isolates were selected as those strains with the absolute lowest and highest biofilm formation of those tested. In addition, *C. albicans* SC5314 and chitinase mutants *cht2Δ* (DSY1768), *cht3Δ* (SPY24), *cht2/3Δ* (DSY1741), including the parental strain CAI4 (*ura3::λimm434/ura3::λimm434*) transformed with Clp10, (gifted by Dr Carol Munro, [Aberdeen Fungal Group]), were used throughout this study (Selvaggin et al., 2004). Isolates were sub-cultured on to SAB agar and incubated at 30°C for 48 h and stored at 4°C. Overnight broths of all *C. albicans* strains were prepared in YPD and incubated with orbital shaking at 30°C with 150 rpm for 18 h, as described previously in 2.3.1.2. Cells were then standardised to the appropriate concentration in RPMI-1640 unless otherwise stated (Ramage et al, 2001).

#### 3.3.2 Growth kinetics

The growth kinetics of *C. albicans* isolates with LBF (n=3) and HBF (n=3) were assessed to determine if growth rate played a role in eDNA release. Each isolate was standardised to  $1 \times 10^4$  cells/mL in YPD with 200 μL added to each well of a 96 well round-bottom plate. Plates were incubated at 37°C for 24 h and absorbance was measured at 530 nm every 1 h, as described in 2.3.4. Each isolate was tested in duplicate and repeated on three independent occasions. Negative controls containing no *C. albicans* were included for background correction.

#### 3.3.3 DNase treatment on preformed biofilms

The role of eDNA in *C. albicans* biofilm formation was first investigated by depletion of eDNA within the biofilm using a hydrolytic enzyme DNase I. DNase I from bovine pancreas (Sigma-Aldrich) was prepared in 0.15 M NaCl supplemented with 5 mM of MgCl<sub>2</sub> to a final concentration of 256 μg/mL. Biofilms of HBF (n=3) and LBF (n=3) isolates were grown in RPMI-1640 as described previously. Following incubation, biofilms were washed with PBS and treated with 256

$\mu\text{g/mL}$  of DNase I (Sigma-Aldrich) for 24 h at  $37^\circ\text{C}$ , a concentration previously shown to decrease fungal biofilm biomass (Martins et al., 2010, Rajendran et al., 2013). Untreated controls were also included for direct comparison. Biofilms were then washed twice with PBS and the biomass was scraped from the surface and passed through  $0.22\ \mu\text{m}$  membrane filters. Biofilms retained on the filters were dried overnight at  $60^\circ\text{C}$  and then dry weight measurements were taken for each isolate, as described previously (Richard et al., 2005). Measurement of each isolate was carried out in duplicate, on three independent occasions.

### 3.3.4 Assessment of eDNA release

*C. albicans* isolates with LBF (n=3), HBF (n=3), CAI4, and chitinase mutants *cht2 $\Delta$* , *cht3 $\Delta$* , *cht2/3 $\Delta$*  biofilms were grown in RPMI-1640 for 4 and 24 h at  $37^\circ\text{C}$  as described previously in 2.3.2. The quantity of eDNA release was measured using a microplate fluorescence assay (MFA) using a DNA binding dye (SYBR<sup>®</sup> Green I), as previously described (Rajendran et al., 2013). Briefly, SYBR<sup>®</sup> Green I (Invitrogen) was added to biofilm supernatants in a black well microtitre plate (Costar3603; Corning) at a ratio of 1:4. Binding of this dye produces fluorescence directly proportional to DNA concentration. The levels of eDNA were quantified using a fluorescence plate reader (Fluostar Optima; BMG Labtech) at 485 and 518 nm, respectively. The concentration of eDNA in the sample was quantified using the DNA standard curve as previously described (Leggate et al., 2006). In addition, optical density of the culture was measured at 530 nm simultaneously for normalising the relative fluorescence units (RFU) data. Each isolate was tested in duplicate, on three separate occasions.

### 3.3.5 Fluorescence microscopy

*C. albicans* CAI4, *cht2 $\Delta$* , *cht3 $\Delta$* , *cht2/3 $\Delta$* , cells were standardised to  $1 \times 10^6$  cells/mL in RPMI-1640 medium and biofilms were grown on Thermanox<sup>™</sup> coverslips within a 24 well plate at  $37^\circ\text{C}$  for 24 h. Following biofilm development, cells were gently washed with PBS and stained with  $5\ \mu\text{M}$  calcofluor white ([CFW] Invitrogen), which binds chitin of *C. albicans* cell walls, and with  $20\ \mu\text{M}$  propidium iodide (PI) (Sigma), which stains the DNA present within a biofilm, according to the manufacturer's instructions. Biofilm growth and accumulation of eDNA was visualised under a fluorescence microscope (Motic BA400 Colorview system) at Ex<sub>350</sub>/Em<sub>400</sub> for CFW and Ex<sub>540</sub>/Em<sub>525</sub> for PI.

Representative images from 10 fields were taken and one from each group was digitally photographed.

### 3.3.6 Quantification of chitinase activity

Previously it has been shown that chitinase activity was an autolytic marker in the investigation behind the mechanism of eDNA release (Rajendran et al., 2013). Therefore, *C. albicans* chitinase activity from isolates with LBF (n=3) and HBF (n=3) was assessed after 4 and 24 h of biofilm formation using a fluorometric chitinase assay kit (Sigma, United Kingdom), as per the manufacturer's instructions. Following biofilm development, supernatants were collected at 4 and 24 h, and an appropriate volume of each sample was incubated with a substrate working solution (4-methylumbelliferyl N-acetyl- $\beta$ -D-glucosaminide) at 37°C for 30 min. Fluorescence was then quantified at Ex<sub>360</sub>/Em<sub>450</sub>. Appropriate positive and negative controls included in the kit were added to each plate. Chitinase activity was calculated and expressed as a ratio between chitinase units and optical density of culture (U/OD). Each isolate was measured in duplicate, on three separate occasions.

### 3.3.7 Quantitative gene expression

*C. albicans* isolates with LBF (n=3) and HBF (n=3) biofilms were grown in 24 well plates as described in 2.3.2 for 4 and 24 h at 37°C. Quantitative analysis of transcriptional changes within these biofilms was performed as previously described in 2.3.9.1 (Ramage et al., 2012a). Briefly, RNA was extracted by mechanical disruption in TRIzol (Invitrogen) and purified using an RNeasy MinElute cleanup kit (Qiagen, Crawley, United Kingdom) as per the manufacturer's instructions. RNA was quantified and its quality determined using a NanoDrop spectrophotometer (ND-1000; Thermo Scientific, Loughborough, United Kingdom). cDNA was synthesised with a High-Capacity RNA-to-cDNA kit (Applied Biosystems) using a MyCycler PCR machine (Bio-Rad, Hertfordshire, United Kingdom), and was stored at -20°C until required for transcriptional analysis. The expression of the chitinase genes *CHT2* and *CHT3* and hyphal cell markers *HWP1* and *ALS3* were then assessed using qPCR with SYBR® GreenER™ (Invitrogen), according to the manufacturer's instructions. Primer sequences for these genes are shown in Table 3.1 and PCR cycling conditions are described in 2.3.8.6. The individual gene expression levels were then calculated using the

$2^{-\Delta CT}$  method for different phases and normalised to the *ACT1* housekeeping gene. All isolates were tested in duplicate for this experiment.

**Table 3.1 - *C. albicans* primers used for qPCR**

| Gene               | Sequence (5' - 3')   | Function            |
|--------------------|--|---------------------|
| <b><i>ALS3</i></b> | F - CAACTTGGGTTATTGAAACAAAAACA<br>R - AGAAACAGAAACCCAAGAACAACCT  | Adhesin             |
| <b><i>HWP1</i></b> | F - GCTCAACTTATTGCTATCGCTTATTACA<br>R - GACCGTCTACCTGTGGGACAGT   | Hyphal wall protein |
| <b><i>CHT2</i></b> | F - TGATTTATTATCCAAAGTCCCCTTG<br>R - TTGAATTGGCCATTGATTGAA       | Chitinase           |
| <b><i>CHT3</i></b> | F - TGCTACTATTCCAGATGACAAAGAAATT<br>R - TTCAGTGATGATAGCAGGTGGTTT | Chitinase           |
| <b><i>ACT1</i></b> | F - AAGAATTGATTTGGCTGGTAGAGA<br>R - TGGCAGAAGATTGAGAAGAAGTTT     | Housekeeping        |

### 3.3.8 Scanning electron microscopy

*C. albicans* single (*cht2Δ*, *cht3Δ*) and double knockout (*cht2/3Δ*) chitinase mutants and their parental strain CA14 were standardised and grown as biofilm in RPMI-1640 directly onto Thermanox™ coverslips (Nunc, Roskilde, Denmark) for 24 h. Following biofilm development, biofilms were carefully washed with PBS and prepared for SEM as described in 2.3.3. Images were assembled using Photoshop software (Adobe, San Jose, CA, USA) at magnification ×1000.

### 3.3.9 Antifungal susceptibility testing

The impact of eDNA and chitinases on AMB sensitivity was assessed using a hydrolytic enzyme DNase I and a chitinase inhibitor acetazolamide ([AZE] Sigma-Aldrich). AMB was chosen *C. albicans* isolates with LBF (n=3), HBF (n=3) and the control strain SC5314 were standardised to  $1 \times 10^6$  cells/mL in RPMI-1640 and biofilms grown in 96 well flat-bottom plates for 4 and 24 h. Preformed biofilms were washed with PBS and treated with serially diluted AMB ± DNase (256 µg/mL) or AZE (256 µg/mL). Biofilms were incubated for a further 24 h at 37°C before metabolic activity assessed using the XTT assay, as previously described (Pierce et al., 2008).

### 3.3.10 RNA Seq

One *C. albicans* clinical isolate with LBF and HBF were standardised to  $1 \times 10^6$  cells/mL in RPMI-1640 and biofilms grown in 75 cm<sup>2</sup> tissue culture flasks for 24 h at 37°C. Following development, biofilms were gently washed with PBS before a cell scraper was used to dislodge the biomass and RNA extracted as previously described in 2.3.9.1. The integrity of the RNA was assessed using an agarose gel to visualise the two distinct rRNA bands. Each isolate was grown in triplicate and a minimum of 10 µg of total RNA was submitted for each sample and sent for sequencing to The GenePool (Edinburgh, UK). RNA integrity was assessed using a Bioanalyzer where an RIN value >7.0 was deemed acceptable for RNA Seq using Illumina 50 base sequencing.

Bioinformatics analysis included the raw read alignment to version A21-s02-m01-r27 of *C. albicans* SC5314 using version 2013-05-09 of a genomic mapping and alignment program (GMAP). Duplicate reads were removed using smatools version 0.1.18 and reads assigned to exons described in *C. albicans* SC5314 version A21-s02-m08-r03 using htseq-count version 0.5.4. Differential expression analysis was performed using the bioconductor package EdgeR (version 2.6.12) and R (version 2.15.0) on LBF and HBF, carried out by Edinburgh Genomics (Edinburgh, UK). The output from the bioinformatics analysis represented the differential expression as log<sub>2</sub> fold change and statistical significance. Genes with a positive log FC value were considered up-regulated in HBF whereas those with a negative value were up-regulated in LBF. All differentially expressed genes were classified based upon their functional groups using GO Slim Mapper (<http://www.Candidagenome.org/cgi-bin/GO/goTermMapper>). In addition, 11 genes from different functional groups, previously investigated in Chapter 2 and 3, were selected to validate the RNA-Seq data. These included *ALS3*, *BCR1*, *HWP1*, *MNN4*, *PMR1*, *CPH1*, *SAP3*, *SAP4*, *SAP6* *PLB1* and *CHT2*.

### 3.3.11 Statistical analysis

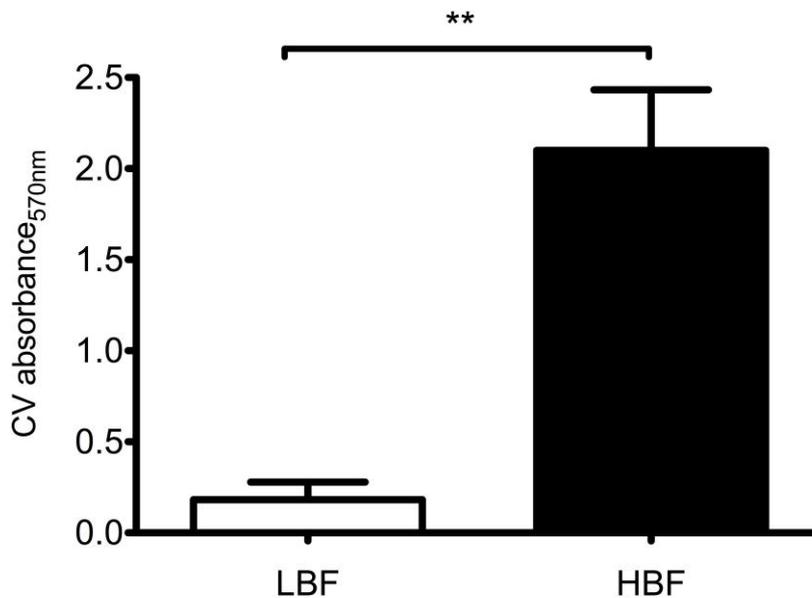
Analysis of variance (ANOVA) and t-tests were used to investigate independent sample data. A Bonferroni correction for multiple comparisons was applied to the data where appropriate. SPSS (Version 11, Chicago, USA) was used for these analysis and GraphPad Prism (Version 4, La Jolla, USA) for the production of the figures. The Spearman's rho correlation coefficient was determined to

investigate the relationship between the parameters measured. A p value of less than 0.05 was considered significant.

### 3.4 Results

#### 3.4.1 eDNA contributes to *C. albicans* biofilm formation

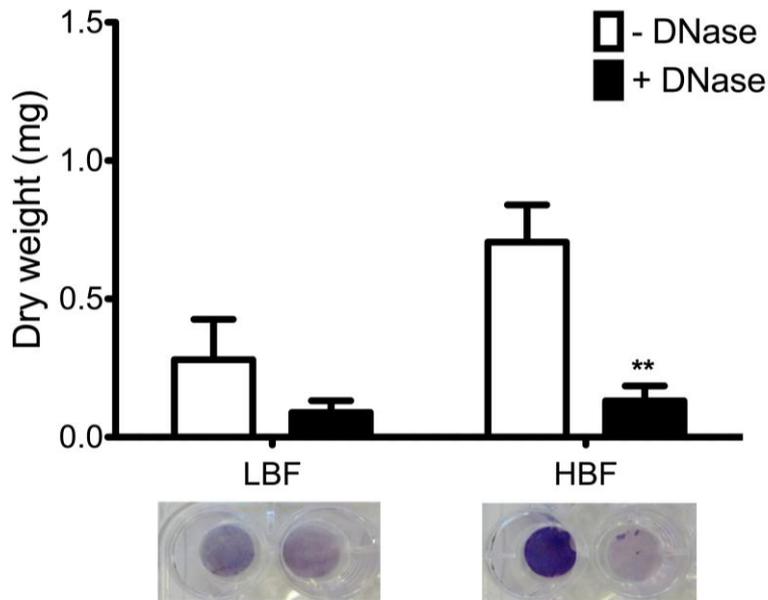
Initially, *C. albicans* biofilm formation was assessed in 6 BSI isolates defined as either LBF (n=3) or HBF (n=3) based on biomass quantification (Figure 3.1).



**Figure 3.1- Variation in *C. albicans* biofilm formation.**

*C. albicans* isolates with LBF (n=3) and HBF (n=3) were grown as biofilms in 96 well flat-bottom microtitre plates at 37°C for 24 h. Biofilm biomass was assessed spectrophotometrically by reading CV absorbance at 570 nm. Each isolate was assessed in triplicate, on at least two independent occasions. Data represents mean ± SD. \*p<0.05.

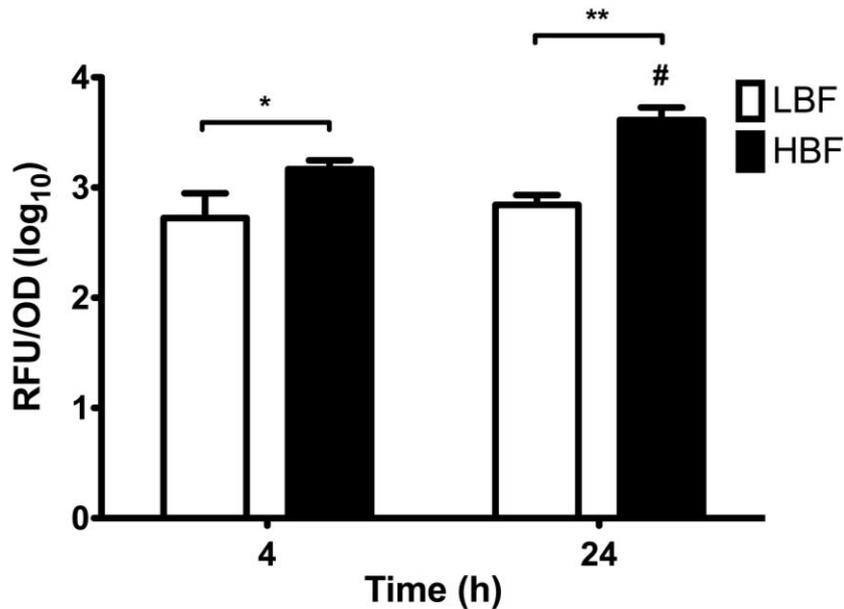
A significant difference in biofilm formation was observed between isolates with LBF and HBF, where 11.6× more (p=0.0035) biomass was observed in the latter group (Figure 3.1). To determine the mechanism behind the variation of biofilm formation in *C. albicans* clinical isolates, the release of eDNA from isolates with LBF and HBF was evaluated by investigating their response to DNase treatment (Figure 3.2).



**Figure 3.2- DNase treatment reduces *C. albicans* biofilm biomass.**

*C. albicans* isolates LBF (n=3) and HBF (n=3) were grown as biofilms at 37°C for 24 h. Biofilms were treated ± 256 µg/mL DNase for a further 24 h before being passed through 0.22 µm membrane filter. Biomass retained on the filters were dried overnight at 60°C and dry weight measurements taken. In addition, biofilms ± DNase treatment were stained with CV and imaged to show the disruptive effect of DNase on the biofilms. Dry weight measurements were carried out in duplicate for each isolate, on three independent occasions. Photographs show representative images from three independent experiments. Data represents mean ± SEM. \*\*p< 0.01.

A significant reduction in dry weight was observed for isolates with HBF with a 5-fold decrease (p<0.01) in biomass compared to untreated controls (Figure 3.2). However, despite isolates with LBF having 3× less biomass following DNase treatment, no significant differences were observed. To further investigate the hypothesis that eDNA preferentially supports biofilm growth, the release of eDNA from both LBF and HBF at 4 and 24 h was investigated using the SYBR® green I assay (Figure 3.3).



**Figure 3.3- eDNA release is greater in isolates with HBF.**

*C. albicans* isolates with LBF (n=3) and HBF (n=3) were grown as biofilms for 4 and 24 h in the presence of the DNA binding dye SYBR<sup>®</sup> Green I. Fluorescence was measured after 4 and 24 h at Ex<sub>485</sub>/Em<sub>518</sub>. Absorbance was measured simultaneously for normalising the fluorescence data. Each isolate was tested in duplicate, on three independent occasions. Data represents mean ± SD. \*,# p<0.05, \*\* p<0.01.

eDNA release was significantly greater in isolates with HBF compared to LBF at both 4 h (2.8-fold, p=0.0252) and 24 h (5.9-fold, p=0.0087), as shown in Figure 3.3. Moreover, eDNA release increased significantly in isolates with HBF by a further 2.8-fold (p=0.0221) between 4 and 24 h. As eDNA has been shown to play a role in *C. albicans* biofilm formation in Figure 3.2 and 3.3, the impact this had on AMB sensitivity was investigated (Table 3.2).

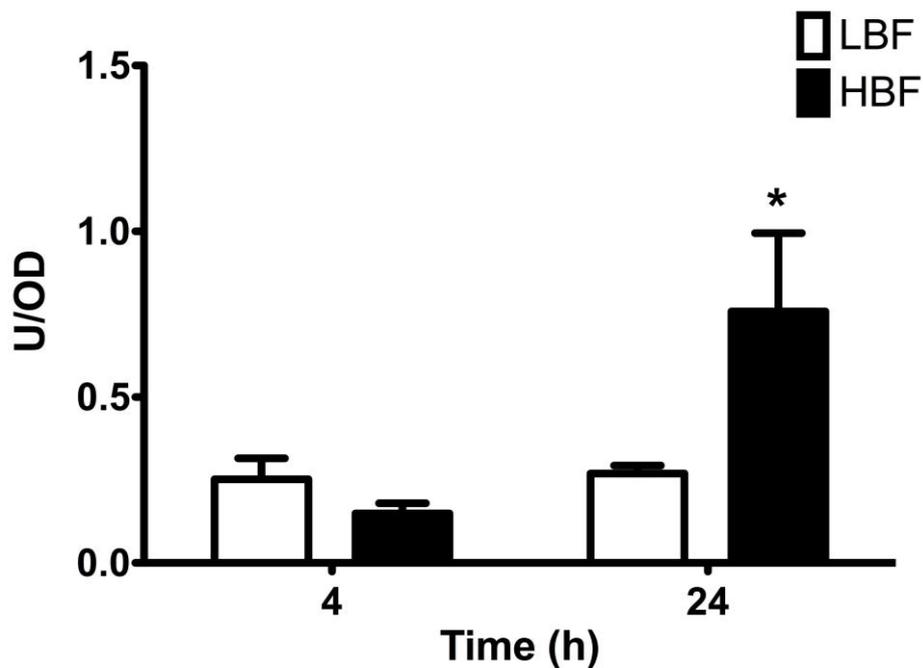
**Table 3.2 - AMB sensitivity ± DNase treatment against *C. albicans* biofilms**

| MIC    |                                 |              |                                 |            |
|--------|---------------------------------|--------------|---------------------------------|------------|
| Strain | 4 h biofilm                     |              | 24 h biofilm                    |            |
|        | AMB MIC <sub>50</sub><br>(mg/L) | + DNase      | AMB MIC <sub>50</sub><br>(mg/L) | + DNase    |
| SC5314 | <0.0313                         | <0.0313      | 0.5                             | 2          |
| LBF    | 0.0313                          | 0.0313       | 0.25 - 0.5                      | 0.25 - 0.5 |
| HBF    | 0.125 - 0.25                    | 0.125 - 0.25 | 0.25 - 1                        | 1 - 8      |

It was shown that isolates with LBF were up to 8-fold more susceptible to AMB than those with HBF at 4 h. However, when comparing these biofilm subsets at 24 h there was only a 2-fold difference in MIC. The addition of DNase during AMB treatment did not have an impact on isolates with LBF at 4 h, with only a 2-fold difference observed at 24 h with DNase treatment. In contrast, isolates with HBF were most susceptible to AMB therapy at both time points, particularly 24 h where a 8-fold decrease in MIC was observed with the addition of DNase. The addition of DNase during AMB treatment increased AMB sensitivity by up to 2 and 8-fold in HBF isolates from 4 and 24 h biofilms, respectively, whereas, those LBF isolates showed no change at 4 h, and only a 2 fold change at 24 h.

### 3.4.2 Differential expression of chitinase activity is correlated with eDNA release and biofilm formation

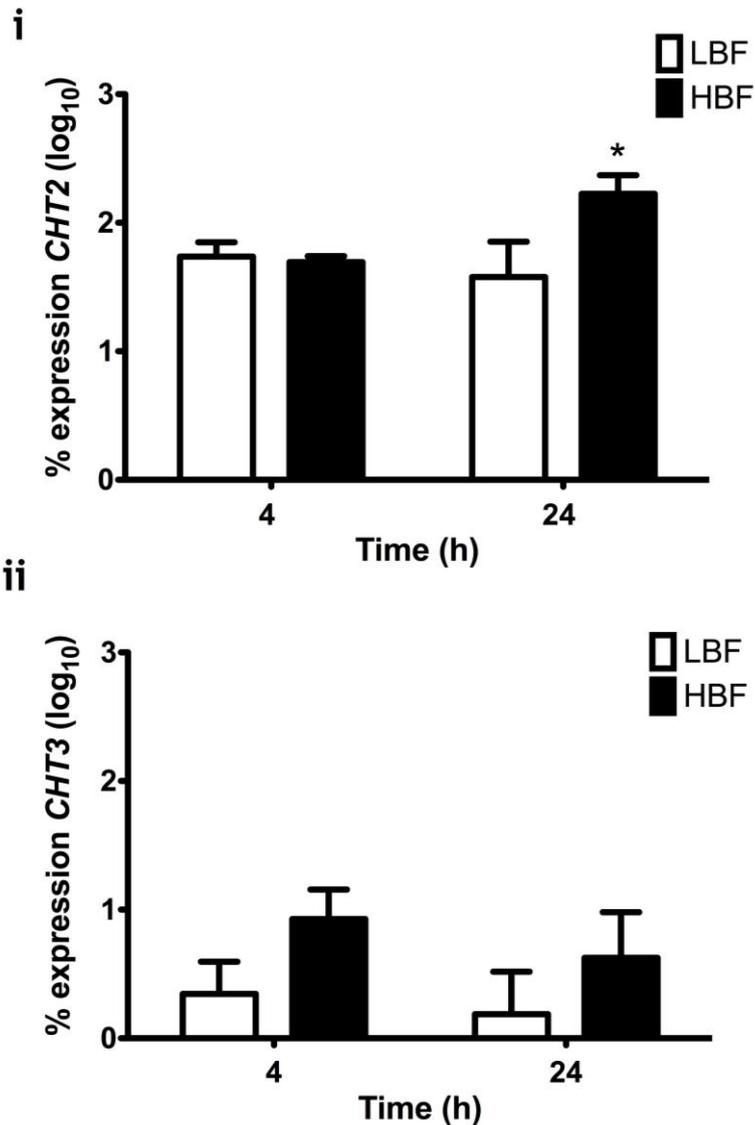
Previous studies demonstrated a positive correlation between chitinase activity in *A. fumigatus* biofilms and eDNA release (Rajendran et al., 2013). Therefore, this property was evaluated for *C. albicans* biofilms (Figure 3.4). No differences in chitinase activity were identified between isolates with LBF and HBF at 4 h, however at 24 h isolates with HBF had ~3-fold greater ( $p < 0.05$ ) chitinase activity than those with LBF (Figure 3.4).



**Figure 3.4 - Chitinase activity is increased in *C. albicans* biofilm formation.**

*C. albicans* isolates with LBF (n=3) and HBF (n=3) were grown as biofilms for 4 and 24 h at 37°C. Supernatants were retained and added to a chitinase substrate working solution for 30 min at 37°C. Fluorescence was measured at Ex<sub>360</sub>/Em<sub>450</sub> and chitinase activity represented as U/OD, normalised to isolate biomass. Each isolate was measured in duplicate, on three independent occasions. Data represents mean ± SEM. \* $p < 0.05$ .

Two well characterised chitinase genes in *C. albicans* *CHT2* and *CHT3* (Selvaggini et al., 2004) were investigated to determine if their expression was related to the chitinase activity observed in Figure 3.4.



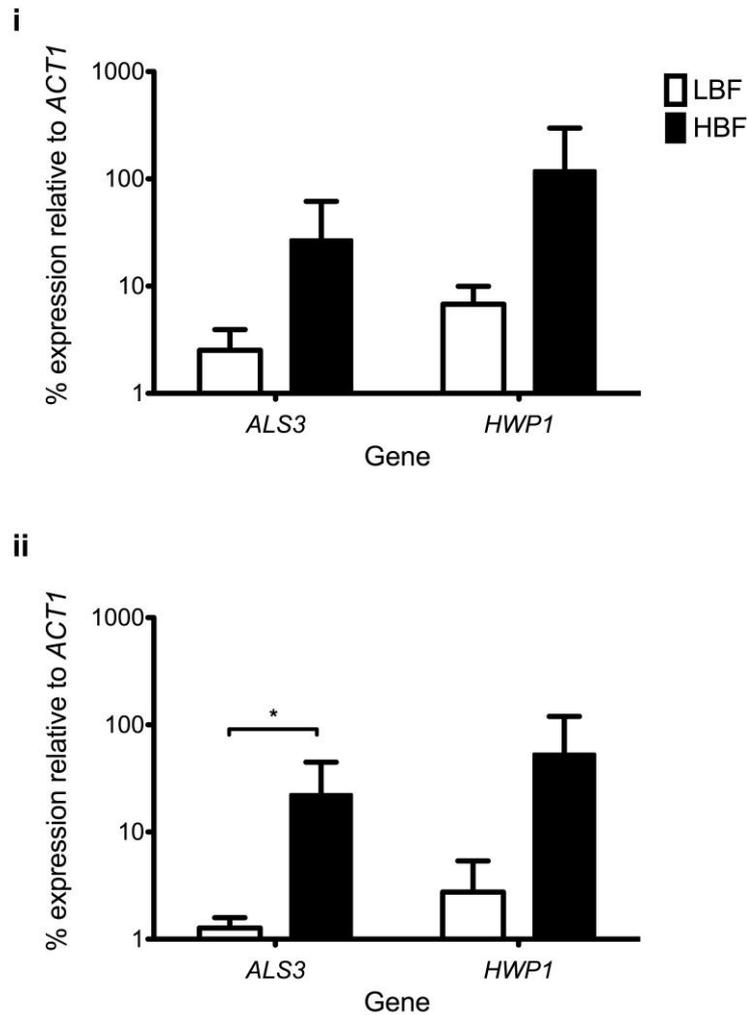
**Figure 3.5 - Expression of chitinases at the mRNA level is up-regulated in *C. albicans* isolates with HBF.**

Biofilms with LBF (n=3) and HBF (n=3) were grown for 4 and 24 h before RNA extracted, cDNA synthesised and qPCR used to measure the expression of *CHT2* (i) and *CHT3* (ii). Each individual isolate was measured in duplicate. Percentage of gene expression was log<sub>10</sub> transformed and represents mean ± SD relative to housekeeping gene *ACT1*. \*p<0.05.

Although no difference was observed in *CHT2* expression at 4 h between the two biofilm subsets, a significant up-regulation by 23% (p<0.05) was found at 24 h in those with HBF compared to those with LBF (Figure 3.5i). Furthermore, despite *CHT3* expression being increased by 4-fold and 3-fold in isolates with HBF than LBF at 4 and 24 h, respectively, no significant difference between the groups was observed (Figure 3.5ii).

In chapter 2 the expression of biofilm-related genes in *C. albicans* biofilms was evaluated, where it was found that these were up-regulated in isolates with

HBF. Here, the expression of *ALS3* and *HWP1* were further investigated for a selective group of isolates with LBF and HBF (Figure 3.6).



**Figure 3.6 - Expression of biofilm related genes at mRNA level is up-regulated in *C. albicans* isolates with HBF.**

Biofilms with LBF (n=3) and HBF (n=3) were grown for 4 (i) and 24 h (ii) before RNA extracted, cDNA synthesised and qPCR used to measure the expression of *ALS3* and *HWP1*. Each individual isolate was measured in duplicate. Percentage of gene expression is represented by mean  $\pm$  SD relative to housekeeping gene *ACT1*. \* $p < 0.05$ .

Despite *ALS3* and *HWP1* expression being increased in HBF by 11 $\times$  ( $p = 0.1143$ ) and 18 $\times$  ( $p = 0.1447$ ) at 4 h (Figure 3.6i) when compared to LBF respectively, the only significant difference in the transcriptional analysis was observed at 24 h where LBF had 18 $\times$  less expression of *ALS3* ( $p = 0.0102$ ) than HBF (Figure 3.6ii).

To investigate the relationship between eDNA release, chitinase activity, biofilm biomass and genes related to biofilm formation (*HWP1* and *ALS3*), a Spearman's rho correlation analysis was performed (Table 3.3).

**Table 3.3 - Spearman's rho correlation analysis**

| Spearman's rho |                         | Biomass            | Chitinase          | <i>HWP1</i>       | <i>ALS3</i>       |
|----------------|-------------------------|--------------------|--------------------|-------------------|-------------------|
| eDNA           | Correlation Coefficient | .943 <sup>**</sup> | .886 <sup>**</sup> | .771 <sup>*</sup> | .829 <sup>*</sup> |
|                | Sig.                    | .002               | .009               | .036              | .021              |
| Chitinase      | Correlation Coefficient | .943 <sup>**</sup> |                    | .771 <sup>*</sup> | .714              |
|                | Sig.                    | .002               |                    | .036              | .055              |

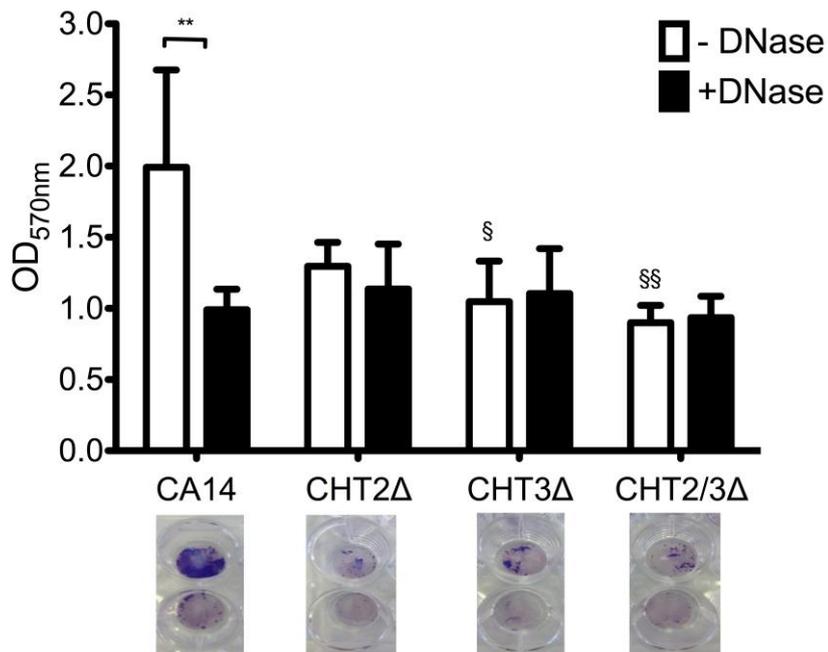
\* Correlation is significant at the 0.05 level

\*\* Correlation is significant at the 0.01 level

A significant correlation was observed between eDNA release and biomass ( $p < 0.01$ ), chitinase activity ( $p < 0.01$ ), and expression of *HWP1* ( $p < 0.05$ ) and *ALS3* ( $p < 0.05$ ) at 24 h. A significant positive correlation was also found between chitinase activity and expression of *HWP1* ( $p < 0.05$ ) in 24 h biofilms (Table 3.3).

### 3.4.3 Compromising chitinase activity affects isolates biofilm formation and antifungal sensitivity

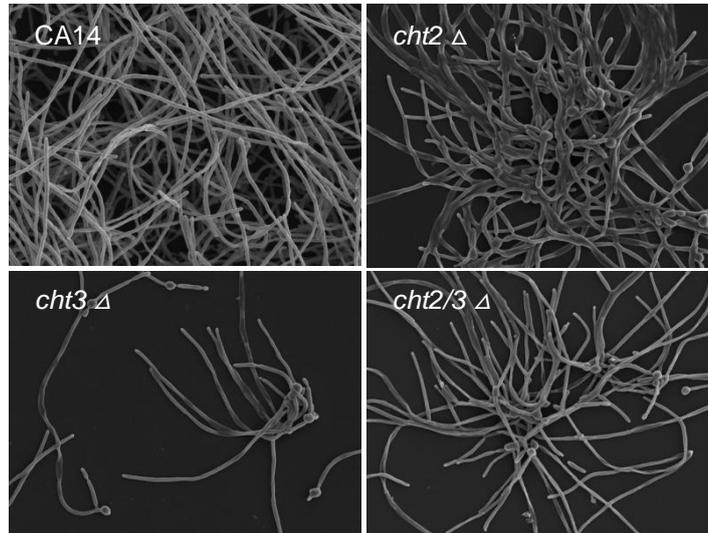
*C. albicans* single and double knockout mutants *CHT2* $\Delta$ , *CHT3* $\Delta$  and *CHT2/3* $\Delta$  were assessed to confirm whether these chitinases played a role in biofilm formation and eDNA release (Figure 3.7). *cht2* $\Delta$ , *cht3* $\Delta$  and *cht2/3* $\Delta$  all showed a reduction in biofilm biomass by 1.5 to 2-fold when compared to the parental strain CAI4. However, a significant reduction was found only with *cht3* $\Delta$  ( $p < 0.05$ ) and *cht2/3* $\Delta$  ( $p < 0.005$ ) (Figure 3.7). To investigate whether this reduction in biomass was caused by presence of eDNA, the biofilms were treated with DNase.



**Figure 3.7 - Chitinase mutants have less biomass and are unaffected by DNase treatment.**

*C. albicans* *cht2Δ*, *cht3Δ*, *cht2/3Δ* biofilms were grown for 24 h at 37°C then treated ± 256 µg/mL DNase for a further 24 h. Biomass was assessed using CV absorbance at 570 nm and stained biofilms were digitally imaged to show the difference in biofilm formation. Each isolate was measured in triplicate on three independent occasions. Data represents mean ± SD. \*\* $p < 0.01$ , § $p < 0.05$ , §§ $p < 0.01$ , compared to parental strain.

Here a significant reduction in biofilm biomass by 2-fold was observed with DNase treatment in CA14 compared to untreated controls ( $p < 0.01$ ). Conversely, no significant reduction was observed with either single or double knockout mutants. The reduction in biofilm formation by all the knockout mutants was confirmed by microscopy (Figure 3.8). SEM imaging showed reduced biomass in these isolates compared to CA14, with *cht3Δ* showing the clearest visual difference, providing evidence that chitinases play a role in *C. albicans* biofilm formation.

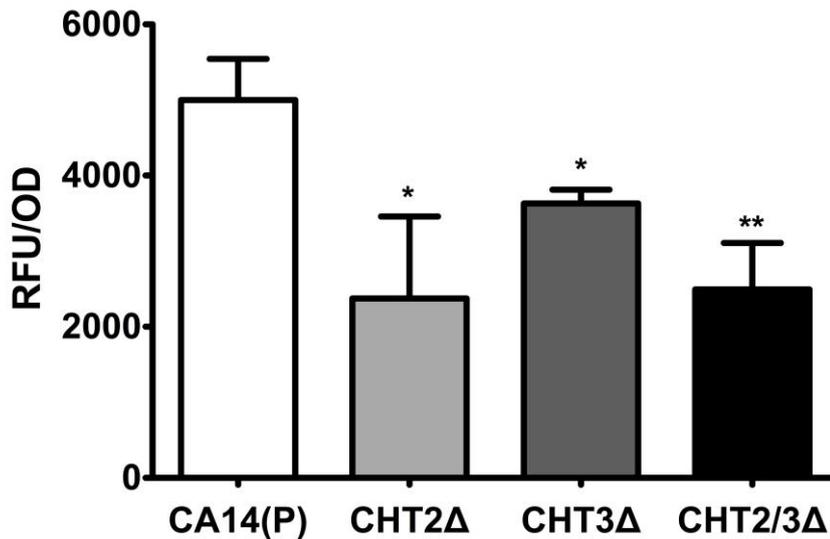


**Figure 3.8 - Chitinase mutants are defective in biofilm formation.**

*C. albicans* *cht2*Δ, *cht3*Δ, *cht2/3*Δ and wild type CA14 (×1000) were grown on Thermanox™ coverslips for 24 h at 37°C. Biofilms were then processed and viewed on a JEOL JSM-6400 scanning electron microscope and images assembled using Photoshop software. Note the reduction in biofilm biomass in *cht3*Δ and *cht2/3*Δ.

#### 3.4.4 Chitinase deficient strains have reduced eDNA release

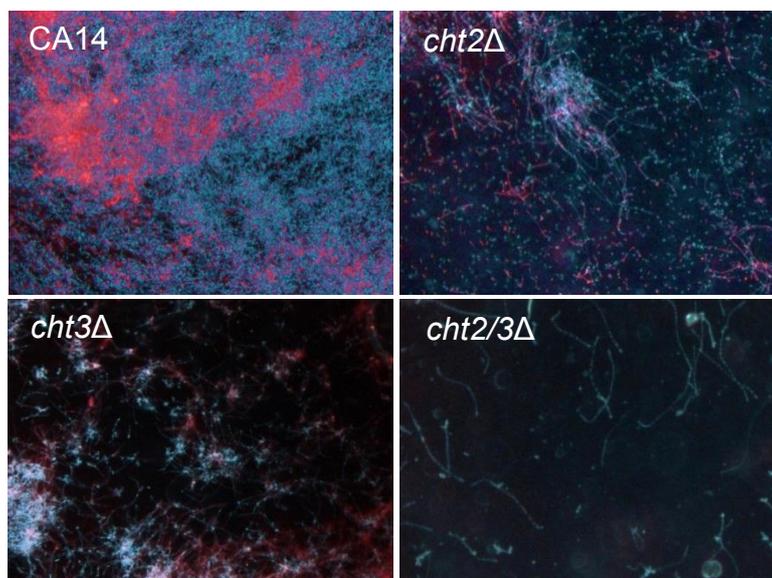
Next, eDNA release was further quantified in these mutants by using the SYBR® green I assay (Figure 3.9). All knockout strains had significantly reduced eDNA release after 24 h. The greatest reduction was in *cht2*Δ, where eDNA was decreased by 53% (p=0.02) compared to the parental strain CA14. This was followed by *cht2/3*Δ with a reduction of 50% (p=0.0061) and *cht3*Δ by 26% (p=0.0161).



**Figure 3.9 - eDNA release is reduced in chitinase mutants.**

*C. albicans* *cht2Δ*, *cht3Δ*, *cht2/3Δ* and their parental strain CA14 were grown in RPMI for 24 h at 37°C. eDNA release was then assessed using SYBR green I assay, normalised to each isolates biomass. Each isolate was measured in duplicate, on three separate occasions. Data represents mean ± SD. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

The level of eDNA present in these knockout mutants was confirmed visually using fluorescence imaging (Figure 3.10). The presence of eDNA in the parental strain CA14 is shown by the abundance of red/pink fluorescence, which was notably reduced in the single knockouts *cht2Δ* and *cht3Δ*, and completely absent from the double knockout *cht2/3Δ* (Figure 3.10).



**Figure 3.10 - eDNA is reduced in chitinase mutants.**

*C. albicans* chitinase mutants *CHT2Δ*, *CHT3Δ*, *CHT2/3Δ* and their parental strain CA14 were grown on Thermanox™ coverslips for 24 h at 37 °C before staining with CFW and PI. The accumulation of eDNA was visualised under a fluorescence microscope (Motic BA400 Colorview system) at an Ex<sub>350</sub>/Em<sub>400</sub> CFW and Ex<sub>540</sub>/Em<sub>525</sub> for PI. One representative from each group was digitally photographed.

#### 3.4.5 Inhibition of chitinase activity improves antifungal sensitivity

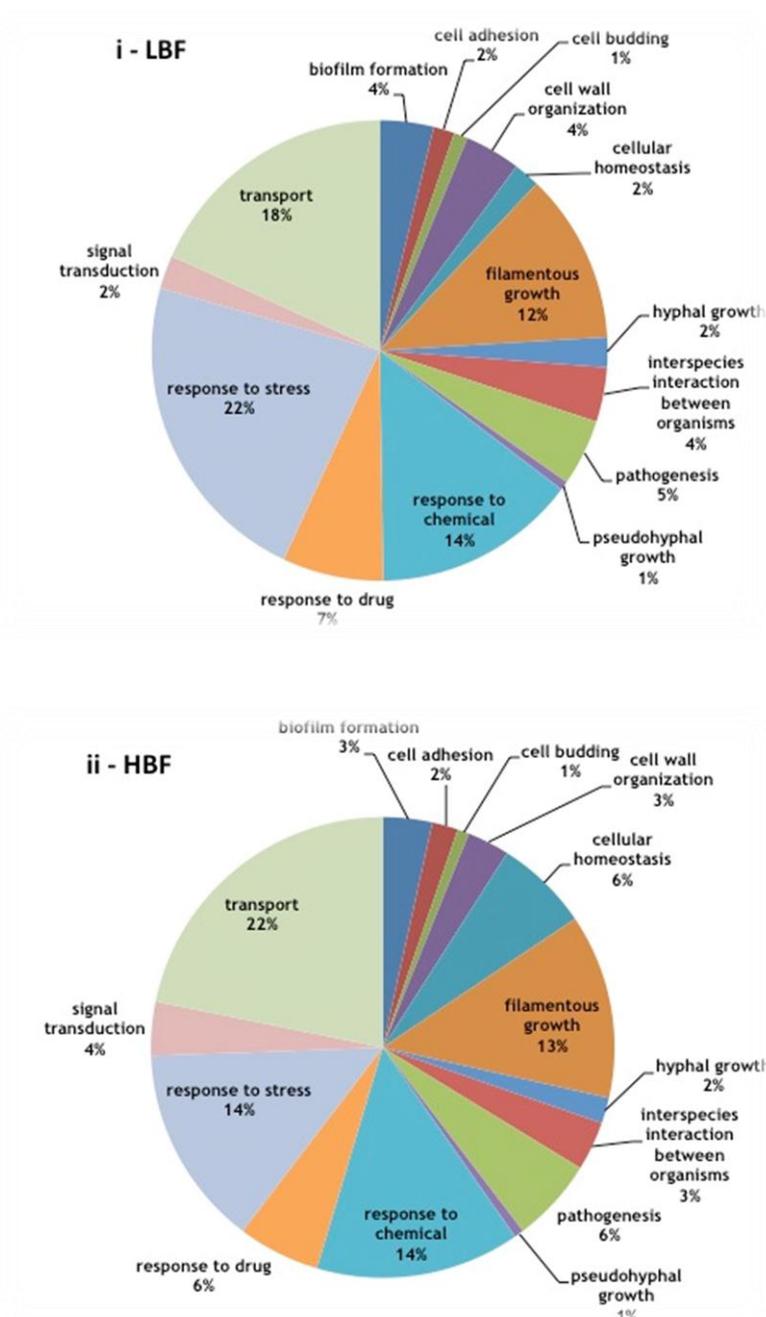
Next, to test whether pharmacological inhibition of chitinases impacted antifungal sensitivity, AZE was used in combination with AMB (Table 3.4). It was shown that the addition of AZE had little impact on AMB sensitivity in any *C. albicans* isolates with LBF and HBF biofilm at 4 h, as the MIC remained unchanged. Furthermore, only a minimal increase in AMB susceptibility was found in HBF isolates at 24 h where a 2-fold decrease in MIC<sub>50</sub> was found.

**Table 3.4 - AMB sensitivity ± AZE treatment against *C. albicans* biofilms**

| MIC    |                                 |              |                                 |            |
|--------|---------------------------------|--------------|---------------------------------|------------|
| Strain | 4 h biofilm                     |              | 24 h biofilm                    |            |
|        | AMB MIC <sub>50</sub><br>(mg/L) | + AZE        | AMB MIC <sub>50</sub><br>(mg/L) | + AZE      |
| SC5314 | <0.0313                         | <0.0313      | 0.5                             | 1          |
| LBF    | 0.0313                          | 0.0313       | 0.25 - 0.5                      | 0.25 - 0.5 |
| HBF    | 0.125 - 0.25                    | 0.125 - 0.25 | 0.25 - 1                        | 0.5 - 2    |

Although chitinases were shown to contribute to *C. albicans* biofilm formation through an increase in eDNA release, these were not shown to play a significant role with regards to antifungal therapy. Differential expression of genes associated LBF and HBF in *C. albicans* were further investigated.

To understand mechanisms that play a role in *C. albicans* biofilm formation in greater detail, RNA Seq was undertaken in *C. albicans* isolates with LBF (n=1) and HBF (n=1). Genes with a positive log<sub>2</sub> FC value were considered up-regulated in HBF whereas those with a negative value were up-regulated in LBF. Figure 3.11 illustrates the up-regulation of genes by *C. albicans* isolates with LBF (i) and HBF (ii). All the differentially expressed genes were grouped into functional categories using the GO Slim Mapper genome database (<http://www.Candidagenome.org/cgi-bin/GO/goTermapper>). These functional categories included a range of processes including filamentous growth, biofilm formation, cellular transport, response to stress, pathogenesis, etc.



**Figure 3.11 - Up-regulation of *C. albicans* functional groups based on RNA-Seq analysis.**

*C. albicans* isolates with LBF (i) and HBF (ii) were grown as biofilms for 24 h before RNA extracted and used for RNA Seq. Up-regulation of each gene was grouped based upon its function. Each isolate was analysed in triplicate.

Notably, the three largest functional groups identified in both LBF and HBF of *C. albicans* were transport, response to chemicals, and response to stress (Figure 3.11), where they accounted for 54% and 50% of the total number of gene up-regulated, respectively (Figure 3.11). A total of 1076 genes were up-regulated in LBF where the greatest increase in regulation was found in genes related to cellular responses to stress, (Figure 3.11i). Genes associated with cell transport

were the functional group mostly highly expressed in the *C. albicans* isolate with HBF, accounting for 22% of the 853 genes (Figure 3.11ii). Of interest, there was no difference in the percentage of genes associated with cell adhesion and hyphal growth in both LBF and HBF, which accounted for only 2% in total. Furthermore, the number of genes related to biofilm formation only represented 4% and 3% in LBF and HBF, respectively. However, when the specific genes within this functional group were investigated, genes associated with a yeast state (*YWP1* [9×], *NRG1* [3.7×]) were up-regulated in LBF, whereas HBF had hyphae specific gene expression increased (*HWP1* [6×], *HYR1* [3.8×] and *ALS3* [2.8×]), which is consistent with their phenotypic presentation.

Next, genes were selected from the RNA Seq data that had shown the greatest regulation within *C. albicans* isolates with HBF (Table 3.5) and LBF (Table 3.6) to determine if other processes were significant in biofilm variation.

Table 3.5 - Genes with greatest regulation in *C. albicans* isolates with HBF by RNA-Seq

| HBF         |              |                                |  |                     |          |
|-------------|--------------|--------------------------------|--|---------------------|----------|
| Gene        | ID           | Function                       | Functional group (Figure 3.11)   | Log <sub>2</sub> FC | p value  |
| <i>RHD2</i> | orf19.2688   | Retrotransposon                | Biological process   | 12.62               | 1.2E-26  |
| <i>TLO4</i> | orf19.7276.1 | Telomere-proximal gene         | Biological process   | 9.85                | 2.21E-39 |
| <i>LDG3</i> | orf19.6486   | Putative LDG family protein    | Biological process   | 8.23                | 7.69E-22 |
| <i>ATO1</i> | orf19.6169   | Putative transmembrane protein | Biological process   | 7.00                | 1.68E-9  |
| <i>HWP1</i> | orf19.1321   | Hyphal cell wall protein       | Biofilm formation, cell adhesion, pathogenesis, cell wall organisation | 6.43                | 6.47E-15 |
| <i>ECE1</i> | orf19.3374   | Hyphae specific protein        | Biofilm formation  | 6.41                | 8.41E-14 |
| <i>SOD5</i> | orf19.2060   | Superoxidase dismutase         | Response to chemicals/stress, interspecies interactions                | 6.18                | 1.42E-9  |
| <i>SAP8</i> | orf19.242    | Protease                       | Other  | 5.98                | 2.03E-16 |
| <i>EXG2</i> | orf19.2952   | Cell wall protein              | Carbohydrate metabolic process   | 5.79                | 7.07E-13 |
| <i>HGT9</i> | orf19.644    | Glucose transporter            | Transport  | 5.66                | 4.57E-11 |

Table 3.6 - Genes with greatest regulation in *C. albicans* isolates with LBF by RNA-Seq

| LBF          |            |                               |   |                     |          |
|--------------|------------|-------------------------------|---|---------------------|----------|
| Gene         | ID         | Function                      | Functional group (Figure 3.11)  | Log <sub>2</sub> FC | p value  |
| <i>POL93</i> | orf19.6078 | Retrotransposon               | DNA metabolic process   | -10.42              | 1.34E-26 |
| <i>YWP1</i>  | orf19.3618 | Yeast wall protein            | Biofilm formation, interspecies interactions  | -9.04               | 4.09E-20 |
| <i>DAG7</i>  | orf19.4688 | Secretory protein             | Response to chemicals/drugs   | -7.83               | 1.91E-19 |
| <i>PHO87</i> | orf19.2454 | Putative phosphate permease   | Regulation of biological process, transport   | -7.38               | 6.43E-23 |
| <i>PGA16</i> | orf19.848  | Putative GPI-anchored protein | Biological process  | -6.98               | 3.28E-6  |
| <i>RBR1</i>  | orf19.535  | Cell wall anchored protein    | Filamentous growth  | -6.90               | 2.93E-11 |
| <i>AQY1</i>  | orf19.2849 | Aquaporin water channel       | Biofilm formation, cell development, transport, response to stress, cell cycle  | -6.85               | 3.52E-16 |
| <i>FMA1</i>  | orf19.6837 | Putative oxidoreductase       | Other   | -6.77               | 3.81E-23 |
| <i>HSP70</i> | orf19.4970 | Chaperone into host cells     | Regulation of biological process, transport, protein folding, interspecies interactions, response to chemicals/stress | -6.54               | 1.92E-10 |
| <i>PCL1</i>  | orf19.2649 | Cyclin homolog                | Response to stress/chemicals/drugs, cell cycle, filamentous growth  | -6.22               | 4.04E-17 |

Although the ten genes with the highest regulation from RNA-Seq are shown in Table 3.5, a number of unknown genes were also shown to have high expression in LBF (n=7) and HBF (n=13). In the HBF group, *HWP1* and *ECE1* were shown to be 6.4 fold greater than in LBF, both of which are known to be associated with hyphal cells. Other genes of interest that were up-regulated in HBF include a number of cell wall and membrane proteins (*EXG2* and *ATO1*). *YWP1* had the second greatest expression (>9×) of all genes up-regulated in *C. albicans* LBF, a gene associated with the presence of yeast cells. Other genes that had increased expression in LBF include a number of proteins, including *PHO87* (7.38×) and *PGA16* (6.98×), a phosphate permease also found in *Saccharomyces cerevisiae* and a predicted cell surface protein, respectively.

### 3.5 Discussion

In the previous chapter it was shown that biofilms formed by different clinical isolates of *C. albicans* did not form biofilms in a homogenous fashion. Instead, there was significant variation in their ability to form and maintain biofilm structure, which was shown not only to impact biofilm formation, but also pathogenicity and antifungal sensitivity. Understanding why these isolates differ would be useful in developing better management strategies, both in terms of antifungal therapy, but potentially also for development of new molecular diagnostics. The data presented herein describes a novel role for eDNA release in conferring heterogeneity during biofilm formation by *C. albicans*, which is in part related to chitinase regulated autolytic events, and which contributes to biofilm associated antifungal resistance.

There have been limited studies to date examining the presence and role of eDNA in *C. albicans* biofilms (Martins et al., 2010, Martins et al., 2012). These studies demonstrate that the quantity of eDNA in biofilms varied considerably with the growth media used, with RPMI-1640 showing a significantly higher accumulation of eDNA in ECM compared to other tested media (Martins et al., 2010). In the previous chapter we showed that biofilm formation was intricately linked with the media in which it was grown, where RPMI-1640 was shown to support the greatest levels of biofilm, and these high biofilm-forming isolates released the greatest quantities of eDNA. Given that eDNA was associated with these high levels of biofilm formation it was hypothesised that this may explain the underlying mechanism for the heterogeneity, i.e. those isolates with the propensity to form biofilms may release more eDNA than isolates forming structurally simple biofilms. This is supported by the data showing that addition of exogenous DNA has been shown to significantly improve biofilm biomass (Rajendran et al., 2013, Shopova et al., 2013). In addition, a recent study by Sapaar and colleagues (2014) showed that addition of homologous eDNA favours yeast to hyphal transition, which in turn enhances biofilm formation by *C. albicans* (Sapaar et al., 2014). Moreover, there are studies highlighting the importance of eDNA within mixed *C. albicans* and bacterial biofilms (Pammi et al., 2013b). Based on two biomass quantification assays in this study, the levels of eDNA observed were greater in isolates with HBF, which also increased with biofilm

maturity, a similar finding to that reported in studies of *A. fumigatus* biofilm development (Rajendran et al., 2013).

The release of eDNA in fungal biofilms is hypothesised to be an end product of autolysis, a process controlled by various hydrolases, including chitinases (Rajendran et al., 2013, White et al., 2002). *C. albicans* has also been shown to possess complex chitinase families that hydrolyse chitin molecules in the fungal cell wall (Gooday et al., 1992). These enzymes are known to be involved in spore formation, hyphal growth, hyphal branching and septum formation (Gooday et al., 1992). There are four chitinase genes known in *C. albicans* (*CHT1-4*), though their function remains unclear. *CHT2* and *CHT3* have been shown to be associated with yeast to hyphal morphogenesis and are more active in the hyphal form (Selvaggini et al., 2004). Here it was reported that these enzymes were transcriptionally expressed and biochemically active during the release of eDNA, which in turn contributes towards biofilm formation. Indeed, correlation analysis demonstrated the relationship between eDNA release, chitinase activity and biofilm formation. Furthermore, a positive correlation between chitinase activity and a hyphal cell marker *HWP1* confirms the involvement of chitinases during hyphal growth, which in turn contributes to eDNA release. This may explain the morphological differences between the HBF and LBF, of which the former demonstrates more filamentous growth. It was also shown that compromising chitinase activity through gene deletion of *cht2Δ*, *cht3Δ* and *cht2/3Δ* significantly affects morphology, biofilm formation and eDNA release, which suggests a role for chitinases in the maintenance and architecture of *C. albicans* biofilms.

Chitinases have also been shown to be involved in tolerance to cell wall stress following antifungal challenge (Kaneko et al., 2010b), and may also influence chitin synthesis. This has implications for the high levels of antifungal resistance in the HBF isolates, particularly in relation to azoles (data not shown). eDNA release was shown to improve AMB sensitivity, demonstrated through DNase combinational treatments, and also through pharmacological inhibition of chitinases that may regulate eDNA release, which was also previously demonstrated in *A. fumigatus* biofilms (Rajendran et al., 2013). Nevertheless, the difference was minimal and

therefore suggests that other mechanisms may also contribute to the variation in biofilms within *C. albicans* clinical isolates.

Using RNA-Seq, a number of genes were identified as having being highly up-regulated in either LBF or HBF, including a number of cell wall proteins. An obvious finding was the high expression of *HWP1* as this is a hyphal specific gene known to be involved in biofilm formation (Staab et al., 2013, Nobile et al., 2006b). Recently, RNA-Seq was used to compare the differential expression of genes in *C. albicans* planktonic cells against those grown in a biofilm, where it was found 1,599 genes were up-regulated by at least 2-fold in the latter (Nobile et al., 2012). Amongst these was *HWP1*, which when knocked out resulted in a significant reduction of biomass, confirming its role in biofilm formation. This study has also identified *SAP8* as a gene highly expressed in *C. albicans* isolates with HBF, a protease previously identified to be highly expressed in mature biofilms of denture stomatitis patients (Ramage et al., 2012a). Another gene with increased expression in *C. albicans* HBF was *HGT9*, a known glucose transporter. The effect of glucose on *C. albicans* adhesion and biofilm formation has shown to be related (Nikawa et al., 1997, Jin et al., 2004, Santana et al., 2013), therefore the up-regulation of *HGT9* expression in this study may be due to the increased utilisation of carbon sources in isolates with HBF.

It was unsurprising that *YWP1* expression was significantly expressed in *C. albicans* LBF as this gene is absent in hyphal cells, where cells lacking this gene are more adhesive and can form thicker biofilms (Granger, 2012). In addition, despite the function of the secretory protein *DAG7* being unknown, the increased expression found in LBF here confirms a previous study that showed up-regulation of *DAG7* in yeast cultures (Sorgo et al., 2010). Interestingly, *PHO87* a gene associated with phosphate uptake was increased in isolates with LBF, with acid phosphatase levels previously shown to be associated with lower virulence *in vivo* (MacCallum et al., 2009b). Furthermore, when *C. albicans* clinical isolates are exposed to phosphate limiting conditions there was a significant increase in biofilm formation (Romanowski et al., 2012). Therefore, the identification of increased gene expression of *YWP1*, *DAG7* and *PHO87* in clinical samples may indicate the presence

of an isolate with LBF, which has implications for more conservative approaches to antifungal therapy, i.e. azole use.

Collectively, this data has provided a base for understanding the mechanisms that may lead to differences in biofilm variation within *C. albicans* isolates, which has been shown not to be reliant on one pathway. The main objective is to identify a potential marker of *C. albicans* biofilm formation that could be transferred into the clinical setting and used as a diagnostic marker, which in turn could help improve antifungal therapy and patient outcomes. Tumbarello and colleagues identified inadequate antifungal therapy as a predictor of patient mortality (Tumbarello et al., 2007), with other studies highlighting the importance of efficient and appropriate treatment of candidaemia cases (Almirante et al., 2005, Garey et al., 2006). Mismanagement of antifungal therapy has important repercussions, as it has been shown that treatment with sub-MIC levels of antifungals causes an increase in chitin content within *Candida* species, which relates to decreased antifungal sensitivity (Walker et al., 2013, Walker et al., 2008). Moreover, a recent study has shown the use of sub-lethal concentrations of antibiotics leads to increased autolysis, eDNA release and biofilm formation in bacteria (Hsu et al., 2011).

Therefore, it is of great interest to develop a molecular diagnostic for *Candida* biofilm infections, as this would be able to help direct more appropriate treatment, preventing an increase in antifungal resistance due to the misuse of antimicrobials and ultimately decrease hospital lengths of stay. Further pathway analysis of the RNA-Seq data may prove useful in identifying specific key pathways associated with the different biofilm groups.

#### CHAPTER FINDINGS:

eDNA is abundant in *C. albicans* isolates with HBF, where its release in biofilms is partly controlled by chitinases.

The addition of DNase in combinational therapy increases the susceptibility of antifungals to isolates with HBF, including AMB.

A number of specific proteins associated with *C. albicans* cell structure were identified for having increased expression within these biofilm-subsets, and therefore could serve as potential diagnostic target.

Chapter 4:  
Assessing the activity of  
a novel antifungal agent

## 4.1 Introduction

Oral diseases such as oral candidosis, are characterised by microbial biofilms (Coco et al., 2008), which are difficult to control with standard chemotherapeutic approaches and host immune defences (Ramage et al., 2010). These biofilm consortia have a community structure, dominant metabolic processes and inter-organism interactions. Antimicrobial resistance is a key characteristic of biofilms, which is associated with exopolymeric matrix, increased cell density, persister cells and up-regulation of efflux pumps (Ramage et al., 2009). Recent studies by our group demonstrated that *C. albicans* biofilms were refractory to prescription antifungal agents, mouthwashes and denture cleansers (Jose et al., 2010, Ramage et al., 2011a), which may explain why oral candidosis is a frequent problem within immunocompromised and elderly populations. Moreover, defined azole resistance mechanisms and the presence of *Candida* spp. that are inherently resistant to azoles (*C. glabrata* and *C. krusei*), complicate clinical management even further (Arendrup, 2013, Guinea et al., 2013). There is now a growing body of literature to demonstrate a key role for *Candida* biofilms in oral candidosis (Coco et al., 2008, Rautemaa and Ramage, 2011), which is associated with inflammation and symptoms such as pain, burning sensation and altered taste (Samaranayake et al., 2009a). Overall, these factors complicate clinical management, therefore alternative agents that elicit antifungal activity are of clinical interest.

At a time where there is growing evidence of antifungal resistance, it is a high priority that alternative novel compounds, such as naturals, are assessed for their ability to prevent and treat fungal infections (Arendrup, 2014, Niimi et al., 2010, Ramage et al., 2012c). Natural compounds have previously been shown to be advantageous for use in the management of fungal infections, including tea tree oil (TTO) and polyphenols (Evensen and Braun, 2009, Ramage et al., 2012b). Fulvic acid is a novel antimicrobial molecule that is reported to have antibacterial and antifungal properties (van Rensburg et al., 2000). It has recently been reported to be non-toxic in a rat wound model, in addition to having anti-inflammatory properties (Sabi et al., 2011). A randomized, double blind, controlled trial indicated that fulvic acid was well-tolerated, whereby side effects were limited and improved

some aspects of eczema (Gandy et al., 2011). This colloidal organic acid is a major constituent of humic acids and has been recognised for its biological significance for many years, yet there is minimal scientific understanding on which to support the claims of its biological properties. Fulvic acid can be isolated from the environment or produced from the oxidation of coal or lignite. However, such preparations contain high levels of heavy metals and potentially toxic elements, making their use in humans unsuitable. Recent innovation has seen the development of carbohydrate derived fulvic acid (CHD-FA), a pure form of fulvic acid produced by a patented process to GMP standards (PA107470/GB). It is characterised by a standard infrared spectrograph and is free of heavy metals and environmental pollutants normally found in fulvic acid from environmental sources.

## 4.2 Aims

The aim of the chapter was to investigate the antifungal effects of the novel compound CHD-FA against clinically relevant isolates associated with different clinical manifestations of candidiasis growing as planktonic and sessile cells, and to compare this to conventional antifungal agents. Furthermore, the mode of action of CHD-FA was investigated, in addition to the impact of known *Candida* resistance mechanisms on CHD-FA sensitivity.

The data represented in this chapter has been published in:

**Sherry L, Jose A, Murray C, Williams C, Jones B, Millington O, Bagg J and Ramage G. 2012.** Carbohydrate Derived Fulvic Acid: An *in vitro* investigation of a novel membrane active antiseptic agent against *Candida albicans* biofilms. *Front Microbiol*, 3, 116.

Work from this chapter has been presented orally at the following conferences and meetings:

**Sherry L, Murray C, Jones B, Williams C and Ramage G.** ‘Carbohydrate-derived fulvic acid is a novel antifungal product.’ ECCMID, Milan, Italy. April 2011.

**Sherry L, Murray C, Jones B, Williams C and Ramage G.** ‘Evaluation of Carbohydrate-derived fulvic acid as a novel oral hygiene product.’ Postgraduate research seminar, Glasgow Dental School, Glasgow, UK. November 2012.

**Sherry L, Nile C, Jones B, Williams C and Ramage G.** ‘The potential use of carbohydrate-derived fulvic acid as an antifungal product.’ Fungal Biofilms Conference, Glasgow, UK. May 2013.

## 4.3 Materials and Methods

To investigate the antimicrobial activity of CHD-FA, minimum inhibitory concentrations were determined against various *C. albicans* clinical isolates in planktonic and sessile states. The mode of action of CHD-FA was assessed using various membrane assays, including the release of intracellular ATP, selective fluorescent dyes and inhibition of fungal cell components. Efflux pump activity, extracellular matrix production and heat shock protein 90 (HSP90) were among the resistance mechanisms investigated to determine if CHD-FA sensitivity was impacted.

### 4.3.1 Culture conditions and standardisation

*C. albicans* type strains SC5314, 3153A, CAF2, ATCC 10231, ATCC 90028 and a range of clinical isolates (n=45) obtained from various oral and bloodstream samples, were used for planktonic and sessile sensitivity testing. All isolates were retrieved from Microbank vials (Pro-Lab Diagnostics, Cheshire, UK) stored at -80°C and sub-cultured on to Sabouraud's dextrose agar (SAB [Sigma-Aldrich, Dorset, UK]). Plates were incubated at 30°C for 24 h and maintained at 4°C. A loopful of each isolate was inoculated in to 10 mL of YPD and placed in the orbital shaking incubator at 200 rpm and 30°C for 18 h, as described in 2.3.1.2. Cells were washed with PBS counted using a Neubauer haemocytometer. *C. albicans* cells were standardised to the desired cellular density in RPMI-1640 medium (Sigma-Aldrich, UK) (Ramage et al., 2001a).

### 4.3.2 Biofilm formation

*C. albicans* cells were standardised to  $1 \times 10^6$  cells/mL in RPMI and 200  $\mu$ L dispensed into each well of a 96 well flat-bottom microtitre plate to allow biofilm formation, as previously described in section 2.3.2. Plates were incubated for 24 h at 37°C. Following incubation, the culture medium was discarded and biofilms were carefully washed twice with PBS to remove planktonic cells. The metabolic activity of the biofilm was measured using the XTT metabolic reduction assay (Pierce et al., 2008, Ramage et al., 2001a) and biofilm biomass quantified using the CV assay (Jose et al., 2010).

#### 4.3.2.1 Quantification of biofilm metabolic activity

A semi-quantitative measure of each biofilm was calculated using a formazan salt-based XTT reduction assay (Hawser et al., 1998b), as described in 2.3.6.2. Briefly, a 100  $\mu$ L aliquot of the XTT/menadione solution was added to each pre-washed biofilm, and to control wells containing no organisms (for measurement of background XTT-reduction levels). Plates were then incubated in the dark for 2 h at 37°C. A colorimetric change in the XTT reduction assay, representing a direct correlation of metabolic activity of the biofilm, was then measured at 492 nm in a microtitre plate reader (FluoStar Omega, BMG Labtech, Aylesbury, Buckinghamshire, UK).

#### 4.3.2.2 Quantification of biofilm formation

Quantification of *C. albicans* biofilms was performed using the CV assay, as described in 2.3.2.1. Briefly, biofilms were grown as previously described in 2.3.2 carefully washed with PBS before quantifying the biomass (Jose et al., 2010). Biofilms were air-dried before staining with 0.05% w/v CV solution and then de-stained with 100% ethanol. The contents of the wells were mixed thoroughly by pipetting and transferred to a new 96 well flat-bottom plate for measurement. The biomass was quantified spectrophotometrically by reading at 570 nm in a microtitre plate reader. All absorbance values were blank corrected based upon the negative control where no biofilms were formed.

#### 4.3.3 Antifungal susceptibility testing

The following antifungal agents were used in the course of this study: CHD-FA (Fulhold, Cape Town, South Africa), voriconazole (VRZ, Pfizer Pharmaceuticals, Sandwich, UK), caspofungin (CSP, Merck Sharp & Dohme, Hertfordshire, UK), amphotericin B (AMB, Sigma-Aldrich, Gillingham, UK). All antifungals were prepared at a stock concentration of 2 mg/mL in sterile water and stored at -20°C until required.

#### 4.3.3.1 Planktonic and sessile inhibitory testing

Antifungal testing to determine minimum inhibitory concentrations (MICs) of planktonic cells was performed using the CLSI M-27A broth microdilution method (CLSI, 2008), as described in 2.3.6.1. In brief, each antifungal was serially diluted two-fold in RPMI-1640 in 96 well round-bottom plate and 100  $\mu$ L of  $1 \times 10^4$  cells/mL *C. albicans* cells added to each well. Plates were incubated at 37°C for 24 h with appropriate positive and negative controls included. For sessile susceptibility testing, biofilms were grown for 24 h at 37°C and treated with two-fold serial dilutions of the appropriate agent (Ramage et al., 2001a). Biofilms were incubated at 37°C for a further 24 h. Sessile minimum inhibitory concentrations (SMICs) were determined at 90% inhibition using an XTT metabolic reduction assay (Pierce et al., 2008). Testing of all planktonic and sessile isolates was performed in triplicate.

#### 4.3.3.2 Kill kinetics of antifungals against *C. albicans*

For time-kill studies two type strains (3153A, SC5314) and 2 clinical bloodstream isolates were selected based on their superior and equivalent biofilm properties. Biofilms were grown for 24 h and treated with CHD-FA, VRZ, CSP and AMB at 1, 2 and  $4 \times \text{SMIC}_{50}$ . At 1, 2, 4, 6, 8 and 24 h the biofilms were carefully washed with PBS and the metabolic activity of the biofilms assessed using the XTT metabolic assay as described in 4.3.2.1. Testing of these four isolates for each drug and each time-point was performed with six replicates and repeated on two separate occasions.

#### 4.3.3.3 Biomass disruption by CHD-FA

*C. albicans* biofilms (n=40) were grown for 24 h and treated with 2 and  $4 \times \text{SMIC}_{50}$  for a further 24 h as these concentrations of CHD-FA were highly effective in the time-kill study above. Following treatment the wells were washed carefully with PBS and the biomass of each biofilm quantified using the CV assay, as described in 2.3.2.1.

#### 4.3.4 Scanning electron microscopy

For scanning electron microscopy (SEM) sessile cells were grown directly on Thermanox™ coverslips (Nunc, Roskilde, Denmark) prior to antifungal treatment as described in 2.3.2. Planktonic *C. albicans* cells were standardised (2.3.1.2) then pre-treated with CHD-FA prior to immobilisation on the coverslip. Treatment was performed for 24 h at  $1 \times \text{SMIC}_{90}$  for both planktonic and sessile cells. Following treatment, *C. albicans* cells were washed with PBS and then fixed with 2% para-formaldehyde, 2% gluteraldehyde and 0.15 M sodium cacodylate, and 0.15% w/v alcian blue, pH 7.4 for 18 h. The fixative was carefully discarded and samples were then prepared for SEM processing as previously described in 2.3.3 (Erlandsen et al., 2004). The specimens were then mounted and sputter-coated with gold in an argon filled chamber and viewed under a JEOL JSM-6400 scanning electron microscope. Images were assembled using Photoshop software (Adobe, San Jose, CA, USA).

#### 4.3.5 Membrane permeabilisation assays

To investigate whether CHD-FA interacted with the membrane a PI uptake and ATP release assay were used. *C. albicans* (SC5314) was grown in an overnight broth as described previously (2.3.1.2), standardised to  $5 \times 10^7$  cells/mL in RPMI-1640 and treated with CHD-FA at 4% for 10, 20, 30, 40, 50 and 60 min.

##### 4.3.5.1 PI uptake

Following treatment, cells were centrifuged at 10,000 rpm to remove CHD-FA, washed twice with PBS, stained with 20  $\mu\text{M}$  of PI (Sigma-Aldrich) and incubated at 37°C for 15 min shielded from light to allow the dye to bind to DNA. One hundred microliters of each sample was transferred to a black microtitre plate (Corning Incorporated, NY, USA) and fluorescence measured using a microtitre plate reader at excitation and emission wavelengths 535/617 nm, respectively. Raw data was corrected for background fluorescence and the assay was carried out in triplicate, on two separate occasions. Alternatively, planktonic cells were standardised in RPMI-1640 and plated onto an Ibidi  $\mu\text{Slide VI}$  (Thistle Scientific, Glasgow, UK) and allowed to settle for 30 min. Cells were grown at 37°C and imaged on an inverted Nikon Eclipse TE300 using a  $\times 20$  objective lens for 1 h. Following incubation, 20  $\mu\text{M}$

PI was added to the cells and imaged for 10 min to confirm cell viability. A 4% CHD-FA was added next and cells imaged every 20 s for 1 h to visualise the uptake of PI. These experiments were performed with the help and assistance from Dr Owain Millington from the University of Strathclyde, Glasgow, UK.

#### 4.3.5.2 Measurement of ATP release

Supernatants were retained from cells treated in the PI studies in order to measure ATP release. The supernatants were buffered to pH 7.0 using 10 mM sodium hydroxide and equal volumes of supernatant were added to ATP assay mix (Sigma-Aldrich, UK) which allowed for the measurement of  $2.0 \times 10^{-6}$  -  $2.0 \times 10^{-12}$  moles/L of ATP. The ATP assay mix (lyophilised powder containing  $\text{MgSO}_4$ , DDT, EDTA, BSA and tricine buffers salts) was reconstituted in 5 mL of sterile water to generate a stock solution at pH 7.8. One hundred microlitres of the ATP assay mix was added to a 1.5 mL tube and incubated at room temperature for 3 min, which would allow any endogenous ATP to be hydrolysed, thereby decreasing background levels. Next, 100  $\mu\text{L}$  of the sample or standard was added to the ATP assay mix, vortexed briskly and the amount of luminescence measured in a white 96 well plate (Corning Incorporated, NY, USA) using a microtitre plate reader. Standards were prepared in ten-fold dilutions starting at  $2.0 \times 10^{-6}$  moles/L and used for the production of an ATP calibration curve. Background light was calculated using sterile water as a blank and subtracting this from all samples tested. This assay was carried out in triplicate and repeated on two separate occasions.

#### 4.3.5.3 Chitin synthase inhibition

To further elucidate the mechanism of action of CHD-FA the role of the cell wall was investigated, specifically the role of chitin. To do so a specific inhibitor of the chitin synthesis pathway, nikkomycin Z ([NKZ] Sigma, Gillingham, UK) was used  $\pm$  CHD-FA to delineate mechanisms of action. For planktonic cells CLSI methodologies were employed, with CHD-FA (0.0625 and 0.125%)  $\pm$  NKZ (0.4  $\mu\text{g}/\text{mL}$ ) (Kaneko et al., 2010a), which was incubated for 24 h at 37°C. Sessile cells were grown in the presence of NKZ for 24 h at 37°C then treated with CHD-FA for further 24 h. This was to allow the formation of biofilms with reduced chitin, in turn allow the cell

membrane to be more accessible. Following incubation of both planktonic cells and biofilms the metabolic activity was quantified using the XTT reduction assay (Pierce et al., 2008). This assay was carried out on two individual occasions, using two type and two clinical strains tested in quadruplicate.

#### 4.3.6 Resistance mechanisms

We next investigated whether well-characterised resistance mechanisms in *C. albicans* biofilms impacted the activity of CHD-FA.

##### 4.3.6.1 Stress response

The first resistance mechanism investigated was heat shock protein 90 (HSP90) as it has been shown to be involved in antifungal resistance (Singh et al., 2009). To do so, a specific inhibitor of the Hsp90p, 17-allylamino-17-demethoxygeldanamycin (geldanamycin [Invivogen, San Diego, CA, USA])  $\pm$  CHD-FA was used. Planktonic cells and 24 h biofilms were treated with CHD-FA (0.0625 and 0.125%)  $\pm$  geldanamycin (12.5  $\mu\text{g}/\text{mL}$ ) and incubated for 24 h at 37°C. The concentration of geldanamycin (GDM) used in this study was adapted from a previous investigation by Robbins and colleagues where they used a checkerboard assay to show the impact of various concentrations of GDM on *C. albicans* biofilms. The highest non-toxic concentration of GDM that retained its activity was 12.5  $\mu\text{g}/\text{mL}$  (Robbins et al., 2011). Following incubation the metabolic activity of the cells was quantified using the XTT reduction assay, as previously described. This assay was carried out on two individual occasions, using two type and two clinical strains tested in quadruplicate.

##### 4.3.6.2 Matrix regulation by *FKS1*

The role of the extracellular matrix in *C. albicans* biofilm resistance was also investigated using strains previously described with altered expression of *fks1* (Nett et al., 2010c). Reference strain (DAY185), *FKS1/fks1* $\Delta$  defective in ECM production and *TDH3-FKS1* a strain overexpressing  $\beta$ -glucans and matrix were gifted by Prof David Andes (University of Wisconsin-Madison, WI, USA) (Nett et al., 2010c). These strains were standardised to  $1 \times 10^6$  cells/mL in RPMI-1640 and inoculated in to 96 well flat-bottom plates as described in 2.3.1.2, and biofilms grown for 8 h at 37°C.

After incubation, biofilms were carefully washed three times with PBS and treated with 0.05 and 0.1% CHD-FA for 24 h. Biofilms were then carefully washed with PBS and metabolic activity was measured using the XTT assay, previously described in 4.3.2.1. This assay was carried out on two separate occasions, in triplicate.

#### 4.3.6.3 Efflux pump activity

A further aim was to evaluate whether CHD-FA treatment of *C. albicans* affected efflux pump expression, as it had been shown to be the case for azoles (White, 1997). Efflux pump activity of planktonic and sessile cells was initially assessed using an alanine  $\beta$ -naphthylamine [MC-005,556 (Ala-Nap)] fluorescent assay, as previously described (Rajendran et al., 2010). Ala-Nap is enzymatically cleaved inside the cells to produce highly fluorescent  $\beta$ -naphthylamine. Higher levels of fluorescence reflect low efflux pump activity and vice versa. *C. albicans* SC5314 was prepared as standardised planktonic cells ( $5 \times 10^7$  cells/mL) in buffer solution ( $K_2HPO_4$  [50 mM],  $MgSO_2$  [1 mM], and glucose [0.4%]) at pH 7.0. Planktonic cells were treated with 0.0313% and 0.0625% CHD-FA for 4 and 24 h before being washed with PBS, resuspended in assay buffer and 100  $\mu$ L transferred to a black flat bottomed microtitre plate. For sessile cells, these were grown directly as biofilms for 24 h within the plates and treated with 0.0313% and 0.0625% CHD-FA for 4 and 24 h. Following incubation, biofilms were washed with assay buffer and 100  $\mu$ L of the buffer solution was added to the biofilms. The reaction was initiated by the addition of Ala-Nap at a final concentration of 100  $\mu$ g/mL. Fluorescence was quantified at 30 s intervals for 1 h at 37°C using a fluorescence plate reader at an excitation and emission wavelengths 355/460nm, respectively. Testing was carried out in triplicate, on three separate occasions.

To determine whether efflux pump activity was detrimental to the effects of CHD-FA, an efflux pump inhibitor (EPI) was used, as previously described (Ramage et al., 2011b). EPI (L-Phe-L-Arg- $\beta$ -naphthylamide [MC-207,110], Sigma-Aldrich, Gillingham, UK) was freshly prepared at a working concentration of 1 mg/mL in distilled water. To determine the effects of MC-207,110 on the MIC of CHD-FA, a checkerboard titration assay was performed (Rajendran et al., 2011). CHD-FA was then tested against 24 h biofilms at a range of 0.002 to 1% in combination with MC-207,110 at a

concentration of 64 µg/mL and incubated overnight at 37°C. Biofilms were washed with PBS and viability was assessed using the XTT reduction assay as previously described. Testing was carried out in triplicate, on three separate occasions.

#### *4.3.7 Statistical analysis*

Data distribution and statistical analysis was performed using GraphPad Prism (version 4; La Jolla, CA, USA). Time kill kinetics were log transformed and analysed using a one-way analysis of variance (ANOVA) with Bonferroni correction to determine significant differences between CHD-FA concentrations at each specific time point and between other antifungals. When comparing CHD-FA to the other classes of antifungals, a one-way ANOVA was used with a Dunnett post-test to compare all antifungals to CHD-FA. Non-parametric data from the membrane assays were analysed using the Kruskal-Wallis test with a Dunn's post-test for comparing all time points to one another. Non-parametric data was also analysed using the Mann-Whitney U test to assess differences between two independent sample groups. Statistical significance was achieved if  $P < 0.05$ .

## 4.4 Results

### 4.4.1 CHD-FA exhibits potent antifungal activity

#### 4.4.1.1 Susceptibility of all classes of antifungals against *C. albicans*

All planktonic *C. albicans* isolates tested were susceptible to all four antifungal drugs, with MIC<sub>90</sub> for CHD-FA of 0.125% and VRZ, AMB and CSP of 0.125 mg/L (Table 4.1). For sessile cells the most effective antifungal compound in ascending order based on MIC<sub>90</sub> was CHD-FA (0.25%), CSP (0.5 mg/L) and AMB (8 mg/L). VRZ was unable to kill any of the biofilms tested, even at a concentration of 256 mg/L. Furthermore, the sessile activity of each compound was compared to planktonic cells in terms of MIC<sub>90</sub> fold-change to calculate how much greater concentration of each drug was required to kill sessile communities. CHD-FA was the most effective compound as only 2 × greater concentration was required to kill biofilms than their planktonic counterpart. This was followed by CSP and AMB where 4 × and 64 × higher concentration was required to achieved the same kill potential. As VRZ was unable to reduce the metabolic activity of *C. albicans* biofilms, the fold change was >2048 × as no MIC could be defined for this drug. Both planktonic and sessile cells showed comparable MICs for all fifty strains tested.

In addition, *C. albicans* clinical isolates with LBF (n=10) and HBF (n=10) were assessed for their susceptibility to CHD-FA. This was to determine if one biofilm subset was more sensitive to treatment by a novel agent (Figure 4.1).

Table 4.1 - Susceptibility of *C. albicans* to four antifungal agents

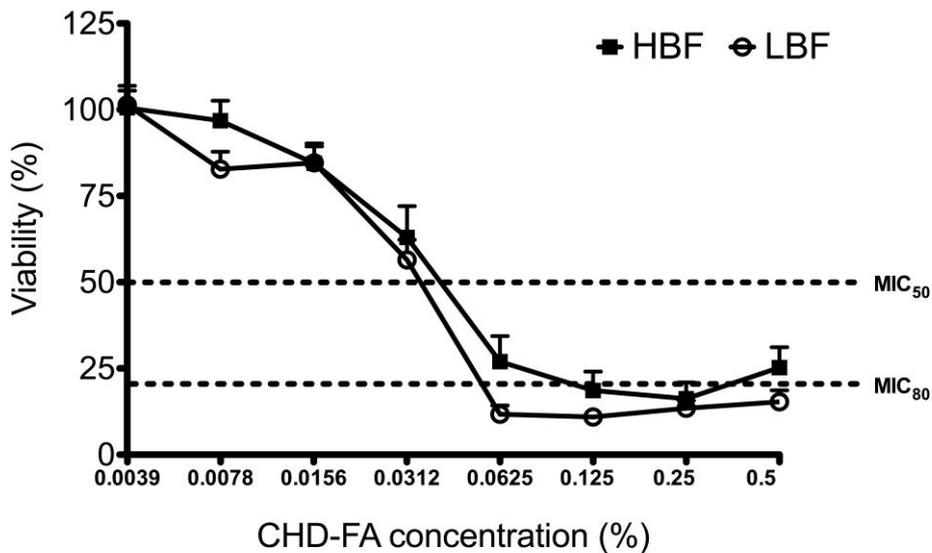
|                   |               | MIC                 |                  |                        |                   |                        |       |                    |  |
|-------------------|---------------|---------------------|------------------|------------------------|-------------------|------------------------|-------|--------------------|--|
|                   |               | VRZ (mg/L)          |                  | AMB (mg/L)             |                   | CSP (mg/L)             |       | CHD-FA (%)         |  |
| n = 50            | PMIC          | SMIC <sub>90</sub>  | PMIC             | SMIC <sub>90</sub>     | PMIC              | SMIC <sub>90</sub>     | PMIC  | SMIC <sub>90</sub> |  |
|                   |               | (fold change)       |                  | (fold change)          |                   | (fold change)          |       | (fold change)      |  |
| Range*            | 0.0625 -<br>2 | >256<br>(>128-4096) | 0.0625 -<br>0.25 | 2 - 256<br>(32 - 1024) | ≤0.0625 -<br>0.25 | ≤0.0625-8<br>(≤1 - 32) | 0.125 | 0.25<br>(2)        |  |
| MIC <sub>50</sub> | 0.125         | >256<br>(>2048)     | 0.0625           | 4<br>(64)              | 0.0625            | 0.25<br>(4)            | 0.125 | 0.125<br>(1)       |  |
| MIC <sub>90</sub> | 0.125         | >256<br>(>2048)     | 0.125            | 8<br>(64)              | 0.125             | 0.5<br>(4)             | 0.125 | 0.25<br>(2)        |  |

\*MIC = range of all isolates

SMIC<sub>90</sub> = 90% inhibition of isolate

MIC<sub>50/90</sub> = % of isolates susceptible to the antifungal concentration

Fold change = difference between planktonic and sessile MIC



**Figure 4.1 - CHD-FA sensitivity is not significantly impacted by biofilm formation.**

Biofilms of ten isolates with LBF (n=10) and HBF (n=10) were grown for 24 h and tested against CHD-FA. Each isolate was tested in duplicate, with data represented the average of each isolate mean + SEM.

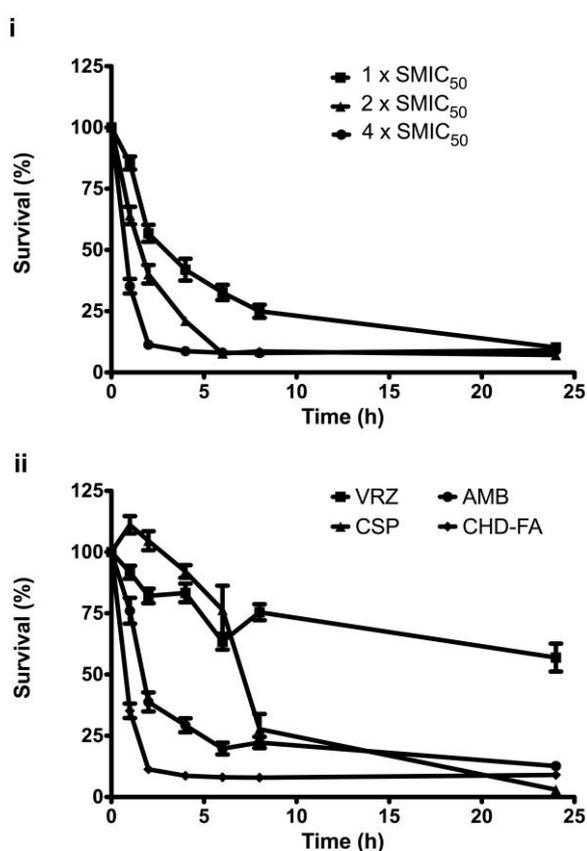
A dose-dependent effect was evident in both biofilm subsets tested with CHD-FA (Figure 4.1). LBF and HBF isolates both had a MIC<sub>50</sub> of 0.0625% CHD-FA, yet isolates with LBF were less viable than those with HBF at this concentration by 16%. In addition, LBF isolates also had and a MIC<sub>50</sub> of 0.0625%, whereas isolates with HBF required 0.125% CHD-FA to reach the same kill. Despite these findings, no significant differences in viability were found between isolates with LBF and HBF at all CHD-FA concentrations. The PMIC and minimum fungicidal concentrations (MFC) was 0.25% for all isolates with LBF (n=10) and HBF (n=10) tested.

#### 4.4.1.2 Kill kinetics of CHD-FA and other antifungals

The biofilm rate of kill of CHD-FA was assessed and dose and time dependent killing was observed (Figure 4.2i). Greater killing was observed after 1, 2 and 4 h at 4 × SMIC<sub>50</sub> in comparison to 1 and 2 × SMIC<sub>50</sub>, where a decrease in viability of 65% and 89% was observed (p<0.0001). Significant differences in cellular viability were observed between all CHD-FA concentrations at each time point except when 2 × and 4 × SMIC<sub>50</sub> were compared at 6, 8 and 24 h and 1 × and 4 × SMIC<sub>50</sub> at 24 h. Furthermore, following a 24 h treatment with 2 × SMIC<sub>50</sub>, *C. albicans*

viability was significantly reduced to 7% compared to 10% achieved by  $1 \times \text{SMIC}_{50}$  ( $p < 0.05$ ).

When each antifungal was compared at  $4 \times \text{SMIC}_{50}$  then CHD-FA displayed the quickest killing compared in ascending order to AMB, VRZ and CSP, which after 2 h was 89%, 61%, 18% and -5% ( $p < 0.0001$ ), respectively (Figure 4.2ii). In fact, CHD-FA was significantly more effective than any other antifungal agent up to 8 h treatment ( $p < 0.0001$ ). However, after 24 h CSP elicited the most potent effect by reducing cell viability by 97% when compared to the 91% reduction achieved by CHD-FA ( $p < 0.05$ ). After 24 h treatment, AMB and VRZ reduced *C. albicans* viability by 88% and 43%, respectively.

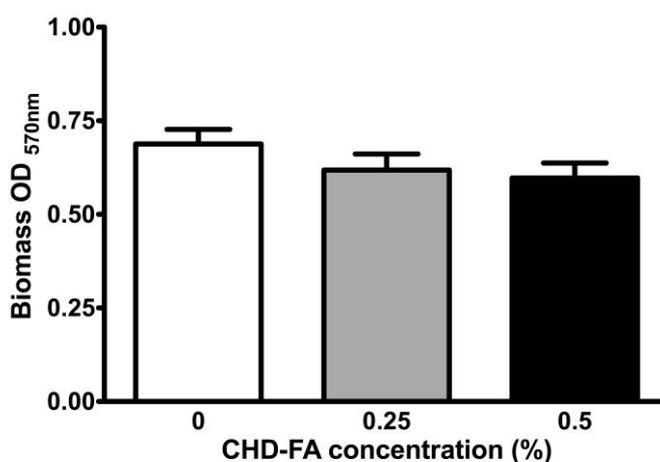


**Figure 4.2 - *C. albicans* biofilm killing by CHD-FA is time and concentration dependent.**

Biofilm time-kill kinetics of CHD-FA at 1, 2 and  $4 \times \text{SMIC}_{50}$  (i). Biofilm time-kill kinetics of VRZ, CSP, AMB and CHD-FA at  $4 \times \text{SMIC}_{50}$  (ii). Standardised *C. albicans* ( $1 \times 10^6$  cells/mL) were grown in flat bottom 96 well plates for 24 h, washed in PBS and treated with antifungal agents at defined concentrations for 1, 2, 4, 6, 8 and 24 h. Metabolic activity of treated biofilms was then quantified using the XTT assay. Four isolates were used for each assay, and this was performed on two independent occasions in triplicate.

#### 4.4.1.3 CHD-FA does not disrupt biofilms

The anti-biofilm activity of CHD-FA has been shown to be superior to other classes of antifungals when compared to planktonic cells. Therefore, the disruptive properties of CHD-FA were next investigated using 2 and 4 × SMIC<sub>50</sub> as these concentrations showed greater activity in the time kill study. Despite the highly fungicidal activity of CHD-FA in the earlier part of this study, there was no significant reduction in biofilm biomass when 2 and 4 × SMIC<sub>50</sub> were used (Figure 4.3), compared to a PBS control (p=0.2318 and 0.1069, respectively).

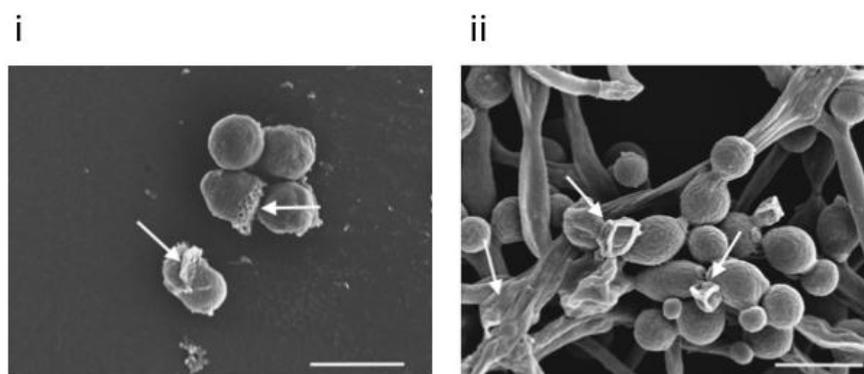


**Figure 4.3 - CHD-FA does not disrupt *C. albicans* biofilms.**

Standardised *C. albicans* ( $1 \times 10^6$  cells/mL) were grown in flat bottomed 96 well plates for 24 h, washed in PBS and treated with 0.25 and 0.5% CHD-FA for 24 h. The biomass was stained with 0.05% w/v crystal violet solution and quantified spectrophotometrically by reading at 570 nm. Forty isolates were tested during this assay, which was performed in triplicate.

#### 4.4.1.4 CHD-FA causes ultrastructural changes in *C. albicans* cells

Planktonic and sessile *C. albicans* cells were treated with CHD-FA and imaged using a scanning electron microscope to visualise any ultrastructural changes in the cells. Deterioration of the general cell structure of both planktonic (Figure 4.4i) and sessile cells (Figure 4.4ii) was observed, of which the latter appear to be crenated.

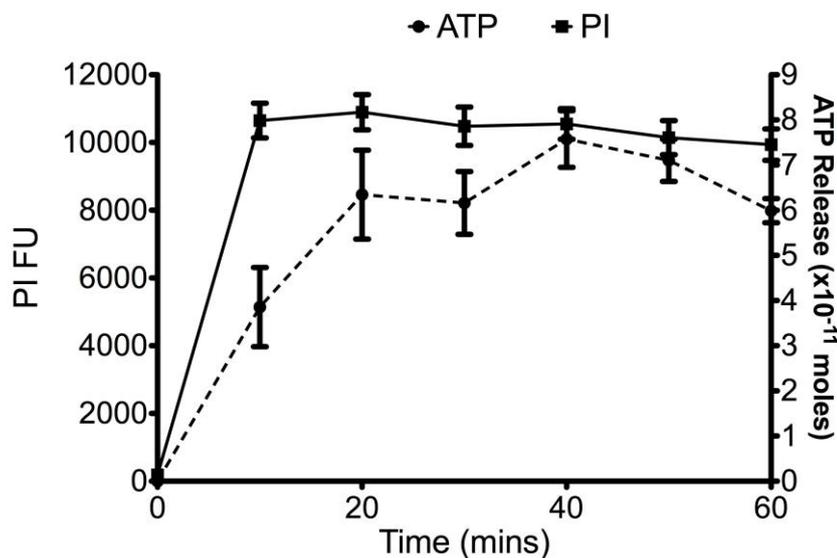


**Figure 4.4 - CHD-FA causes crenation of *C. albicans* planktonic and sessile cells.**

Planktonic (i) and sessile (ii) cells were treated at their respective MIC<sub>90</sub> for 24 h either in solution or on Thermanox™ coverslips, respectively. These were then processed and viewed on a JEOL JSM-6400 scanning electron microscope. Note the apparent disruption of the cell wall denoted by arrows on both the planktonic and sessile cells. Scale bars represent 5 μm.

#### 4.4.2 CHD-FA permeabilises *C. albicans* cell membranes

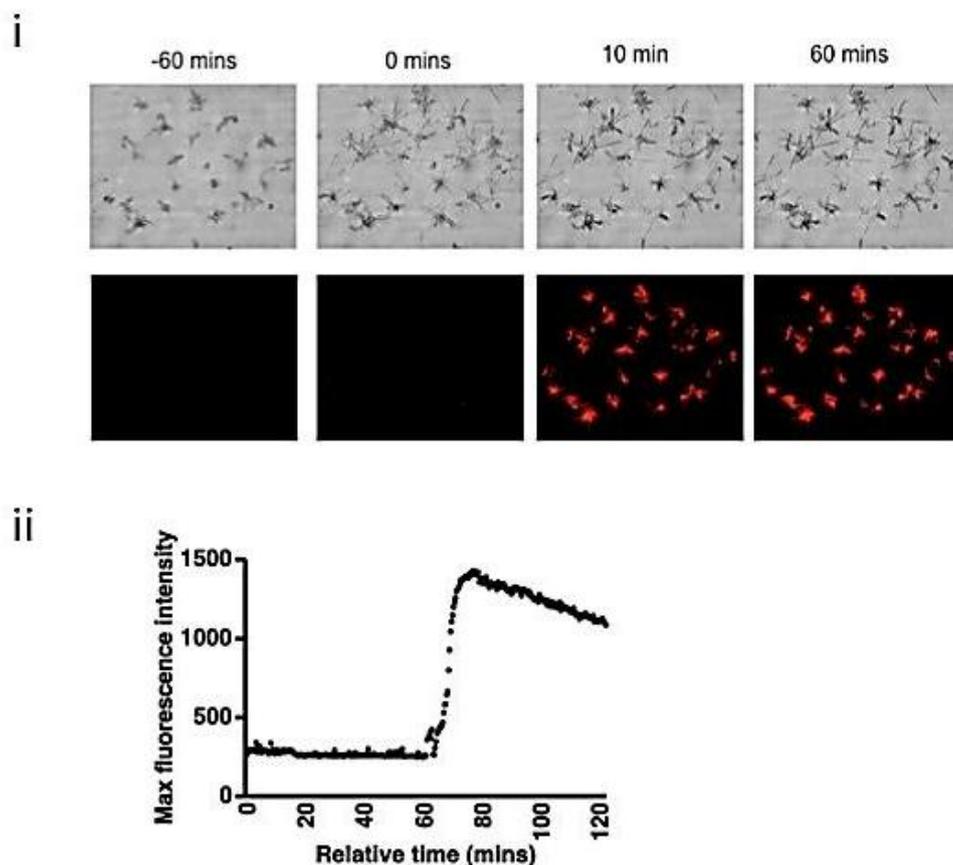
Based on microscopy images it was hypothesised that the cell membrane may have been destabilised, so membrane permeability assays were performed including the measurement of ATP release and uptake of PI.



**Figure 4.5 - CHD-FA permeabilises cell membranes.**

*C. albicans* (SC5314) planktonic cells were treated with 4% CHD-FA for 0 to 60 min. For PI experiments the cells were washed and resuspended in 20 μM PI and fluorescence measured at Ex<sub>485</sub>/Em<sub>620</sub>. For ATP release the cells were quantified using a luminescent plate reader. Each assay was performed on at least two independent occasions in triplicate.

ATP was released in a time dependent manner, with a significant increase ( $p=0.0012$ ) after 40 min exposure to CHD-FA (Figure 4.5). These observations were confirmed through quantification of PI, where uptake was shown to be rapid over the first 10 min ( $p<0.0001$ ), after which this plateaued and no further significant increase in PI fluorescence was seen at later time points. The uptake of PI by the cells was also confirmed visually using time-lapse microscopy.



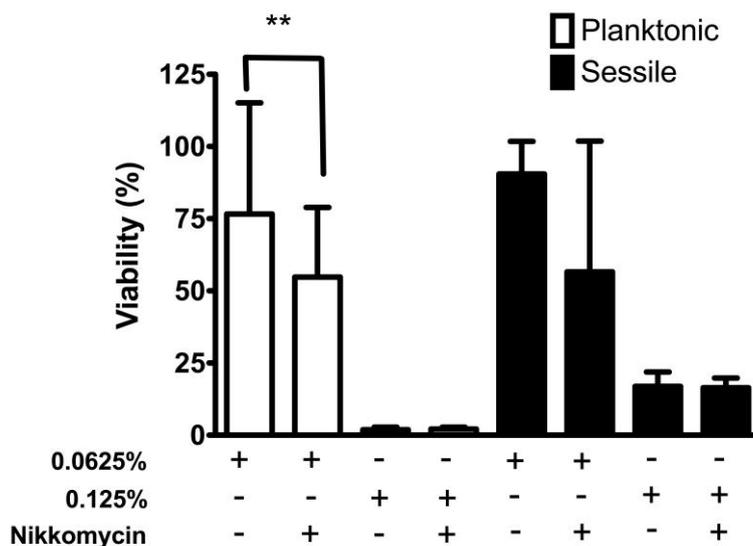
**Figure 4.6 - *C. albicans* cell membranes become rapidly compromised with CHD-FA treatment.**

*C. albicans* cells were allowed to grow on an Ibidi  $\mu$ Slide VI for 60 min before 20  $\mu$ M PI and 2% CHD-FA was added. PI uptake was measured over 60 min using time-lapse microscopy, with images taken every 20 sec (i). Note the sudden uptake of PI into *C. albicans* cells after exposure to CHD-FA for 10 min. Fluorescence was also quantified during CHD-FA treatment (ii).

Using time-lapse microscopy, uptake of PI was shown to occur rapidly following addition of CHD-FA, with detectable membrane permeability within 10 min of exposure to CHD-FA, with all cells becoming PI-positive and cell growth attenuated (Figure 4.6i, ii).

To further elucidate the mechanisms of action, the impact of chitin was investigated using its inhibitor NKZ. It was hypothesised that by weakening the

Ch. 4 Assessing the activity of a novel antifungal agent  
chitin layer this would enable CHD-FA to have greater activity, if indeed the cell  
membrane was the target.



**Figure 4.7 - *C. albicans* has increased sensitivity to CHD-FA when the cell membrane is accessible.**

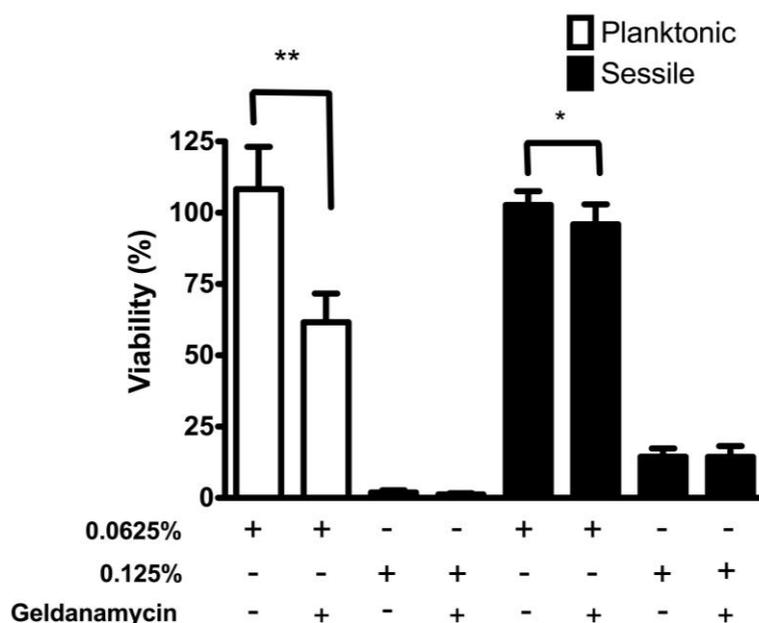
*C. albicans* was grown as planktonic and biofilm cells  $\pm$  NKZ (0.4  $\mu\text{g}/\text{mL}$ ) for 24 h and treated with 0.0625% and 0.125% CHD-FA for 24 h. Metabolic activity of treated biofilms was then quantified using the XTT assay. Four isolates were used for each assay, and this was performed on two independent occasions in quadruplicate. Data represents mean  $\pm$  SD. \*\* $p < 0.01$ .

*C. albicans* planktonic cells treated with 0.0625% CHD-FA in the presence of NKZ were found to be significantly more sensitive to CHD-FA ( $p = 0.0022$ ) than NKZ free cells (Figure 4.7), which is demonstrated by a 21% reduction in cell viability. Treatment of *C. albicans* biofilm treatment showed a ~34% reduction in cell viability, however, this was not statistically significant ( $p = 0.1057$ ). In both planktonic and sessile states, NKZ showed no effect on cell viability (data not shown).

#### 4.4.3 The impact of *C. albicans* resistance mechanisms on CHD-FA

##### 4.4.3.1 *C. albicans* stress responses

The roles of key resistance mechanisms utilised by *C. albicans* were next investigated to determine whether they were likely to impact the effectiveness of CHD-FA. A Hsp90 inhibitor was used to determine if *C. albicans* stress responses were responsible for reduced CHD-FA sensitivity.



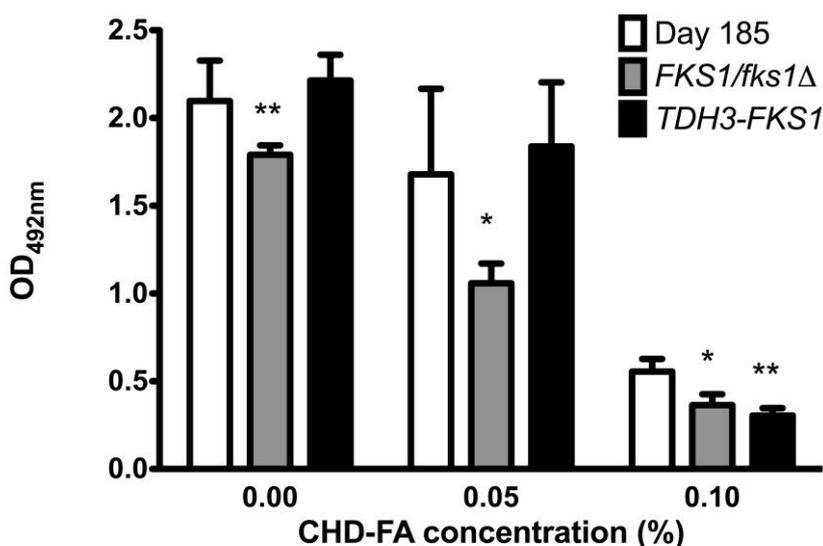
**Figure 4.8 - *C. albicans* stress responses play a limited role in CHD-FA activity.**

*C. albicans* was grown as biofilm  $\pm$  GDM (12.5  $\mu\text{g}/\text{mL}$ ) for 24 h and treated alongside planktonic cells with 0.0625 and 0.125% CHD-FA for 24 h. Metabolic activity of treated biofilms was then quantified using the XTT assay. Four isolates were used for each assay, and this was performed on two independent occasions in quadruplicate. \* $p < 0.05$ , \*\* $p < 0.01$ .

*C. albicans* susceptibility to 0.0625% CHD-FA (sub-MIC) in the presence of the Hsp90 inhibitor GDM was significantly increased in planktonic ( $p = 0.0009$ ) and sessile ( $p = 0.0133$ ) cells (Figure 4.8), reducing the cell viability by 49% and 7%, respectively. No significant differences were observed for both planktonic and sessile cells treated at MIC levels (0.125%) in the presence of GDM. In both cell states, GDM showed no effect on cell viability.

#### 4.4.3.2 The impact of extracellular matrix

The role of *FKS1* expression in *C. albicans* biofilm resistance was assessed to determine if CHD-FA was sequestered by extracellular matrix  $\beta$ -1, 3 glucan (Figure 4.9).



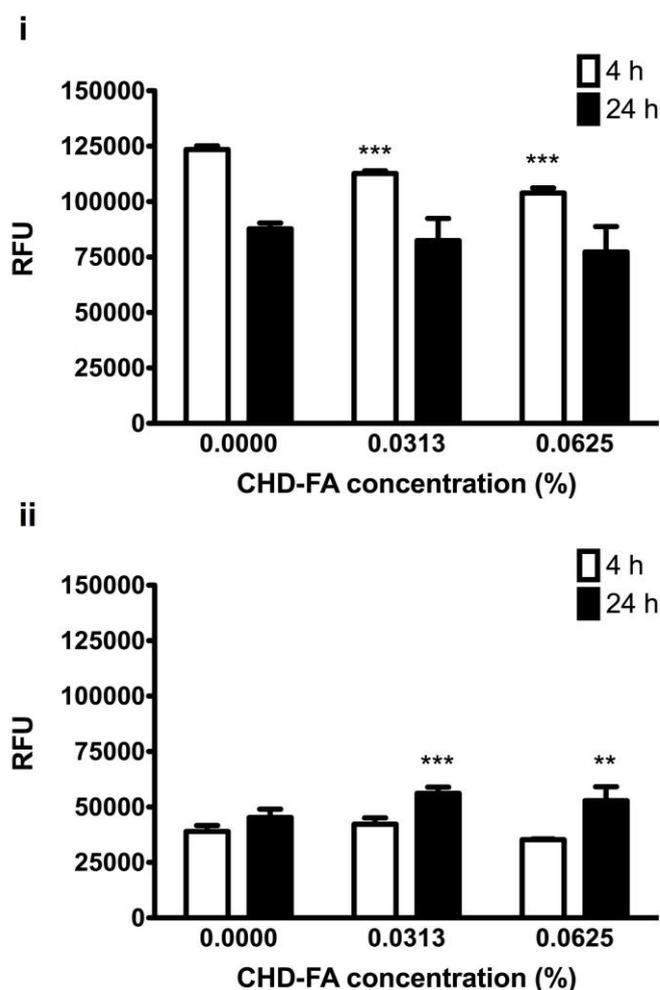
**Figure 4.9 - Extracellular matrix does not sequester CHD-FA.**

*C. albicans* DAY185, *FKS1/fks1Δ* and *TDH3-FKS1* were grown as biofilms for 8 h and treated with 0.05 and 0.1% CHD-FA for 24 h. Metabolic activity of treated biofilms was then quantified using the XTT assay. This was performed on two independent occasions in triplicate. Statistical significance represents the comparison of *FKS1/fks1Δ* to the reference strain DAY185. Data represents mean + SD. \* $p < 0.05$ , \*\* $p < 0.005$ .

Figure 4.9 shows that *FKS1/fks1Δ* biofilms were significantly more susceptible to CHD-FA at sub-MIC levels (0.05%) compared to the treated parental strain DAY185 ( $p = 0.0126$ ), with cellular metabolic activity being reduced by 40%. In comparison, the over-expressing strain, *TDH3-FKS1*, was less sensitive to 0.05% CHD-FA, with cell survival 10% greater than the reference strain ( $p = 0.3095$ ).

#### 4.4.3.3 Efflux pump activity

Finally, the role of efflux pumps in CHD-FA sensitivity was investigated using a fluorescent dye (Ala-Nap) and an efflux pump inhibitor (Figure 4.10).



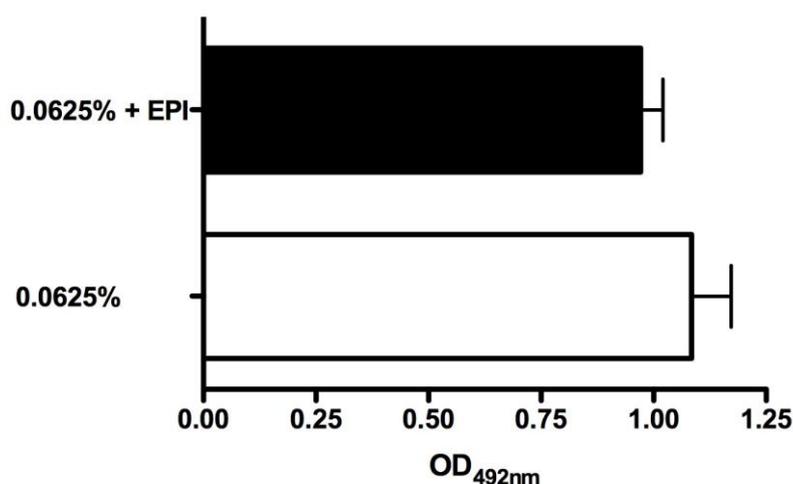
**Figure 4.10 - Efflux pumps have a limited role in CHD-FA sensitivity.**

*C. albicans* SC5314 was grown as planktonic (i) and sessile (ii) cells and treated with 0.0313 and 0.0625% CHD-FA for 4 and 24 h. Cells were resuspended 100 µg/mL of Ala-Nap fluorescence read at 30 s intervals over 60 min ( $Ex_{355}/Em_{460}$ ). For biofilms the relative fluorescence is presented, which is normalised to dry weight of biofilms (RFU/mg). High fluorescence values indicate low efflux activity and vice versa. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

CHD-FA treated planktonic cells exhibited a time and dose dependent up-regulation of efflux pump activity compared to untreated cells (Figure 4.10i). As the CHD-FA concentration increased from 0% to 0.0313% and 0.0625% there was significant up-regulation of efflux pump activity, indicated by low fluorescence, after treatment for 4 h ( $p < 0.0001$ ), but not after 24 h ( $p = 0.2581$  and  $0.0625$ , respectively). For sessile cells a general increase in efflux pump activity was observed compared to planktonic cells. However, no significant increase in efflux pump activity was reported when the biofilms were exposed for 4 h to 0.0313% and 0.0625% CHD-FA ( $p = 0.3401$  and  $0.0503$  respectively). Conversely, 24 h treated biofilms showed a minor down-regulation of efflux pumps compared to

Ch. 4 Assessing the activity of a novel antifungal agent the untreated control at these concentrations ( $p=0.0003$  and  $0.0019$ , respectively) (Figure 4.10ii).

Due to differential activity of efflux pumps in *C. albicans* cells treated with CHD-FA, an efflux pump inhibitor (L-Phe-L-Arg- $\beta$ -naphthylamide [MC-207,110], combined with CHD-FA was used to determine if cells became more sensitive to CHD-FA when efflux pumps were inhibited.



**Figure 4.11 - An efflux pump inhibitor does not increase the sensitivity of CHD-FA to *C. albicans* cells.**

*C. albicans* was grown as biofilms for 24 h and treated with 0.0625% CHD-FA  $\pm$  EPI for a further 24 h. Metabolic activity measured using XTT assay. Testing was carried out in triplicate and was performed on three independent occasions.

There was no significant reduction in cell activity when 0.0625% CHD-FA and EPI were used in combination ( $p=0.0569$ ), despite a 10% reduction in viability (Figure 4.11).

## 4.5 Discussion

*C. albicans* biofilm-related diseases are generally refractory to prescription antibiotics and antifungal agents (Ramage et al., 2012c). Studies from our group recently reported that commercial mouthwashes were superior to antifungals and a more favourable chemotherapeutic option for treating oral candidal biofilm infections (Ramage et al., 2011a). However, even with chlorhexidine containing products, residual viable biofilm cells are retained, so novel compounds are still urgently required. For the first time it is reported that a potential novel antifungal agent, CHD-FA, has potent antifungal activity against *C. albicans* biofilms. Furthermore, it is proposed that this compound is effective through disruption of the cellular membrane and is unaffected by biofilm resistance mechanisms.

Initial studies aimed to evaluate the activity of CHD-FA and the three major classes of antifungal agent, namely azoles, polyenes and echinocandins, using a panel of clinical *C. albicans* strains. This was primarily to assess the anti-biofilm activity of each drug compared to planktonic cells rather than direct antifungal drug comparison. CHD-FA was shown to kill rapidly and be similarly effective against both planktonic and sessile cells for all strains, whereas the existing antifungal agents exhibited increased resistance in the biofilm phenotype. This is in agreement with earlier studies by our group, and others, who reported high level azole resistance and decreasing sensitivity of polyenes and echinocandins (Kuhn et al., 2002b). Furthermore, CHD-FA sensitivity was not impacted by the presence of an isolate with LBF or HBF. This is in contrast to AMB where a significant difference in MIC was observed in Chapter 2 between these biofilm subsets.

Disruption of the biofilm was also assessed, but despite CHD-FA being highly fungicidal no significant reduction of the biofilm biomass was observed. Nevertheless, given the effectiveness of this compound then it would be possible to combine CHD-FA with a known *C. albicans* biofilm disrupting compound, such as lauroyl glucose (Dusane et al., 2008). Lauroyl glucose is a sugar ester that exhibits antimicrobial and emulsification properties (Ferrer et al., 2005), which contribute to the disruption of pre-formed *C. albicans* biofilms by up to 45%

Ch. 4 Assessing the activity of a novel antifungal agent (Dusane et al., 2008). Therefore, these and other novel biofilm disruption agents could possibly be used to augment the activity of CHD-FA.

The use of SEM demonstrated the appearance of crenated cells, both planktonically and in biofilms. Visually, these appear similar to polyene treated cells, which integrates into and actively disrupts cell membranes, lysing their cytoplasmic contents (Gale, 1963). It was therefore hypothesised that CHD-FA activity was potentially based on interaction with the cell membrane; an idea supported from the activity of CHD-FA against bacteria (van Rensburg et al., 2000). Membrane permeabilisation assays were therefore used to confirm our hypotheses.

The measurement of intracellular ATP release has been previously described as an appropriate method to measure membrane permeability, as ATP leakage is not observed in viable cells (Smelt et al., 1994). It was shown that ATP release occurred in a steady manner with maximum release being observed after 40 min CHD-FA exposure, with a slight reduction observed thereafter; a similar phenomenon to a previous report (Smelt et al., 1994). PI uptake was in agreement with ATP release, again showing a time dependent disruption of the cell membrane. It was demonstrated that *C. albicans* membrane integrity is compromised rapidly after exposure to CHD-FA, data supported visually from our fluorescent images showing PI uptake in as little as 1 min post-exposure (Ahmad et al., 2011).

To further test this cell membrane hypothesis the cell wall was weakened by inhibiting chitin biosynthesis using the chitin synthase inhibitor NKZ, which has been shown to inhibit all three Chs isoenzymes in *C. albicans*, and in turn results in a lack of chitin in fungal cells (Kim et al., 2002). It was reasoned that if the membrane were the active target then the weakened cell would become hyper-susceptible to CHD-FA. It was shown that NKZ in combination with CHD-FA made both planktonic and sessile cells more susceptible to CHD-FA, which is similar to previous work showing that NKZ in combination with azoles increased *C. albicans* biofilm susceptibility (Kaneko et al., 2010a).

Our group, amongst others, have shown the importance of biofilms in antimicrobial resistance, a multifactorial process (Ramage et al., 2009). A

further aim was therefore to evaluate the potential effects of key biofilm resistance mechanisms on CHD-FA. The major regulator of stress in *C. albicans* is Hsp90, which has been shown to potentiate the emergence and maintenance of resistance to azoles and echinocandins in *C. albicans* biofilms, at least in part via calcineurin (Robbins et al., 2011). This study has shown that Hsp90 plays a role in stress induced resistance, as planktonic and sessile *C. albicans* cells were more susceptible to CHD-FA at a sub-MIC level when GDM, the Hsp90 inhibitor, is used in combination. Therefore, stress induced resistance mechanisms appear to play a role in *C. albicans* response to CHD-FA, however, this is more apparent in planktonic cells.

Next, the role of biofilm ECM was investigated, a key component and defining characteristic of fungal biofilms (Baillie and Douglas, 2000). Recent work by the Andes group has shown that ECM  $\beta$ -1,3 glucan, synthesised from Fks1p, is responsible for sequestering antifungal drugs and acting as a 'drug sponge' (Nett et al., 2010c), therefore conferring resistance on *C. albicans* biofilms. This was used as the basis to determine whether CHD-FA was sequestered by ECM  $\beta$ -1,3 glucan in the same way as other classes of antifungal agents. For this, strains used in the previous investigations were used (Nett et al., 2010c), where it was shown that *FKS1/fks1* $\Delta$  biofilms sensitivity to fluconazole was exquisite in comparison to the biofilm resistant parental strain and the ECM  $\beta$ -1,3 glucan over-expressing strain *TDH3-FKS1*, which were highly resistant to fluconazole. These investigations showed that the *FKS1/fks1* $\Delta$ , *TDH3-FKS1* and the reference strain, DAY185, were equally sensitive at  $1 \times \text{MIC}_{50}$  to CHD-FA. This is surprising given that Nett *et al* (2010) had shown other classes of antifungals were impacted by ECM. This suggests that ECM  $\beta$ -1,3 glucan does not sequester CHD-FA, unlike azoles, polyenes and echinocandins (Nett et al., 2010c).

Finally, investigation of whether efflux pumps were affected by CHD-FA was performed, as these have previously been shown to be expressed in *C. albicans* biofilms (Ramage et al., 2002a). In this study it was shown that efflux pump activity in both planktonic and sessile *C. albicans* cells was increased, but only significant up-regulation after 4 h treatment in planktonic cells at sub-MIC levels of CHD-FA. However, overall efflux pump activity in *C. albicans* biofilms was found to be higher when compared to planktonic cells, which was predicted

Ch. 4 Assessing the activity of a novel antifungal agent based on previous literature (Ramage et al., 2002a). In order to determine their contribution to potential resistance to CHD-FA efflux pump activity was inhibited using a competitive substrate. Efflux pump inhibitor studies have previously shown, with the use of mutants with single and double deletion mutations, that *C. albicans* have reduced mRNA expression for various multidrug resistant efflux pumps (Ramage et al., 2002a). Here it was demonstrated that this compound did not alter the MIC. This data indicates that CHD-FA is not impacted by efflux pump activity, despite these pumps being induced by treatment. This is unsurprising given that CHD-FA acts on the cell membrane and not through an internal cellular mechanism.

CHD-FA is a fungicidal compound that acts non-specifically on the cell membrane. In the context of oral and systemic health, individuals suffer from a range of candidal biofilm diseases that cause high levels of morbidity and therefore, CHD-FA may serve as a potential novel antiseptic agent for the treatment of oral candidiasis and other candidal biofilm infections. Moreover, there are many other oral diseases of microbial biofilm origin, such as caries and periodontitis, which would benefit from novel antiseptic molecules, assuming these meet appropriate safety standards. Whilst CHD-FA appears to have appropriate biological properties of an antiseptic, further cellular and *in vivo* studies are required.

## CHAPTER FINDINGS

CHD-FA is fungicidal against biofilms, but does not disrupt biomass.

CHD-FA elicits its effects by non-specifically targeting the cell membrane.

Known *C. albicans* resistance mechanisms do not play a role in CHD-FA sensitivity towards biofilms.

Chapter 5:  
Evaluating CHD-FA for the  
management of oral biofilm  
infections

## 5.1 Introduction

Inflammation of the oral cavity in candidal associated biofilm diseases such as denture stomatitis is not strictly only caused by *Candida* spp (Pereira et al., 2013, Chopde et al., 2012). Many manifestations of oral candidosis are polymicrobial, in which consortia of different species of bacteria coaggregate with yeasts and hyphal elements, which helps support the physical infrastructure of these complex biofilms. In addition to oral candidosis, dental caries, gingivitis and periodontitis are amongst the most common polymicrobial diseases of the oral cavity (Peyyala et al., 2013, Yamakami et al., 2013).

Antimicrobial mouthwashes are one of the main therapeutic and preventative strategies currently used in the management of complex oral biofilm diseases, of which chlorhexidine (CHX) is widely accepted as the ‘gold standard’ (Herrera, 2013). This antiseptic agent has superior activity to its comparators, and is both cidal and static against microorganisms present in oral biofilms with roles in the pathogenesis of oral disease. CHX has been shown to have a broad-spectrum of activity against bacteria, fungi and viruses, highlighting its usefulness in a number of diseases, particularly polymicrobial infections (Lim and Kam, 2008, Salim et al., 2013, Machado et al., 2011). Moreover, its substantivity provides prolonged activity through its ability to adsorb onto the pellicle found on enamel surfaces of teeth (Najafi et al., 2012). Despite this, various studies have shown long-term use of CHX may not be practical as it is associated with staining of the teeth and taste alterations (Quirynen et al., 2001, Jones, 1997). Furthermore, recent reports of adverse events, including anaphylactic reactions, to this compound have been described (Pemberton and Gibson, 2012). It has also been shown recently to be ineffective against biofilms grown from clinical isolates (Smith et al., 2013).

The prevention and treatment of oral biofilm diseases, such as periodontal disease and mucosal infections, may benefit from a compound that has the potency of CHX with minimal side effects, but also elicits adjunctive biological properties, such as alteration of inflammatory pathways, which are clearly important in the pathogenesis of oral biofilm disease (Turkoglu et al., 2009, Liu et al., 2013). A previous study has shown CHX is able to down-regulate bacterially induced expression of proinflammatory mediators (Hour-Haddad et

Ch. 5 Evaluating CHD-FA for the management of oral biofilm infections al., 2008), although the toxicological aspects of CHX may have been the reason for this observation.

Recent studies have shown the benefit of using natural agents in the management of oral infections. Tea tree oil (TTO) was shown to be non-toxic and effectively dampened the host immune response to a fungal stimulus (Ramage et al., 2012b). Furthermore, as outlined in the previous chapter, the antiseptic activity of CHD-FA was evaluated, where it was shown that the compound was equally effective against *C. albicans* planktonic and biofilm cells (Sherry et al., 2012). Mechanistically this was identified as a membrane disruption process that was not impacted by defined biofilm adaptive resistance mechanisms (Sherry et al., 2012). A purified form of CHD-FA has recently been produced by a patented process, which has been shown to be non-toxic in a rat wound model, with suggestions of anti-inflammatory activity (Sabi et al., 2012). Moreover, a recent randomized, double blind, controlled trial indicated that it was well-tolerated in a clinical study of eczema (Gandy et al., 2011). Collectively, these studies suggest that CHD-FA could have broader implications for oral healthcare applications.

## 5.2 Aims

The aims of this chapter were to investigate whether CHD-FA has a broad-spectrum of activity against microbial biofilms of oral relevance and to determine whether it could be used as an alternative to CHX based mouthwashes, which are known to have side effects from prolonged use. In addition, to determine whether CHD-FA had any adjunctive immunomodulatory properties, as reported elsewhere (Gandy et al., 2011, Sabi et al., 2011).

The data represented in this chapter has been published in:

Sherry L, Millhouse E, Lappin DF, Murray C, Culshaw S, Nile CJ and Ramage G. 2013. Investigating the biological properties of carbohydrate derived fulvic acid (CHD-FA) as a potential novel therapy for the management of oral biofilm infections. *BMC Oral Health*, 13, 47.

Work from this chapter has been presented orally at the following conferences and meetings:

Sherry L, Murray C and Ramage G. 'The potential use of carbohydrate-derived fulvic acid as a broad-spectrum antimicrobial.' Scottish and Northern Periodontal Research Group, Newcastle, UK. April 2011.

Sherry L, Murray C, Jones B, Williams C and Ramage G. 'Evaluation of Carbohydrate-derived fulvic acid as a novel oral hygiene product.' BSODR, Sheffield, UK. September 2011.

## 5.3 Methods

### 5.3.1 Culture conditions and standardisation

A selection of laboratory strains of commensal and pathogenic bacteria associated with oral biofilm disease were used in this study, including *Porphyromonas gingivalis* ATCC 33277 and *Fusobacterium nucleatum* ATCC 10596, which were maintained at 37°C on fastidious anaerobic agar (FAA [Lab M, Lancashire, UK]) under anaerobic conditions (85% N<sub>2</sub>, 10% CO<sub>2</sub> and 5% H<sub>2</sub>, [Don Whitley Scientific Limited, Shipley, UK]). *Streptococcus mutans* 10449, *Streptococcus mitis* NCTC 12261, *Aggregatibacter actinomycetemcomitans* OSM 1123 and *Enterococcus faecalis* NCTC 5957 were grown and maintained at 37°C on colombia blood agar (CBA [Oxoid, Hampshire, UK] in 5% CO<sub>2</sub>). All isolates were stored in Microbank<sup>®</sup> vials (Pro-Lab Diagnostics, Cheshire, UK) at -80°C until required.

*P. gingivalis* and *F. nucleatum* were propagated in 10 mL Schaedler's anaerobic broth (Oxoid), *S. mitis* and *A. actinomycetemcomitans* were grown in 10 mL tryptic soy broth (TSB [Sigma-Aldrich, Dorset, UK]) supplemented with 0.6% yeast extract and 0.8% glucose. *E. faecalis* was grown in TSB with 0.25% glucose, and *S. mutans* was grown in 10 mL brain heart infusion (BHI [Sigma-Aldrich]), all at 37°C and at appropriate atmospheric conditions. Overnight cultures were washed by centrifugation (3,000 rpm) and resuspended in 10 mL PBS. All bacteria were then standardised and adjusted to a final working concentration of  $5 \times 10^4$  cells/mL and  $1 \times 10^7$  cells/mL for planktonic and sessile susceptibility testing, respectively.

### 5.3.2 Antibacterial susceptibility testing of planktonic and biofilm cells

During the course of this study two active compounds from the oral hygiene products Dentrudent (Fulhold Ltd, Cape Town, South Africa) and Corsodyl (GlaxoSmithKline Consumer Health Care, UK) were tested; CHD-FA and CHX, respectively.

Antimicrobial testing to determine minimum inhibitory concentrations (MICs) of planktonic cells (PMIC) was performed using the CLSI M11-A8 broth microdilution method for anaerobic bacteria (CLSI, 2012) and CLSI M7-A9 for bacteria grown

Ch. 5 Evaluating CHD-FA for the management of oral biofilm infections in 5% CO<sub>2</sub> (CLSI, 2011). Minimum bactericidal concentrations (MBC) were also determined by standard plating methods. From each well of the PMIC plates that contained no growth, 20 µL was plated out in duplicate on the appropriate medium and incubated for 24 h at the relevant atmospheric conditions. The lowest concentration with no growth was deemed the MBC.

For biofilm testing standardised *P. gingivalis*, *F. nucleatum*, *S. mitis* and *A. actinomycetemcomitans* were grown for 72 h and *E. faecalis* for 24 h in their respective media and atmospheric conditions, with the exception of *S. mutans* which was grown in BHI supplemented with 2% sucrose for 48 h. Biofilms were grown statically in commercially available 96-well flat bottomed microtitre plates (Corning Incorporated, NY, USA) and sessile susceptibility testing was performed as described elsewhere (Pierce et al., 2008). Following antimicrobial treatment, biofilms were washed twice with PBS and 10% alamarBlue<sup>®</sup> (Invitrogen, Paisley, UK) was added to the biofilms prior to incubation for 4 h in the dark (Kirchner et al., 2012). The alamarBlue<sup>®</sup> is a colorimetric assay whereby an oxidation/reduction reaction occurs resulting in a colour change based upon cellular metabolic activity. Resazurin is the active ingredient of alamarBlue<sup>®</sup> that is reduced in the mitochondria of viable cells to produce resorufin, seen by a blue to pink colour change. Sessile minimum inhibitory concentrations (SMIC) were read visually and no change in colour was defined as the SMIC. Testing of all planktonic and sessile isolates was performed in quadruplicate on two separate occasions.

### **5.3.3 Antibacterial susceptibility testing of a multi-species periodontal biofilm**

A multi-species periodontal biofilm model consisting of *P. gingivalis*, *F. nucleatum*, *S. mitis* and *A. actinomycetemcomitans* was previously developed (Jose, 2012) for antimicrobial testing. All bacterial species were standardised to  $1 \times 10^7$  cfu/mL in artificial saliva (AS) as previously described (Pratten et al., 1998). This was comprised of porcine stomach mucins (0.25% w/v), sodium chloride (0.35 w/v), potassium chloride (0.02 w/v), calcium chloride dihydrate (0.02 w/v), yeast extract (0.2 w/v), lab lemco powder (0.1 w/v) and proteose peptone (0.5 w/v) in ddH<sub>2</sub>O. Urea was diluted in PBS (40% w/v) and added to a final concentration of 0.05% (v/v) in AS. Biofilms were prepared in 24 well

Ch. 5 Evaluating CHD-FA for the management of oral biofilm infections plates (Corning, NY, USA) containing customised Thermanox™ coverslips (13 mm diameter, Fisher Scientific). For the addition of each bacterial species to the biofilm a standardised bacterial suspension was prepared in 500 µL of AS. Initially, *S. mitis* biofilms were grown for 24 h. Media was then removed and standardised *F. nucleatum* was then added and biofilms incubated anaerobically for a further 24 h. The supernatant was again removed and standardised *P. gingivalis* and *A. actinomycetemcomitans* in AS added to the biofilm. This was then incubated at 37°C in an anaerobic chamber for a further 4 days; each day supernatants were replaced with fresh AS. CHD-FA was shown to be active at 0.5% v/v against all bacterial biofilms tested in this study, therefore this concentration was used in this study. In addition, ‘the gold standard’ 0.2% v/v CHX was also used to treat multispecies biofilms. Biofilms were treated for 30 min, before being carefully washed with PBS, and biofilm viability determined using alamarBlue®. The absorbance was read at 570 nm and the reference wavelength at 600 nm. The percentage reduction in biofilm viability was calculated according to the manufacturer’s instructions. To calculate the percentage reduced by the untreated and treated cells the following calculation was used;

$$[(\epsilon_{\text{OX}})\lambda_2 A \lambda_1 - (\epsilon_{\text{OX}})\lambda_1 A \lambda_2 / (\epsilon_{\text{RED}})\lambda_1 A' \lambda_2 - (\epsilon_{\text{RED}})\lambda_2 A' \lambda_1] \times 100$$

$$\lambda_1 = 570 \text{ nm}, \lambda_2 = 600 \text{ nm}$$

$$(\epsilon_{\text{OX}})\lambda_2 = 117,216, (\epsilon_{\text{OX}})\lambda_1 = 80,586, (\epsilon_{\text{RED}})\lambda_1 = 155,677, (\epsilon_{\text{RED}})\lambda_2 = 14,652$$

$$A \lambda_1 = \text{OD reading for test well}, A \lambda_2 = \text{OD reading for test well}$$

$$A' \lambda_2 = \text{OD reading for negative control}, A' \lambda_1 = \text{OD reading for negative control}$$

This study was performed in triplicate on three separate occasions.

### 5.3.3.1 DNA extraction of periodontal biofilms

Following the antimicrobial treatment, biofilms were retained and used to quantify the number of each bacterial species found after CHD-FA and CHX

Ch. 5 Evaluating CHD-FA for the management of oral biofilm infections treatment compared to the untreated control. Once biofilms were treated with CHD-FA or CHX and washed with PBS, samples were sonicated in 1 mL of PBS for 10 min and the supernatant transferred to a 1.5 mL microfuge tube and centrifuged at 13,000 rpm for 10 min to pellet each sample. DNA was subsequently extracted using the MasterPure Gram Positive DNA Purification Kit (Epicentre<sup>®</sup>, Cambridge, UK). The supernatant was discarded and 150  $\mu$ L of TE Buffer was added to each sample to resuspend the pellet and 1  $\mu$ L of lysozyme was added to break down the bacterial cells. Samples were incubated at 37 $^{\circ}$ C for 2.5 h. Next, a mixture containing 1  $\mu$ L proteinase K (50  $\mu$ g/mL) and 150  $\mu$ L Gram positive cell lysis solution was added to each sample, mixed thoroughly and incubated at 65 $^{\circ}$ C for 15 min. Samples were allowed to cool to 37 $^{\circ}$ C and then stored on ice for 5 min before the DNA precipitation stage. To each lysed sample, 175  $\mu$ L of MPC protein precipitation reagent was added and vortexed for 10 s. Samples were then centrifuged at 13,000 rpm for 10 min at 4 $^{\circ}$ C with the resultant supernatant transferred to a clean microfuge tube with 1  $\mu$ L of RNase A (5  $\mu$ g/mL). All samples were incubated at 37 $^{\circ}$ C for 30 min and 500  $\mu$ L of isopropanol added to each supernatant, which were then mixed by inverting each tube 40 times. The DNA was pelleted by centrifugation at 13,000 rpm for 10 min at 4 $^{\circ}$ C. The pellet was washed with 70% ethanol and the DNA pellet was resuspended in 35  $\mu$ L of TE buffer.

### **5.3.3.2 Quantification of periodontal bacteria following treatment**

One microlitre of extracted DNA, as outlined in section 5.3.3.1, was added to a mastermix containing 12.5  $\mu$ L SYBR<sup>®</sup> GreenER<sup>™</sup>, 9.5  $\mu$ L of UV-treated RNase-free water and 1  $\mu$ L of 10  $\mu$ M forward/reverse primers for *S. mitis* (Periasamy et al., 2009), *F. nucleatum* (Sanchez et al., 2014b), *P. gingivalis* (Boutaga et al., 2003) and *A. actinomycetemcomitans* (van der Reijden et al., 2010). The sequences for each primer set used are shown in Table 5.1.

**Table 5.1 - Primer sequences used for PCR**

| Target                          | Primer sequence (5'-3')                                     |
|---------------------------------|---|
| <i>A. actinomycetemcomitans</i> | F - GAACCTTACCTACTCTTGACATCCGAA<br>R - TGCAGCACCTGTCTCAAAGC |
| <i>F. nucleatum</i>             | F - GGATTTATTGGGCGTAAAGC<br>R - GGCATTCTACAAATATCTACGAA     |
| <i>P. gingivalis</i>            | F - GCGCTCAACGTTTCAGCC<br>R - CACGAATTGCCTGC                |
| <i>S. mitis</i>                 | F - GATACATAGCCGACCTGAG<br>R - CCATTGCCGAAGATTCC            |

Three independent replicates were analysed in triplicate using a MxProP Quantitative PCR machine and MxProP 3000 software (Stratagene, Amsterdam, Netherlands). The thermal profile consisted of 95°C for 10 min for DNA polymerase activation and 40 amplification cycles of 95°C denaturation for 30 s, 55°C annealing for 60 s and 72°C extension for 60 s. A dissociation curve was performed following the final amplification cycle for confirmation of only one product. This cycle consisted of 95°C for 60 s, 55°C for 30 s and 92°C for 30 s. A non-template control (NTC) replacing DNA with sterile water was included to rule out the presence of contamination. The cycle threshold (Ct) was set automatically and Ct values for all samples of interest were used for the quantification of bacteria. Quantification was carried out by standardising each bacterial species to  $1 \times 10^8$  CFU/mL, DNA extracted as described in 5.3.3.1, ten-fold dilutions prepared ranging from  $1 \times 10^3$  to  $1 \times 10^8$  CFU/mL and qPCR performed as described previously in this section. Standard curves were constructed by plotting the Ct value against the equivalent DNA dilution allowing unknown bacterial counts to be quantified. Each dilution of DNA was assessed in duplicate for each primer set.

#### 5.3.4 Ultrastructural changes of bacterial biofilms

Scanning electron microscopy (SEM) was performed on *S. mutans*, *E. faecalis*, and the multispecies biofilms. Cells were standardised in appropriate media, as described above and biofilms grown directly onto Thermanox™ coverslips (Nunc, Roskilde, Denmark). Following maturation biofilms were carefully washed with

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PBS before their respective treatments. Biofilms were then carefully washed twice with PBS and then fixed in 2% paraformaldehyde, 2% glutaraldehyde and 0.15 M sodium cacodylate, and 0.15% w/v alcian blue, pH 7.4, and prepared for SEM as previously described (Erlandsen et al., 2004). The specimens were sputter-coated with gold and viewed under a JEOL JSM-6400 scanning electron microscope. Images were assembled using Photoshop software (Adobe, San Jose, CA, USA).

### *5.3.5 Cytotoxicity of CHD-FA*

#### **5.3.5.1 Growth and maintenance of OKF6/TERT2**

OKF6/TERT2 cells (gift of the Rheinwald laboratory, Brigham and Woman's Hospital, Boston, USA) are an immortalised human oral keratinocyte cell line developed through the forced expression of telomerase (Dongari-Bagtzoglou and Kashleva, 2003). OKF6/TERT2 cells were maintained in keratinocyte serum-free medium (KSFM) [37010-022 Invitrogen, Paisley, UK] supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 25 µg/mL bovine pituitary extract (BPE), 0.2 ng/mL epidermal growth factor (EGF) and 0.4 mM CaCl<sub>2</sub>. BPE and EGF were filter sterilised (0.2 µm) in to KSFM. Cells were seeded at 5 x 10<sup>4</sup> cells/mL in a 75 cm<sup>2</sup> cell culture flask (Corning, NY, USA) and passaged when 90% confluence was reached. The adherent monolayer of cells was detached using 0.05% trypsin EDTA which was then neutralised with 15 mL of Dulbecco's modified Eagle's media ([DMEM] Sigma, Poole, UK) containing 10% FCS (Sigma, Poole, UK). Next, cells were washed in Hanks balanced salt solution (Sigma, Poole, UK) and resuspended in 4 mL KSFM before counted using a haemocytometer with 20 µL of cells mixed with 10 µL trypan blue (Sigma, Poole, UK) to give a dilution factor of 1.5. Cells were viewed under the microscope and round, clear healthy cells counted using the appropriate grid. Trypan blue is able to stain dead cells blue due to the loss of cell wall integrity and were therefore omitted during the counting process. Cells were finally re-seeded at 1 x 10<sup>5</sup> cells/flask in KSFM.

Frozen stocks of OKF6/TERT2 epithelial cells were prepared for long-term storage in liquid nitrogen. OKF6/TERT2 cell suspension were standardised to 2 x 10<sup>6</sup> cells/mL in DMEM supplemented with 20% FCS. Equal volumes of 20% DMSO and the OKF6-TERT2 cells were transferred to a cryo-tube and stored in an insulated box at -80°C, overnight. This was to ensure the cells were cooled

Ch. 5 Evaluating CHD-FA for the management of oral biofilm infections slowly before finally being stored in liquid nitrogen. Frozen stocks of these cells were then revived by thawing the cells at 37°C before transferring them into a cell culture flask containing KSFM, pre-heated to 37°C. OKF6/TERT2 cells were maintained until the fourth passage by which point they could be used in experiments.

For toxicity and immunomodulatory studies, OKF6/TERT2 cells were seeded in defined-KSFM (D-KSFM) where BPE is replaced with defined growth-promoting additives. This was due to BPE only being stable within media for 4 weeks, compared to the 3 months associated with D-KSFM and to also reduce the influence on the results of the variability found between batches of BPE.

#### **5.3.5.2 Toxicity of CHD-FA against the OKF6/TERT2 oral epithelial cell line**

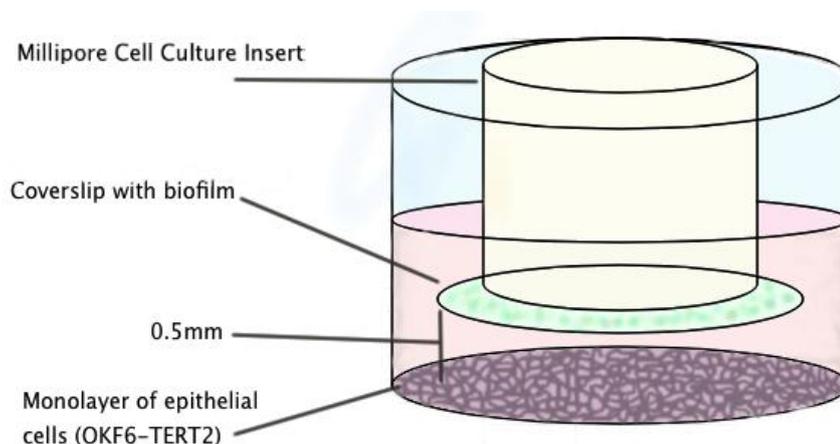
OKF6/TERT2 cells were used for determining the cytotoxicity of CHD-FA. Cells were grown to 90% confluence in KSFM at 37°C in 5% CO<sub>2</sub> and seeded at a density of  $1 \times 10^5$  cells/mL in D-KSFM in a 24 well plate, as described in 5.3.5. Once the cells reached 80-90% confluence, the cells were carefully washed with PBS before treatment with 0.5% (v/v) CHD-FA at the native pH 2.0 and a neutral pH of 7.0 and with 0.2% (v/v) CHX for 30 min. After 30 min, the compounds were removed and the cells carefully washed with PBS to remove any residual actives. Cells were incubated in KSFM for 4 and 24 h before cell viability was assessed using the alamarBlue<sup>®</sup> assay, as described in 5.3.3. Viability studies were carried out in triplicate, on three separate occasions.

#### **5.3.6 Assessing immunomodulatory properties of CHD-FA**

##### **5.3.6.1 Stimulation of epithelial cells with periodontal biofilms**

OKF6/TERT2 cells were grown to 90% confluence in 24 well plates in D-KSFM then pre-treated with 0.5% CHD-FA (pH 7.0) for 30 min before washed with PBS to remove residual CHD-FA. CHD-FA at pH 2.0 was toxic against the OKF6/TERT2 cell line used in this study and therefore could not allow us to analyse any potential immunomodulatory properties of this compound. In addition, 0.2% CHX was also shown to be toxic to the epithelial cells and therefore these compounds were not further investigated. The multispecies periodontal biofilm was attached to the underside of a hanging cell culture insert (Millipore,

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Massachusetts, USA) using Vaseline<sup>®</sup>, then laid adjacent to the OKF6/TERT2  
monolayer. Figure 5.1 displays a schematic of the co-culture system.



**Figure 5.1 - Epithelial biofilm co-culture model system.**

Hanging inserts with the Thermanox coverslips containing multispecies biofilms were introduced into each well of a 24 well plate. The inverted coverslip was secured to the hanging insert using sterile Vaseline<sup>®</sup> with a 0.5 mm space between the biofilm and the monolayer of epithelial monolayer.

Epithelial cells were incubated with the periodontal biofilm for 4 and 24 h at 37°C in 5% CO<sub>2</sub>. Epithelial cells not pre-treated with CHD-FA, or not challenged with biofilms, served as appropriate controls. Following stimulation, supernatants and cell lysates were retained to assess changes in the expression and release of a panel of pro-inflammatory mediators.

#### **5.3.6.2 Extraction of RNA from OKF6/TERT2 cells**

Following stimulation, RNA was extracted using the RNeasy Mini Kit (Qiagen, Crawley, UK), in accordance with the manufacturers' instructions. Epithelial monolayers were lysed with the addition of 350 µL of buffer RLT to each well of the 24 well plate. The resultant lysate was transferred into an RNase free microfuge tube and mixed with 350 µL of 70% ethanol. Each 700 µL sample was then transferred to a RNeasy spin column placed within a 2 mL collection tube and centrifuged at 13,000 rpm for 15 s, with the flow-through discarded.

DNA contamination of isolated total RNA was addressed using a DNase kit, as per manufacturer's instructions (Qiagen Ltd, Crawley, West Sussex, UK). For DNase

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digestion, DNase I stock solution (Qiagen, Crawley, UK) was initially prepared by injecting 550  $\mu\text{L}$  of RNase free water into the DNase I vial using a needle and syringe and mixed with gentle inversion. Aliquots of the stock solution were made and stored at  $-20^{\circ}\text{C}$  for no longer than 9 months. Firstly, 350  $\mu\text{L}$  of buffer RW1 was added to the spin column and centrifuged at 13,000 rpm for 15 s and flow-through discarded. Ten microlitres of DNase I was gently mixed with 70  $\mu\text{L}$  of buffer RDD and added directly on to the RNeasy column membrane and incubated at room temperature for 15 min. Buffer RW1 was then added to the column and centrifuged at 13, 000 rpm for 15 s and flow-through discarded.

Following the DNase digestion stage, 700  $\mu\text{L}$  of buffer RW1 was added to the spin column, centrifuged at 13,000 rpm for 15 s and flow-through discarded. Next, two wash stages of the membrane occurred with 500  $\mu\text{L}$  of buffer RPE with centrifugation at 13,000 rpm for 15 s. The spin column was then placed in a new 2 mL collection tube and centrifuged at 13,000 rpm for 1 min to dry the membrane. The column was finally placed in a 1.5 mL microfuge tube and 30  $\mu\text{L}$  of RNase free water added directly to the membrane and centrifuged at 13,000 rpm for 1 min to elute the RNA. The resultant RNA can then be placed back on membrane and centrifuged one final time to ensure maximum release of the RNA from the column. RNA was collected and quantified using a spectrophotometer.

To assess the concentration and quality of the RNA extracted from OKF6/TERT2 cells or DNA from bacterial biofilms, a NanoDrop 1000 spectrophotometer (Thermo Scientific, DE, USA) was used. Before RNA was measured, the pedestal was wiped clean and background corrected with 1.5  $\mu\text{L}$  of RNase free water. Each RNA sample was then subsequently measured and recorded as ng/ $\mu\text{L}$ . The purity of the RNA was also determined using the ratio of the absorbance at 260 and 280 nm, where RNA with an  $A_{260/280}$  ratio greater than 1.8 was deemed to be of high enough quality for gene expression studies.

### **5.3.6.3 Real time PCR analysis using RT<sup>2</sup> Profiler PCR Array**

Initial gene expression analysis was carried out using real time PCR (qPCR) with a custom designed RT<sup>2</sup> Profiler PCR Array (Qiagen, Crawley, UK). RT<sup>2</sup> Profiler arrays

Ch. 5 Evaluating CHD-FA for the management of oral biofilm infections are a SYBR<sup>®</sup> GreenER<sup>™</sup> based real-time PCR that allow for the detection of several genes of interest, simultaneously.

#### **5.3.6.3.1 Reverse transcription by RT<sup>2</sup> First Strand kit**

For the generation of complementary DNA (cDNA), genomic DNA (gDNA) was eliminated using the RT<sup>2</sup> First Strand kit (Qiagen, Crawley, UK). A mixture consisting of 2  $\mu$ L of 5  $\times$  gDNA elimination buffer, 55 ng of RNA and the remainder of the 10  $\mu$ L final volume made up with RNase-free water was incubated at 42 °C for 5 min before immediately chilled on ice. Next, the reverse transcription (RT) cocktail consisting of 4  $\mu$ L of 5  $\times$  RT buffer, 1  $\mu$ L of primer and external control mix, 2  $\mu$ L of RT enzyme mix and 3  $\mu$ L of RNase free water was mixed with 10  $\mu$ L of the gDNA elimination mixture and incubated at 42 °C for 15 min. The reaction was stopped by heating the samples to 95 °C for 5 min followed by the addition of 91  $\mu$ L of RNase free water to each reaction tube. cDNA was then stored at -20 °C until required.

#### **5.3.6.3.2 Gene expression analysis using RT<sup>2</sup> Profiler**

A mastermix was prepared with SYBR<sup>®</sup> GreenER<sup>™</sup>, cDNA and RNase-free water and 24  $\mu$ L was added to each well of the custom RT<sup>2</sup> Profiler plate, which already contained the forward and reverse primers for the genes of interest (*IL-1a*, *IL-1B*, *IL-6*, *TNF*, *CSF2*, *CSF3*, *IL-8*, *CXCL1*, *CXCL3*, *CXCL5*, *CCL1* and *GAPDH*). Thermal cycler conditions were as follows: 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Two replicates of each condition were used in the RT<sup>2</sup> Profiler and the experiment was carried out on two separate occasions.

Gene expression was then quantified using the  $2^{-\Delta\Delta Ct}$  method to calculate the expression of gene transcripts relative to the media control. *GAPDH* was used as the endogenous control. The  $\Delta Ct$  value was calculated [ $\Delta Ct = Ct$  (gene of interest) –  $Ct$  (*GAPDH*)]. This was performed for each gene tested. The relative expression of each gene was then calculated with the formula  $\Delta\Delta Ct = \Delta Ct$  (test sample) –  $\Delta Ct$  (unstimulated control sample). This was the expressed in terms of fold change relative to the unstimulated control sample with the formula [fold change =  $2^{-\Delta\Delta Ct}$ ] (Livak and Schmittgen, 2001).

**5.3.6.4 IL-8 qPCR analysis using SYBR<sup>®</sup> GreenER<sup>™</sup>**

IL-8 gene expression was analysed using SYBR<sup>®</sup> Green (Invitrogen, Paisley, UK) based qPCR with *GAPDH* as a housekeeping gene (Ramage et al., 2012b). The primers used are shown in Table 5.2.

**Table 5.2 - Primer sequences used qPCR**

| Target | Primer sequence (5'-3')   |
|--------|---------------------------|
| IL-8   | F - CAGAGACAGCAGAGCACACAA |
|        | R - TTAGCACTCCTTGGCAAAC   |
| GAPDH  | F - CAAGGCTGAGAACGGGAAG   |
|        | R - GGTGGTGAAGACGCCAGT    |

RNA was extracted from cell lysates and cDNA synthesised using the RT<sup>2</sup> First Strand cDNA synthesis kit as described in 5.3.6.1 and 5.3.6.4.1, respectively. 1  $\mu$ L of cDNA was added to a mastermix containing 12.5  $\mu$ L of SYBR<sup>®</sup> GreenER<sup>™</sup>, 10.5  $\mu$ L of RNase-free water and 0.5  $\mu$ L of forward/reverse primers. The thermal profile were as follows: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 60 s at 60°C. A dissociation curve was also carried out to confirm the presence of one product, the thermal cycles was as follows: 60 s at 95°C, 30 s at 55°C and 30 s at 95°C. Three independent replicates from each parameter were analysed in duplicate using MxProP Quantitative PCR machine and MxProP 3000 software (Stratagene, Amsterdam, Netherlands) and gene expression normalised to the housekeeping gene *GAPDH* according to the  $2^{-\Delta Ct}$  method (Livak and Schmittgen, 2001).

**5.3.6.5 IL-8 protein analysis by Enzyme Linked Immunosorbent Assay (ELISA)**

Supernatants from OKF6/TERT2 cells challenged with multispecies biofilms for 4 and 24 h,  $\pm$  pre-treatment with CHD-FA, were retained to assess the release of interleukin 8 (IL-8) by ELISA (Invitrogen, Paisley, UK), as per manufacturer's instructions. Capture antibody (1  $\mu$ g/mL) was prepared in Na<sub>2</sub>HCO<sub>3</sub> and 100  $\mu$ L was added to each well of a Nunc Maxisorp<sup>®</sup> flat-bottomed microtitre plates (Fisher, Loughborough, UK). Plates were sealed and incubated at 4°C overnight and contents discarded and plates washed with 300  $\mu$ L of wash buffer (8 g NaCl,

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0.2 g KCl, 1.44 g Na<sub>2</sub>HPO<sub>4</sub>, 0.24 g KH<sub>2</sub>PO<sub>4</sub> and 500 µL Tween 20 in 1 L of distilled water (dH<sub>2</sub>O). The plate was then blocked with 300 µL of assay buffer (0.5% bovine serum albumin [BSA]) for 1 h at room temperature to block non-specific protein interactions, before being aspirated and 100 µL of each sample loaded in triplicate. In addition, standards of known concentrations of IL-8 (31.25 - 2000 pg/mL) were prepared and included on each plate in duplicate. Detection antibody was diluted to 0.04 µg/mL in assay buffer and added to each well containing a sample or standard. Plates were sealed and incubated for 2 h at room temperature with shaking at 700 rpm. Following incubation, the plate was washed and streptavidin-HRP (1/2500 dilution) was added to each well before incubation at room temperature with shaking at 700 rpm for 30 min. Finally, the plate was washed and 3,3',5,5'-tetra-methylbenzidine (TMB, R&D systems, Abingdon, UK) was added to each well and incubated in the dark for 30 min. Absorbance was read using a plate reader (FLUOstar Omega BMG Labtech, VA, USA) at 630 nm. A standard curve was constructed by plotting the mean absorbance for each standard against IL-8 protein concentration and R-squared calculated. Results were calculated using a 4-parameter curve fit to determine the concentration of IL-8 in the samples tested. All samples were tested in triplicate, on three individual occasions.

#### **5.3.6.6 Stimulation of epithelial cells with an inflammatory fungal agonist**

OKF6/TERT2 cells were grown to 90% confluence in 24 well plates in D-KSFM and pre-treated with 0.5% CHD-FA (pH 7.0) for 2 min (to simulate an oral rinse) before being washed with PBS to remove any residual agent. Cells were then stimulated with zymosan (extracted from *Saccharomyces cerevisiae*), a ligand found within the cell walls of yeast such as *C. albicans*, for a further 4 h and 24 h at 37°C in 5% CO<sub>2</sub>. Un-stimulated cells, cells pre-treated with CHD-FA only or cells stimulated with zymosan for 4 h and 24 h only served as appropriate controls. Following stimulation, supernatants and cell lysates were retained to assess the regulation of a panel of pro-inflammatory mediators. RNA was extracted as described in 5.3.6.2 and subsequent IL-8 ELISA techniques performed as described in 5.3.6.7.

##### **5.3.6.6.1 qPCR analysis using using Taqman<sup>®</sup> Low Density Array (TLDA)**

For gene expression analysis custom-designed ABI microfluidic Taqman<sup>®</sup> Low Density Array ([TLDA] Applied Biosystems, Foster City, CA, USA) was used in a qPCR process using the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). This catered for 384 qPCR reactions to be carried out simultaneously in order to determine the transcription of several genes. The TLDA used in this study was designed to measure the expression of *CCL3L1*, *CSF2*, *CSF3*, *CXCL1*, *CXCL10*, *CXCL11*, *CXCL13*, *ICAM1*, *IL-1 $\alpha$* , *IL-1 $\beta$* , *IL-6*, *IL-8*, *ITGB2*, *NF $\kappa$ B*, *S100A8* and *TNF $\alpha$* . *GAPDH* was included as the housekeeping gene. The reaction mixture contained 20  $\mu$ L of cDNA template, 30  $\mu$ L of RNase free water and 50  $\mu$ L of TaqMan<sup>®</sup> universal master mix (Applied Biosystems, Foster City, CA, USA) and was added to each port of the TLDA card. The thermal profile conditions were as follows: 10 min at 94.5°C and 30 s at 97°C and 1 min at 59.7°C for 40 cycles. The thermal cycling and fluorescence detection was performed on Applied Bio-Systems ABI Prism 7900HT Sequence Detection System with ABI Prism 7900HT SDS Software 2.1. Gene expression was calculated based upon normalisation to *GAPDH* and relative to the un-stimulated control, as described in 5.3.6.5.2.

### 5.3.7 Statistical analysis

Graph production, data distribution and statistical analysis were performed using GraphPad Prism (version 4; La Jolla, CA, USA). After assessing whether data conformed to a normal distribution by before and after data transforms, ANOVA and t tests were used to investigate significant differences between independent groups of data that approximated to a Gaussian distribution. A Bonferroni correction was applied to the p value to account for multiple comparisons of the data. Non-parametric data was analysed using the Mann-Whitney U-test to assess differences between two independent sample groups. Student t-tests were used to measure statistical differences between the  $\Delta$ Ct values of the two independent groups assessed in gene expression studies, although data may be represented as percentage or fold change in the figures. Statistical significance was achieved if  $P < 0.05$ .

## 5.4 Results

### 5.4.1 CHD-FA has rapid and broad-spectrum antibacterial activity

Corsodyl<sup>®</sup> (0.2% CHX) and Dentrudent (0.8% CHD-FA) are oral formulations containing the active ingredients CHX and CHD-FA, respectively. All agents tested had antimicrobial activity against the oral bacteria evaluated in this study, however CHD-FA and CHX had greater activity than their respective formulations against planktonic and sessile cells (Table 5.3). When focusing on these active ingredients, both were shown to be not only inhibitory but highly cidal against these organisms (Table 5.3). PMICs for the oral isolates ranged from 0.0625% to 0.25% for CHD-FA and from  $\leq 0.00039\%$  to 0.00078% for CHX. The PMBC/PMIC ratio for CHD-FA and CHX were  $\leq 4$ , indicating both compounds displayed bactericidal activity. None of the bacterial species tested were notably more sensitive or resistant to either of the compounds. Both CHD-FA and CHX showed activity against mature biofilms, with SMICs of 0.5% for CHD-FA and from 0.003% to 0.025% for CHX. Interestingly, although CHX was effective at lower concentrations, the fold-change from PMIC to SMIC ranged from 2 to 64 whereas CHD-FA ranged from 2 to 8. In addition, all bacterial biofilms were equally susceptible to CHD-FA with a SMIC of 0.5%.

When considering the formulation, the PMIC for Dentrudent, that contains the active ingredient CHD-FA, ranged from 6.25% to 25% and the SMIC ranged from 25% to 50%. There was no greater than a 4-fold increase in concentration required for killing the bacterial biofilms when compared to planktonic cells. In contrast, despite Corsodyl being highly effective against all species in this study, the PMIC/SMIC ratio varied greatly and in some cases was up to 64-fold higher. Overall, *P. gingivalis* was the most susceptible organism to the antimicrobial therapies tested, particularly for planktonic cells.

Table 5.3 - Susceptibility profiles of clinically relevant bacteria to four antimicrobial agents

| MIC (%)              |        |       |      |            |      |      |         |        |        |          |       |       |
|----------------------|--------|-------|------|------------|------|------|---------|--------|--------|----------|-------|-------|
|                      | CHD-FA |       |      | Dentrudent |      |      | CHX     |        |        | Corsodyl |       |       |
|                      | PMIC   | PMBC  | SMIC | PMIC       | PMBC | SMIC | PMIC    | PMBC   | SMIC   | PMIC     | PMBC  | SMIC  |
| <i>A. A*</i>         | 0.25   | 0.25  | 0.5  | 25         | 50   | 50   | 0.0008  | 0.0031 | 0.025  | 0.39     | 0.78  | 12.5  |
| <i>E. faecalis</i>   | 0.125  | 0.25  | 0.5  | 12.5       | 25   | 50   | 0.0016  | 0.025  | 0.003  | 0.39     | 6.25  | 1.56  |
| <i>F. nucleatum</i>  | 0.125  | 0.5   | 0.5  | 12.5       | 50   | 25   | 0.0004  | 0.0008 | 0.0125 | 0.195    | 0.195 | 12.5  |
| <i>P. gingivalis</i> | 0.0625 | 0.5   | 0.5  | 6.25       | 50   | 25   | ≤0.0004 | 0.0008 | 0.0063 | ≤0.098   | 0.195 | 3.125 |
| <i>S. mitis</i>      | 0.0625 | 0.125 | 0.5  | 12.5       | 25   | 50   | 0.0008  | 0.0016 | 0.0125 | 0.39     | 0.39  | 3.125 |
| <i>S. mutans</i>     | 0.25   | 0.25  | 0.5  | 25         | 50   | 50   | 0.0004  | 0.0008 | 0.025  | ≤0.098   | 0.195 | 3.125 |

\**A. actinomycetemcomitans*

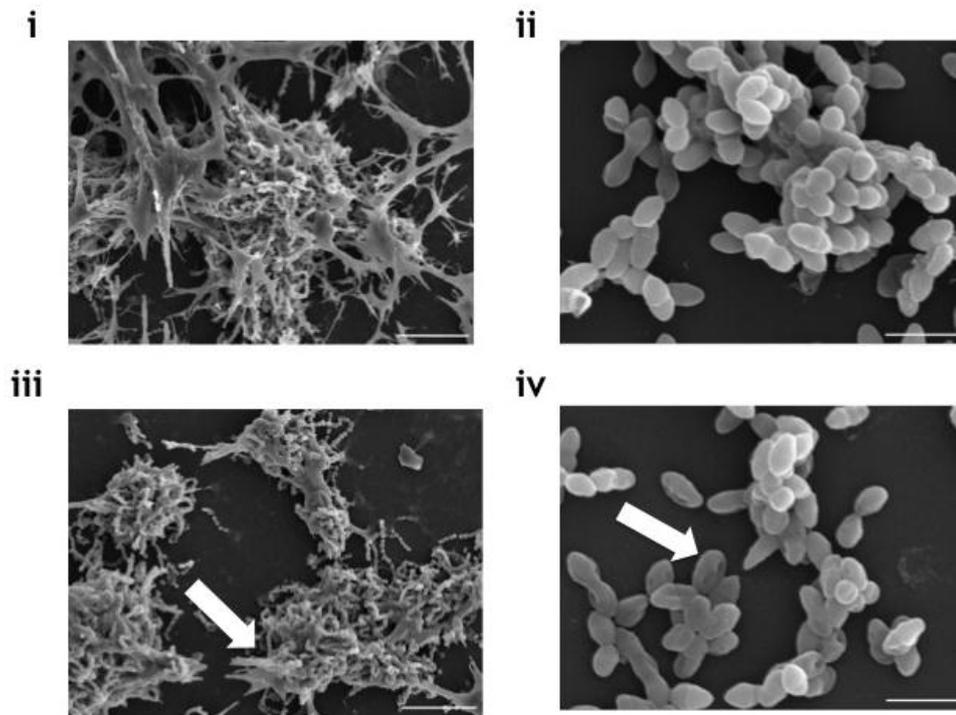
PMIC = planktonic minimum inhibitory concentration

PMBC = planktonic minimum bactericidal concentration

SMIC = sessile minimum inhibitory concentration

### 5.4.2 CHD-FA physically alters biofilm structure

Further analysis of the impact of CHD-FA upon the physical cellular structure of *S. mutans* (dental caries) and *E. faecalis* (endodontic infection) biofilms was assessed using SEM (Figure 5.2).



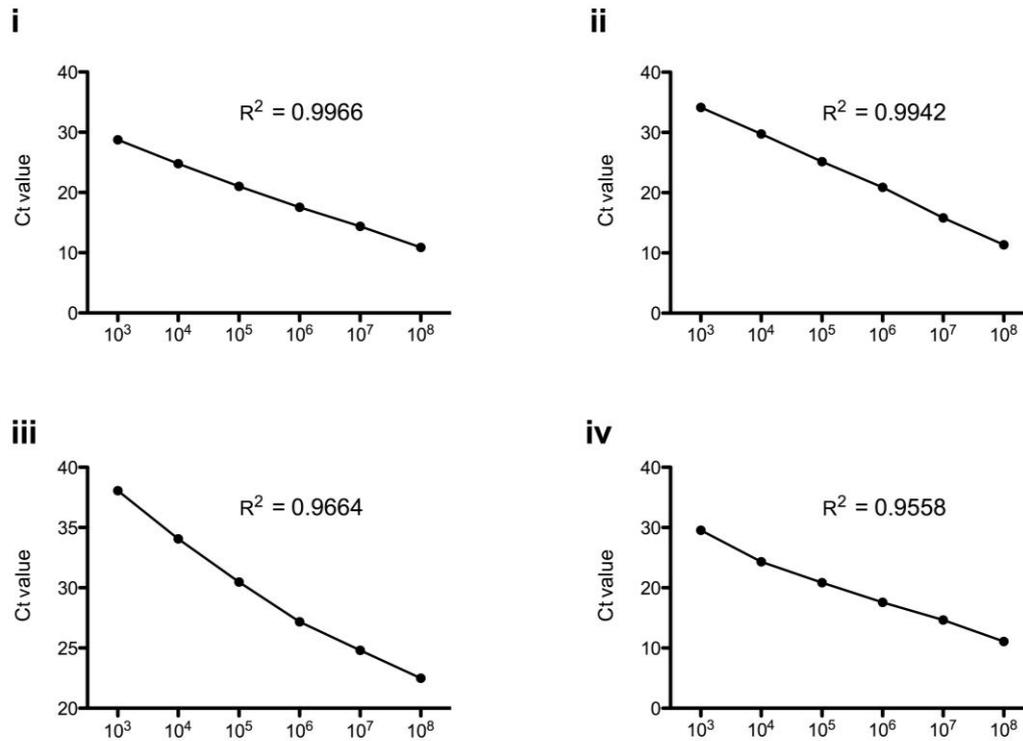
**Figure 5.2 - CHD-FA reduces ECM and compromises cell membrane structure.**

*S. mutans* ( $\times 2000$ ) and *E. faecalis* ( $\times 10,000$ ) biofilms were either untreated (i and ii, respectively) or treated with 0.5% (v/v) CHD-FA (iii and iv, respectively) for 24 h on Thermanox™ coverslips. These were then processed and viewed on a JEOL JSM-6400 scanning electron microscope. Note the reduction in extracellular matrix (iii) and perturbation of bacterial cell membranes (iv), denoted by arrows on sessile cells. Images are representative of three areas of the field. Scale bars represent 10  $\mu\text{m}$  and 2  $\mu\text{m}$  for *S. mutans* and *E. faecalis*, respectively.

Treatment with CHD-FA was able to reduce the overall quantity of *S. mutans* ECM (Figure 5.2iii) compared to the untreated control (Figure 5.2i), as denoted by arrows. Furthermore, perturbation of the cell membrane in *E. faecalis* cells was apparent (Figure 5.2iv), with visible punctures up on the cell surface unlike the biofilm that was not exposed to CHD-FA (Figure 5.2ii).

### 5.4.3 CHD-FA is effective against periodontal biofilms

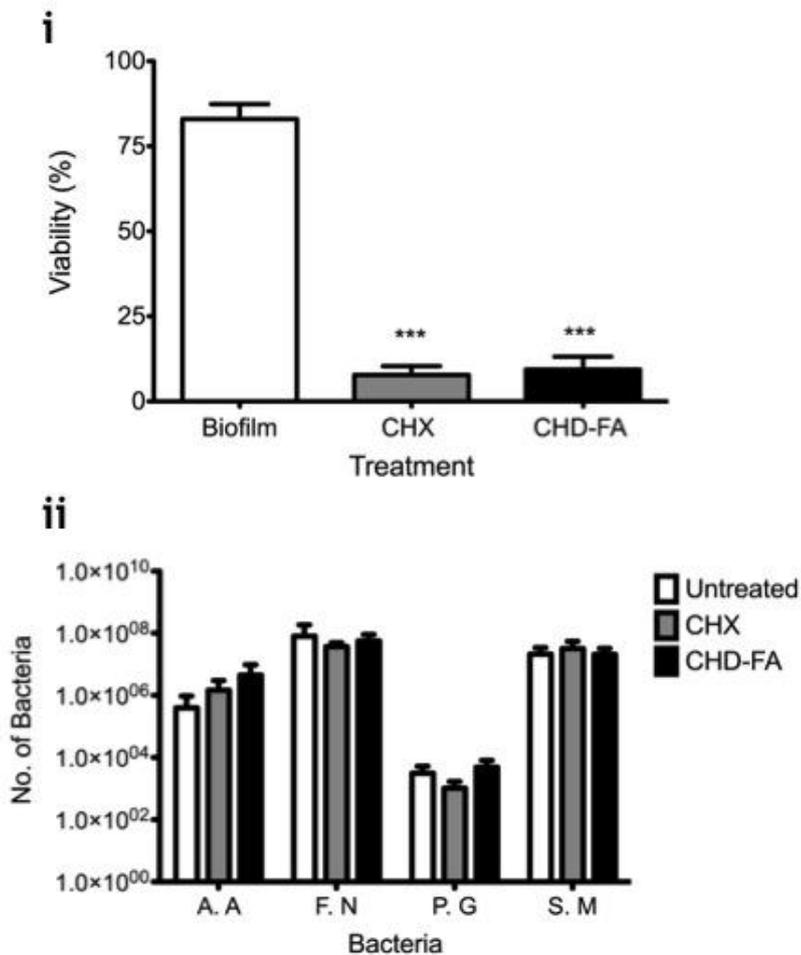
Before multispecies periodontal biofilms could be quantified ( $\pm$  treatment) by qPCR methodologies, DNA from known concentrations of each bacterial species were used to prepare a standard curve that could be extrapolated to determine unknown bacterial counts (Figure 5.3).



**Figure 5.3 - Standard curves of each species within multispecies biofilms.**

Ten-fold dilutions of DNA from known concentrations of *A. actinomycetemcomitans* (i), *F. nucleatum* (ii), *P. gingivalis* (iii) and *S. mitis* (iv) were used for qPCR and their equivalent Ct used for the preparation of a standard curve. Unknown bacterial counts could then be quantified by extrapolating from the appropriate curve. Each DNA dilution was assessed in duplicate for each of the four primer sets.

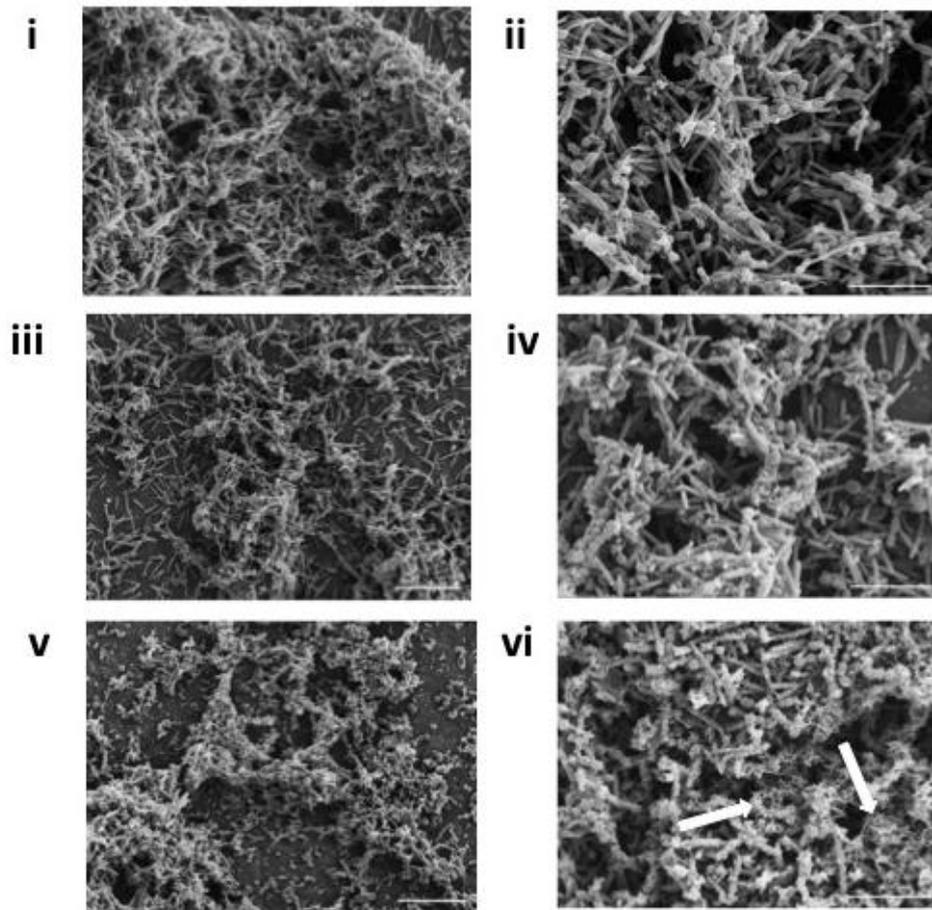
Given that biofilms of the oral cavity are polymicrobial in nature, a simple and reproducible multi-species model representative of sub-gingival plaque was developed to test the activity of CHD-FA using a number of techniques including the assessment of viability using alamarBlue<sup>®</sup> and the level of biomass following treatment with qPCR techniques (Figure 5.4).



**Figure 5.4 - CHD-FA kills multi-species periodontal biofilms.**

Multi-species periodontal biofilms were grown on Thermanox™ coverslips and treated with 0.5% (v/v) CHD-FA and 0.2% (v/v) CHX for 24 h and metabolic activity measured using the alamarBlue® (i). Biofilms were retained after treatment with CHD-FA or CHX and DNA was extracted for quantification of each species using SYBR® GreenER™ based qPCR (ii). All samples were assayed in triplicate, on three separate occasions. Data represents mean ± SD (\*\*\*) $p < 0.0001$ ).

The antimicrobial activity of CHD-FA at 1 × SMIC was shown to significantly reduce cell viability (Figure 5.4i) to less than 10% ( $p < 0.0001$ ) and was comparable to CHX treatment, which also significantly reduced cell viability to 8% ( $p < 0.0001$ ). No significant difference was found between CHD-FA and CHX treatment. Despite this reduction in viability, biofilm biomass remained unchanged following treatment with both compounds. The number of each bacterium was quantified using qPCR and showed no significant difference in bacterial numbers present after CHD-FA and CHX treatment, compared to the untreated control (Figure 5.4ii). SEM analysis of these biofilms was then performed to evaluate any effect on the biofilm architecture (Figure 5.5).



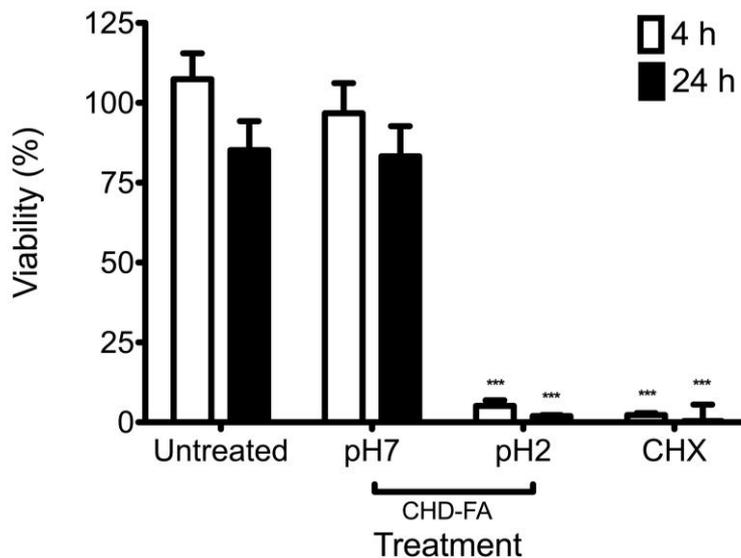
**Figure 5.5 - CHD-FA alters multi-species periodontal biofilm architecture.**

Biofilms were also analysed by SEM at both 2000 × (i, iii, v) and 5000× (ii, iv, vi). Biofilms were processed and viewed on a JEOL JSM-6400 scanning electron microscope. Untreated multispecies biofilms were first compared at low magnification (i) to biofilms treated with 0.2% (v/v) CHX (iii) and 0.5% (v/v) CHD-FA (v) for 24 h. At higher magnification the biofilms treated with 0.5% (v/v) CHD-FA for 24 h resulted in a fibrous ECM, as denoted by arrows (vi), as compared to the control (ii) and CHX (iv). Scale bars represent 10 µm and 5 µm for 2000× and 5000×, respectively.

At low magnification (2000×) both CHX and CHD-FA were shown to disrupt biofilm architecture (Figure 5.5iii and v) when compared to the untreated control (Figure 5.5i), as shown by areas of sparse disaggregated biofilms. Moreover, at high magnification (5000×) CHD-FA also appeared to alter the overall physical appearance of the biofilm matrix with greater quantities of fibrous ECM observed as denoted by the arrows (Figure 5.5vi), compared to the control and CHX (Figure 5.5ii and iv).

#### 5.4.4 CHD-FA is non-toxic against OKF6/TERT2 cells

Toxicity was assessed using an orally relevant epithelial cell line to determine whether there were any detrimental effects of the compounds tested prior to investigating immunomodulatory investigations (Figure 5.6).



**Figure 5.6 - CHD-FA is non-toxic against an oral epithelial cell line.**

An orally relevant epithelial cell line (OKF6/TERT2) was used for toxicity studies. Cells were treated with 0.5% (v/v) CHD-FA at pH 2.0 and 7.0 and with 0.2% CHX for 30 min and cellular viability assessed using the alamarBlue® assay. All samples were assayed in triplicate, on three independent occasions. Data represents mean  $\pm$  SD (\*\*\*)  $p < 0.0001$ ).

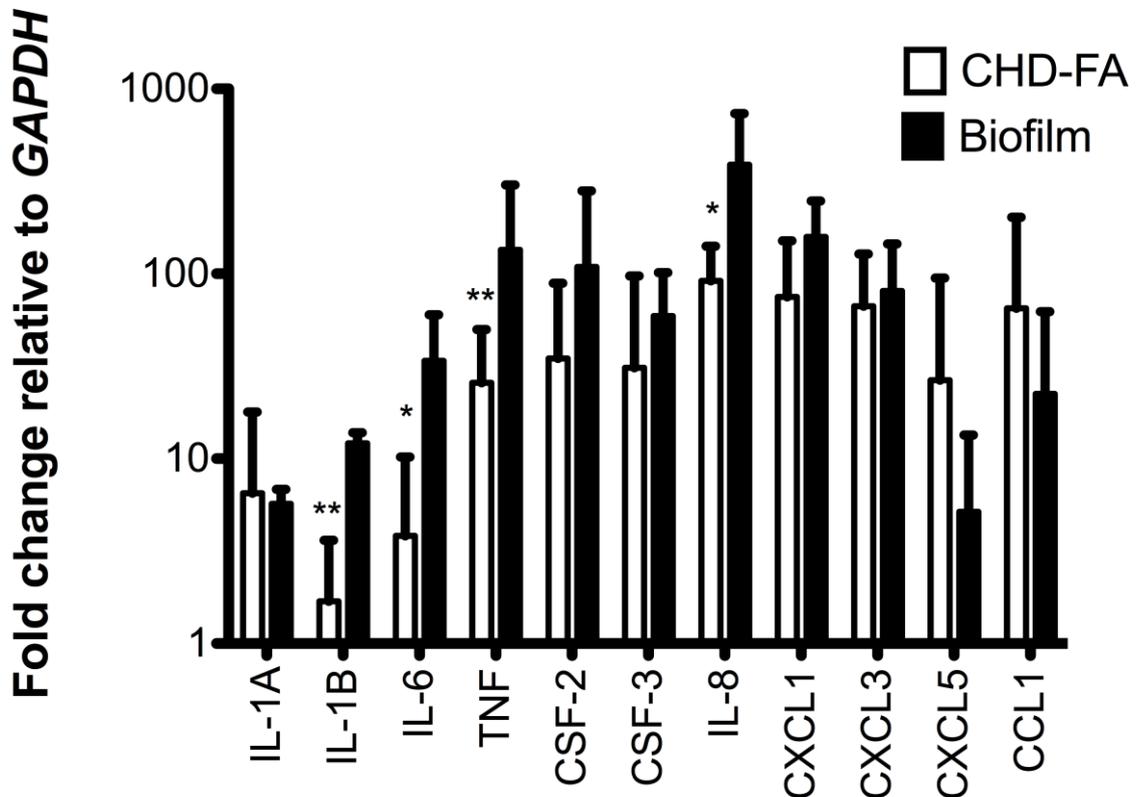
Both CHX and CHD-FA, at its native pH of 2.0, were shown to be highly toxic towards epithelial cells after 30 min exposure. Treatment with CHX and CHD-FA at pH 2 reduced viability to 2% and 5% after a 4 h recovery time, respectively, when compared to the untreated control (Figure 5.6). Furthermore, after a 24 h recovery, both compounds reduced viability to less than 2%. However, when CHD-FA was buffered to a neutral pH of 7.0, no toxicity was observed and cellular viability remained at 97% and 83% after a 4 and 24 h recovery period, respectively.

#### 5.4.5 CHD-FA alters the expression of pro-inflammatory mediators

##### 5.4.5.1 Regulation of inflammation associated with periodontal biofilms

Next it was important to determine whether or not CHD-FA was able to induce a biological response from the oral epithelial cell line, principally by measuring changes in immune mediators. To evaluate this, the four-species biofilm model

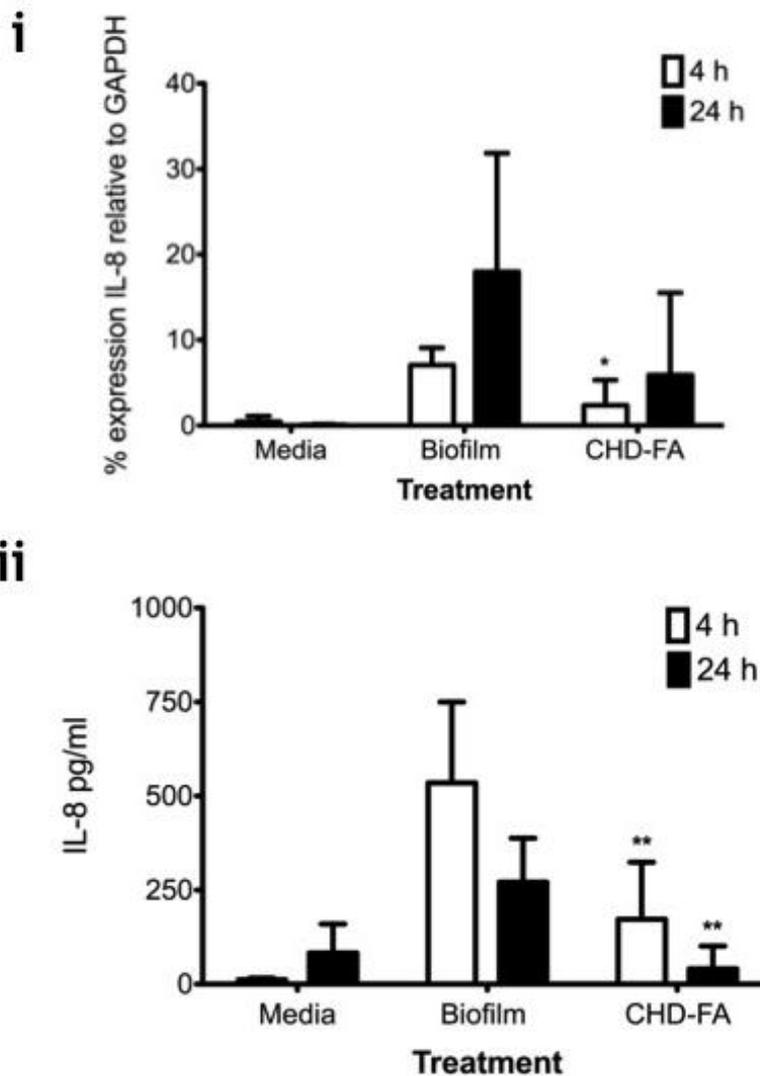
previously described in 5.3.3 was used as an agonist  $\pm$  CHD-FA treatment (Figure 5.7).



**Figure 5.7 - CHD-FA modulates biofilm mediated expression of key inflammatory mediators by OKF6/TERT2 cells.**

OKF6/TERT2 epithelial cells were used for the effect of CHD-FA on the host immune response. Cells were pre-treated with 0.5% (v/v) CHD-FA pH 7.0 for 30 min and stimulated with the multi-species periodontal biofilm for 4 h. The RT<sup>2</sup> Profiler was used to analyse the expression of a panel of pro-inflammatory mediators. Duplicate samples from two independent experiments were used in the RT<sup>2</sup> Profiler. Data is represented by percentage expression mean + 95% CI, relative to untreated control. \*p < 0.05, \*\*p < 0.01.

Initial gene expression studies using the RT<sup>2</sup> Profiler on OKF6/TERT2 cells pre-treated with CHD-FA prior to biofilm challenge showed a general down-regulation of pro-inflammatory mediators (Figure 5.7). Significant down-regulated genes included IL-6 (8.5 fold [p=0.018]), IL-1 $\beta$  (7.05 fold [p=0.012]), TNF $\alpha$  (5.22 fold [p=0.013]) and IL-8 (4.24 fold [p=0.021]). The down-regulation observed in the RT<sup>2</sup> Profiler was assessed further by means of IL-8 expression at the mRNA and protein level (Figure 5.8).



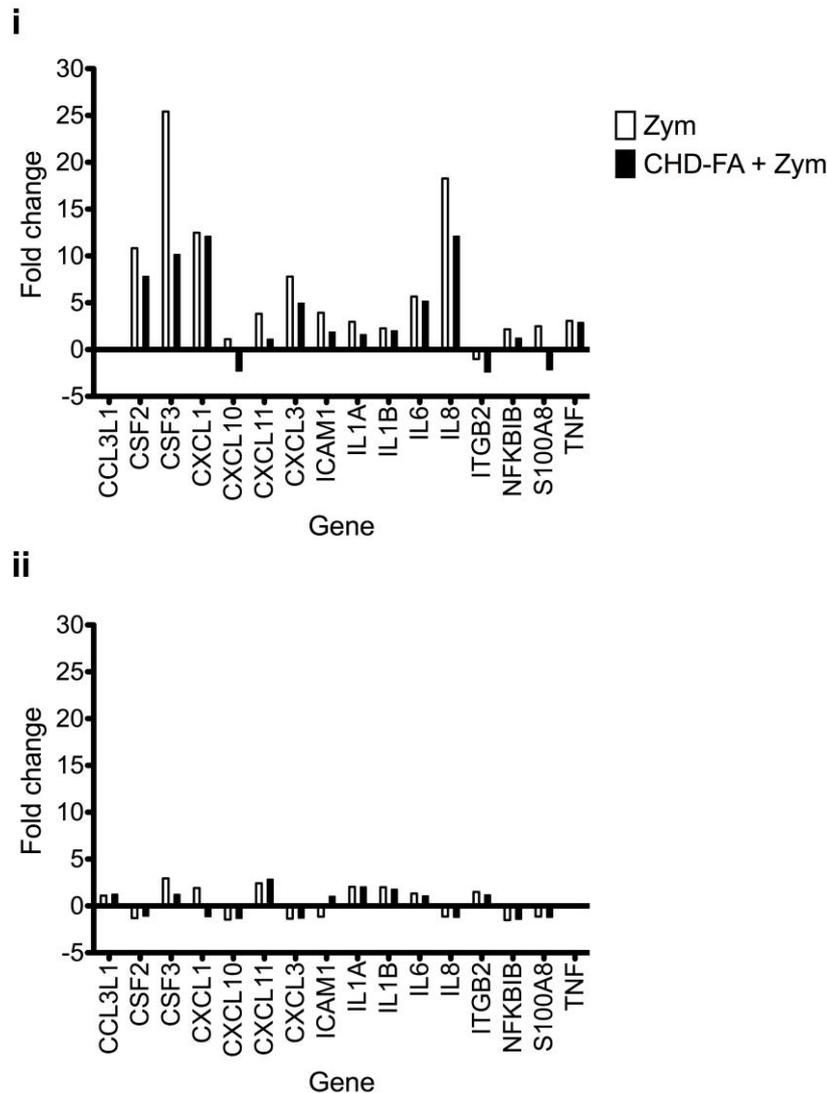
**Figure 5.8 - CHD-FA alters biofilm mediated expression of IL-8 by OKF6/TERT2 cells.**

OKF6/TERT2 epithelial cells were pre-treated with 0.5% (v/v) CHD-FA pH 7.0 for 30 min and stimulated with the multi-species periodontal biofilm for 4 h and 24 h. IL-8 gene expression was assessed using SYBR<sup>®</sup> GreenER<sup>™</sup> based qPCR (i), relative to *GAPDH*. IL-8 protein release was measured by ELISA (ii). All samples were assayed in triplicate, on three independent occasions. Data represents mean  $\pm$  SD (\* $p < 0.05$ , \*\* $p < 0.01$ ).

IL-8 is one of the significantly affected genes and a key mediator of periodontal inflammation. At the mRNA level, biofilm-induced *IL-8* expression was significantly down-regulated in cells pre-treated with CHD-FA after 4 h, when compared to the untreated control ( $p=0.0383$ ) (Figure 5.8i). No statistically significant difference was observed after 24 h stimulation ( $p=0.1712$ ). In addition, at the protein level, IL-8 release was shown to be significantly down-regulated when cells were pre-treated with CHD-FA, after 4 h ( $p=0.008$ ) and 24 h ( $p=0.0037$ ) biofilm stimulation (Figure 5.8ii).

### 5.4.5.2 Regulation of pro-inflammatory mediators associated with fungal stimulation

Finally it was investigated whether CHD-FA impacts the regulation of the same pro-inflammatory mediators tested in 5.4.5.1 when a fungal agonist is used to stimulate the oral epithelial cell line (Figure 5.9).

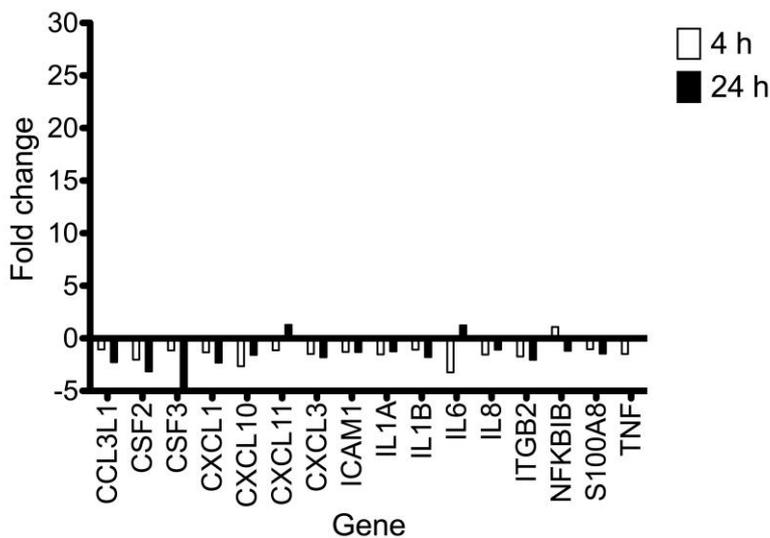


**Figure 5.9 - CHD-FA modulates the zymosan induced mRNA expression of inflammatory mediators by OKF6/TERT2 cells.**

OKF6/TERT2 cells were pre-treated  $\pm$  0.5% (v/v) CHD-FA for 2 min. Zymosan was then used to stimulate the cells for 4 (i) and 24 h (ii). The TLDA was used to analyse the expression of a panel of pro-inflammatory mediators. Each sample was carried out in triplicate on two independent experiments, with two replicates pooled from each experiment used in the TLDA. Data is represented by mean, relative to unstimulated control and *GAPDH*.

As identified by the RT<sup>2</sup> Profiler, a general trend of inhibition of zymosan induced expression of the panel of pro-inflammatory mediators was observed after 4 h zymosan stimulation with CHD-FA pre-treatment, compared to zymosan only (Figure 5.9i). The greatest reduction with CHD-FA pre-treatment was found in *CSF3* (2.5×), *CXCL3* (1.6×) and *IL-8* (1.5×). Of further interest, the expression of transcription factor *NFκB* was 1.7× greater in zymosan only stimulated cells compared to cells pre-treated with CHD-FA. In contrast, differences between ± CHD-FA pre-treatment were observed after 24 h zymosan stimulation (Figure 5.9ii).

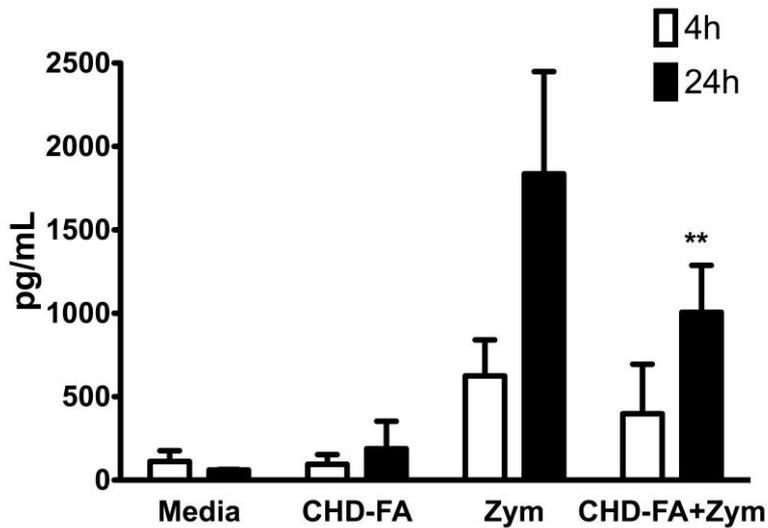
To show CHD-FA does not have an effect on the epithelial cell line in the absence of an agonist, pre-treatment with the agent was carried out without zymosan stimulation. Here it was shown that CHD-FA treatment alone does not have an effect on the host immune response after 4 h and 24 h (Figure 5.10).



**Figure 5.10 - CHD-FA does not modulate the OKF6/TERT2 cells in the absence of an agonist.**

OKF6/TERT2 cells were pre-treated ± 0.5% (v/v) CHD-FA for 2 min and incubated in media for 4 h and 24 h. The TLDA was used to analyse the expression of a panel of pro-inflammatory mediators. Each sample was carried out in triplicate on two independent experiments, with two replicates pooled from each experiment used in the TLDA. Data is represented by mean, relative to unstimulated control and *GAPDH*.

As interleukin-8 (IL-8) was shown to be down-regulated with CHD-FA treatment for both the RT<sup>2</sup> Profiler and TLDA, the expression of this chemokine was further investigated by ELISA (Figure 5.11).



**Figure 5.11 - CHD-FA down-regulates IL-8 release from OKF6/TERT2 cells stimulated with zymosan.**

OKF6/TERT2 epithelial cells were pre-treated with 0.5% (v/v) CHD-FA pH 7.0 for 2 min and stimulated with zymosan for 4 h and 24 h. IL-8 protein release was measured by ELISA. All samples were assayed in triplicate, on two independent occasions. Data represents mean  $\pm$  SD (\*\*p < 0.01).

Figure 5.11 shows IL-8 release was inhibited when cells were pre-treated with CHD-FA, after 4 h zymosan stimulation (1.5  $\times$ ) and significantly reduced following 24 h zymosan stimulation (1.8  $\times$  [p < 0.001]). No significant differences were observed between CHD-FA treatment alone and the media control.

## 5.5 Discussion

Oral microbial diseases are typically mediated by biofilms; communities of microorganisms that co-aggregate as sticky and tenacious structures and which characteristically have increased resistance to antimicrobials (ten Cate and Zaura, 2012). Our group recently reported that mouthwashes, including CHX were ineffective against a range of clinical MRSA strains (Smith et al., 2013), suggesting that alternative antimicrobial agents ought to be investigated. Indeed, recent studies by our group, including data presented in the previous chapter, have shown that the use of naturally derived molecules are effective against both orally and systemically derived isolates of *C. albicans* (Ramage et al., 2012b, Sherry et al., 2012). The data presented herein show that CHD-FA, a naturally derived antiseptic molecule, displays rapid broad-spectrum antimicrobial activity, and also elicits immunomodulatory activity.

Firstly, a comparative assessment of CHD-FA and CHX against a range of important bacteria associated with oral biofilm infections was undertaken. Both molecules were shown to effectively inhibit and kill planktonic cells, and both compounds were also effective against biofilms. Antimicrobial activity against planktonic bacteria has been reported previously for oxifulvic acid, a derivative of CHD-FA, where inhibition was observed against a range of important clinical pathogens, including *P. aeruginosa*, *S. aureus* and *Streptococcus pyogenes* (van Rensburg et al., 2000). Notably, only marginally higher concentrations of CHD-FA were required to kill the biofilm as compared to planktonic cells, whereas for CHX the fold change was up to 64 times, a phenomenon also found in chapter 4. Moreover, CHD-FA showed a rapid rate of kill for the periodontal pathogens tested as polymicrobial biofilms, as after 30 min treatment cellular viability was reduced by  $\geq 90\%$ , which was also observed for time kill studies in *C. albicans* biofilms in the previous chapter. It is recognised that a potential limitation of this study is that it was performed on biofilms produced from laboratory strains of the periodontal organisms *in vitro*, and it is conceded that further investigation may be required to assess the anti-microbial properties against the most virulent of clinical strains and biofilms formed *in vivo*. Equally, it would have been useful to test mixed *C. albicans* and bacterial biofilms. Nevertheless, collectively, these data suggest that CHD-FA has potent and broad-spectrum activity against polymicrobial biofilms. Furthermore, the SEM images indicate an

action against the bacterial cell membrane. Interestingly, the biofilms appeared to be disaggregated and displayed a fibrous appearance, presumably as a consequence of cell lysis and release of intracellular components. The previous studies on *C. albicans* biofilms in chapter 4 do not corroborate this observation, where no disruption of biofilms was observed. However, the filamentous nature of *C. albicans* biofilms may explain why the compound was unable to disaggregate these. Despite this finding, there was no significant difference in the number of each species when treated with CHD-FA, when compared to the untreated control.

Given that CHD-FA displayed an excellent antimicrobial profile, the next step was to ascertain whether it possesses any other biological properties, as has also been demonstrated for other naturals including tea tree oil (Carson et al., 2006). It has been reported that CHD-FA has no toxicity in rats and humans, and it has been further suggested that it elicits anti-inflammatory and wound healing promoting properties (Gandy et al., 2011, Sabi et al., 2011). Periodontitis is characterised by chronic inflammation that leads to tissue and bone destruction (Berker et al., 2013), and inflammatory processes play a key role in erythematous forms of oral candidosis. Controlling inflammation is therefore an attractive option for clinical management. The use of *in vitro* multi-species oral biofilms to study the inflammatory processes driven by complex biofilms have been shown to be important (Peyyala et al., 2013), therefore a similar system was developed to test CHD-FA and other bioactive molecules. Both CHX and CHD-FA in their native forms were shown to be toxic, therefore, in order to demonstrate subtle biological effects CHD-FA was buffered to pH 7.0 in order to test the hypothesis that it was immunomodulatory *in vitro*. Using an orally relevant epithelial cell line stimulated with a polymicrobial biofilm and a fungal agonist, it was demonstrated that at the transcript level cells pre-treated with CHD-FA showed a significant inhibition of pro-inflammatory molecules when induced by an inflammatory agonist, including the chemokine IL-8 (CXCL8). Analysis of the IL-8 protein also showed a significant reduction in its release from oral epithelial cells. These data indicate that CHD-FA has bioactivity against mammalian cells, as has been reported elsewhere (Gandy et al., 2011, Sabi et al., 2011). However, it is accepted that these differences are only observed when CHD-FA is adjusted to a neutral pH therefore, further studies are

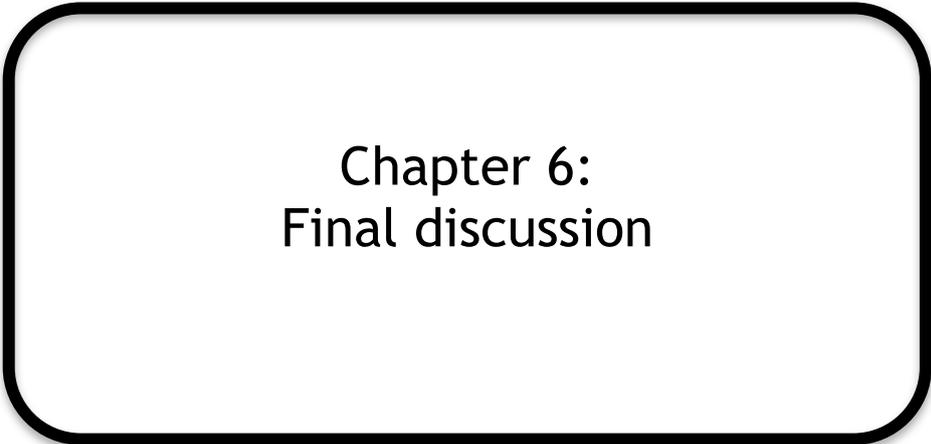
required to determine the most suitable formulation of CHD-FA to potentially be used clinically, which at present is at pH 2.0. However, the precise mechanism of action remains unknown, but it can only be speculated that CHD-FA interacts with membrane proteins resulting in blocking signaling pathways, which leads to the inhibition of pro-inflammatory mediators upon stimulation with biofilms. Further investigations are required to determine the specific mechanism of action of CHD-FA. Specifically, NF $\kappa$ B was shown to be down-regulated when CHD-FA was used as a pre-treatment therefore, studies investigating if the agent has a direct effect on NF $\kappa$ B or whether it blocks binding at the cell surface will be carried out next.

## CHAPTER FINDINGS

CHD-FA has broad-spectrum antimicrobial activity against a range of clinically relevant oral biofilms, including multispecies biofilms.

CHD-FA is non-toxic when buffered from its native pH 2.0 to pH 7.0.

Pre-treatment of an oral epithelial cell line with CHD-FA down-regulates a panel of pro-inflammatory mediators normally elevated in oral disease.



Chapter 6:  
Final discussion

## 6.1 Introduction

In recent years, *Candida* species have been identified as the most common fungal pathogen in BSI in patients within the ICU (Wisplinghoff et al., 2004), where the presence of an indwelling catheter has been associated to biofilm formation (Kojic and Darouiche, 2004, Douglas, 2003). Many studies have previously identified mechanisms involved in *C. albicans* biofilm development (Blankenship and Mitchell, 2006, Finkel and Mitchell, 2011, Nobile et al., 2012), however, these have been restricted by the use of well characterised laboratory strains, with few studies investigating the impact of biofilm formation in clinical isolates. Recent studies have shown a correlation between *C. albicans* biofilm formation and patient mortality, identifying specific risk factors associated with these infections (Tumbarello et al., 2012, Tumbarello et al., 2007). As not all clinical isolates have the ability form biofilms to the same degree as one another, the mechanisms behind this must be fully understood to improve the clinical management of these infections, which has implications both for diagnostics and treatment.

## 6.2 Improvement *C. albicans* diagnostics to detect biofilm-associated infections

The underlying molecular mechanisms responsible for *C. albicans* biofilm formation have been studied extensively, where elevated transcription of several key genes has been identified as playing a critical role in biofilm development (Blankenship and Mitchell, 2006, Cao et al., 2005, Garcia-Sanchez et al., 2004, Nobile et al., 2006a). However, to date with exception of a few studies very little has been explored with respect to clinical isolates, where differential levels of biofilm formation has been shown to exist that significantly impact antifungal therapy and clinical outcomes (Tumbarello et al., 2012, Walraven and Lee, 2013). As *C. albicans* is a commensal in over 50% of the healthy population (Coronado-Castellote and Jimenez-Soriano, 2013), it would be useful to understand whether specific isolates from these individuals have the capacity to be more pathogenic than one another and lead to disease, and methods to distinguish between these opportunistic pathogens and their levels of biofilm growth would be advantageous. Herwald and Kumamoto (2014) analysed a number of studies that investigated the regulation of genes in pathogenic

(Andes et al., 2004, Garcia-Sanchez et al., 2004, Nobile et al., 2012, Yeater et al., 2007) and commensal *C. albicans* cells (Rosenbach et al., 2010, Pierce et al., 2013b), and concluded that 1,444 genes were up-regulated in biofilm cells that were related to protein translation (Herwald and Kumamoto, 2014). Further studies are required *in vitro* and *in vivo* to identify a potential set of *C. albicans* genes that could be used as a molecular target for diagnosing biofilm infections.

Currently, the identification of *Candida spp.* clinically is reliant on culture based tests or the detection of B-1,3 glucans and mannans, all of which do not elude to the isolates biofilm forming ability (Mokaddas et al., 2011, Held et al., 2013). In addition, a traditional and rapid test also used diagnose *C. albicans* infections is the germ tube test (GTT), whereby isolates are viewed microscopically to determine their hyphae forming ability with positive tests indicating the presence of *C. albicans* or *C. dubliniensis* (Rimek et al., 2008, Sheppard et al., 2008). Although this test is able to identify the presence of hyphal cells it does not give an indication as to whether this isolate is able to adhere to a surface and produce ECM, defining features of a biofilm. Furthermore, germ tubes can also be found in isolates with LBF albeit not in a great abundance and therefore are not indicative of an isolate with HBF.

The use of molecular diagnostics such as PCR over standard culture methods has been shown to be advantageous, with *Candida* being detected in blood samples 3 days prior to blood culture techniques, therefore enabling the administration of antifungal therapy earlier (Wellingshausen et al., 2009). Molecular methods including qPCR have been compared to the XTT assay and although they show similar data at early phases of biofilm development, qPCR could quantify differences in biomass  $\pm$  treatment that were undetected by XTT (Xie et al., 2011). Therefore, the development of a diagnostic that would distinguish *C. albicans* biofilm forming ability is of great importance as it could help direct more appropriate antifungal therapy. In this thesis, a number of transcriptional targets were investigated with the aim to identify a potential marker for *C. albicans* biofilm formation. Although preliminary data did not identify a defined biofilm target, subsequent RNA-Seq analysis concluded 853 genes were significantly up-regulated in isolates with HBF, many being cell wall and membrane proteins including *HWP1*. This cell surface protein is highly expressed in hyphal cells and is essential for biofilm formation *in vitro* and *in vivo* (Fanning

et al., 2012, Nobile et al., 2006b). Therefore, there is potential to use this protein as a biomarker for diagnosing *C. albicans* biofilm infections, though further studies are required to take this forward.

### 6.3 Current anti-biofilm strategies

In the absence of a biofilm diagnostic, biofilm-related infections must be managed efficiently to prevent increased morbidity and mortality rates, as well as controlling antifungal therapy. Based on a comprehensive study by the WHO investigating antimicrobial resistance it was reported that the overuse of fluconazole has led to the increases in azole resistant species, including *C. albicans* and notable *C. glabrata* (WHO, 2014). Moreover, although azoles are active against planktonic cells, they are highly ineffective against *C. albicans* biofilms (Ramage et al., 2012c, Taff et al., 2013). Despite this, these agents remain as the first choice antifungal in *C. albicans* infections, due to safety within the host and cost effectiveness compared to other classes of antifungal. Studies have investigated the potential of combining azoles with various inhibitors of stress responses, including calcineurin (Shinde et al., 2012, Uppuluri et al., 2008) and Hsp90 (Cowen et al., 2009, Robbins et al., 2011), where it has been shown to improve fluconazole activity against *C. albicans* biofilms. Therefore, the use of azoles in *C. albicans* biofilm-related infections may need to be used as part of a combinational therapy to kill the biofilm.

Although current guidelines state the removal of an indwelling catheter is the most appropriate treatment of invasive candidiasis (Andes et al., 2012, Cornely et al., 2012), this is not possible for all patients due to the location of the medical device and host status. ALT has received interest as a potential alternative treatment for device-associated infections, where polyene agents have shown high activity with >60% success rates when used (Angel-Moreno et al., 2005, Buckler et al., 2008, Wu and Lee, 2007). Although these agents have excellent biofilm activity, it tends to be at concentrations that are also toxic to the host (Pierce et al., 2013a, Ramage et al., 2002c). Therefore, alternative methods of administering amphotericin B are under investigation. These include the incorporation of AMB within a nanoparticle, which have been shown to be successful in a topical *in vivo* model, where reduced fungal burdens and low toxicity was observed (Sanchez et al., 2014a). The relatively new class of

antifungals, echinocandins, were initially shown to be highly effective against *C. albicans* biofilms (Bachmann et al., 2002), yet they do not completely kill the biofilm and have slower kill kinetics compared to other agents (Cateau et al., 2011, Ramage et al., 2013). Furthermore, a paradoxical effect is observed in CSP that makes it more difficult to determine an appropriate treatment towards *C. albicans* biofilm-related infections, as too little or high concentration would be ineffective (Bizerra et al., 2011, Imtiaz et al., 2012, Stevens et al., 2005).

Tumbarello and colleagues (2012) had identified inadequate antifungal therapy as one of the key predictors of patient mortality and hospital length of stay in patients with biofilm forming isolates (Tumbarello et al., 2012). One of the main challenges with the use of antifungal therapy is that clinicians tend to class all *C. albicans* infections as one and do not consider how individual strain characteristics impact treatment. This emphasises the importance of identifying isolate characteristics including biofilm susceptibility, before administering therapy that could be completely ineffective.

In the absence of molecular diagnostics and increasing antifungal resistance, the use of alternative agents with high biofilm activity is sought after, particularly those derived from a natural source as these tend to be cheap and less toxic to the host. Agents with broad-spectrum activity are advantageous due to many infections caused by polymicrobial biofilms.

## **6.4 Can CHD-FA be used as alternative antimicrobial?**

Increasingly *Candida* biofilms are becoming clinically important (Ramage et al., 2009, Tumbarello et al., 2007), particularly because of inappropriate or misuse of broad-spectrum antimicrobials that are ineffective against recalcitrant biofilms (Niimi et al., 2010). Therefore, the use of natural antimicrobials is highly sought after as it is thought to be relatively inexpensive and less toxic than conventional agents currently used clinically. Tea tree oil (TTO) is one of the most documented naturals currently used as an alternative oral hygiene agent (Vazquez and Zawawi, 2002, Soukoulis and Hirsch, 2004), with many studies showing its broad-spectrum antimicrobial activity (Thompson et al., 2008, Hammer et al., 2003b, Garozzo et al., 2011, Hammer et al., 2003a). Furthermore terpinen-4-ol, the active agent of TTO, is equally active against

*Candida* planktonic and sessile cells (Ramage et al., 2012b), an advantageous property of an antifungal as it has been reported that up to 1000-fold greater concentrations of agents are required to kill biofilms than their planktonic counterparts (Di Bonaventura et al., 2006, Ramage et al., 2001a).

Other natural compounds shown to be of clinical importance include garlic where it was used as a topical treatment in patients with oral candidiasis and was shown to be equally effective as clotrimazole (Sabitha et al., 2005). Furthermore, the use of naturals in combinational therapy has also shown to be beneficial, whereby curcumin was able to work synergistically with fluconazole to inhibit the resistance and restore the sensitivity of the azole (Garcia-Gomes et al., 2012). Moreover, curcumin was able to inhibit the adhesion of *Candida* species to buccal epithelial cells to a greater level than fluconazole (Martins et al., 2009).

It is known that fulvic acids are one of the components that make up humic substances, which are ubiquitous in the environment and are formed during plant decay (Snyman et al., 2002, van Rensburg et al., 2001). A previous study has shown oxifulvic acid elicits broad-spectrum activity against a range of planktonic bacteria and fungi including *E. faecalis*, *S. aureus* and *C. albicans* (van Rensburg et al., 2000) whereby all organisms tested were successfully inhibited at  $\leq 1.5\%$ , however its activity against biofilms was unknown. This thesis has provided evidence that CHD-FA could potentially be used as an alternative natural agent for the treatment of biofilm infections as it is highly effective against a panel of oral pathogens, where 0.5% CHD-FA was able to inhibit planktonic and sessile growth. Furthermore, this concentration was equally effective against a multispecies periodontal biofilm *in vitro*, reducing viability to the same level as 'the gold standard' chlorhexidine (Herrera, 2013). However, it is accepted that although a four species mixed biofilm model was used in this study, antimicrobial activity against this model does not fully represent all mixed biofilms that are found within the oral cavity, but only a few of species relevant in periodontal disease. Currently our group are developing various co-culture models that contain up to 11 species of prokaryotes and eukaryotes. This provides a greater understanding to how the immune response is regulated in the presence of such complex biofilm models (Guggenheim et al., 2009, Peyyala et al., 2013), as well as providing a situation that is more reflective of the

clinical environment. Furthermore, as CHD-FA is unable to disrupt the biomass of biofilms, the combination of this novel agent with a compound such as lauroyl glucose could enhance the breakdown of biofilms and improve antifungal therapy (Dusane et al., 2008, Martins et al., 2011). Moreover, eDNA has is a known component of bacterial and fungal biofilms (Martins et al., 2010, Whitchurch et al., 2002), the use of DNase in conjunction with an antimicrobial would enhance biofilm disruption (Martins et al., 2011).

Overall, this data demonstrates that CHD-FA is effective against polymicrobial biofilms, independent from biofilm disruption, suggesting it is a useful antimicrobial. CHD-FA has already shown to have no sign of toxicity in rats and humans, and in fact was reported to have anti-inflammatory and wound healing properties, possibly through a free radical scavenging mechanism (Gandy et al., 2012, Gandy et al., 2011, Sabi et al., 2012), which has been further supported by this thesis. One inconsistency of all these studies is the variation in pH of CHD-FA ranging from 2.0 to 7.0. This study has shown low pH is toxic against an oral epithelial cell line with a similar reduction in viability to CHX treatment, an issue reported in many cell types (Chang et al., 2001, Mariotti and Rumpf, 1999, Lee et al., 2010). Furthermore, CHX has been responsible for anaphylaxis in two individuals within the UK following routine dental treatment (Pemberton and Gibson, 2012), resulting in fatalities. This emphasises the extreme side effects of this antiseptic agent but awareness of such cases are needed to possibly prevent such hypersensitivity reactions in the future.

In addition to its broad-spectrum antimicrobial properties, this study has shown CHD-FA can modulate the immune response and down-regulate the biofilm induced expression of pro-inflammatory mediators known to be up-regulated in oral keratinocytes (Peyyala et al., 2013). However, a limitation of this study was only a selected number of inflammatory mediators were investigated. Moreover, this model does not take host factors in to consideration, which may influence the inflammatory response even further. Therefore, further studies that determine the effect of CHD-FA on the host as well as assessing its ability to clear the infection *in vivo* are of great value. However, it does not take away from the fact that CHD-FA is able to modulate the immune response and complements other studies that have shown an anti-inflammatory effect of CHD-FA when used as a topical agent in humans and rats (Gandy et al., 2011, Sabi et

al., 2012). Collectively, these properties make CHD-FA an attractive option for the development of a topical agent such a mouthwash or denture cleanser to treat microbial oral disease. Moreover, the use of this compound as a broad-spectrum antiseptic agent is desirable, whereby it could be used for the treatment of systemic disease as well as potentially being incorporated in to biomaterials and wound dressings. However, further studies *in vitro* and *in vivo* are first required to further define the mode of action of this unique compound.

## 6.5 Future work

This body of work has assessed *C. albicans* biofilm formation by clinical isolates by using a number of techniques and demonstrated that this varies between strains. Although the presence of eDNA was identified as a contributing factor towards biofilm formation, the depletion of this only led to a minimal impact with regards to antifungal susceptibility. Therefore, other mechanisms must be involved in *C. albicans* biofilm variation, and the identification of these could be found with further analysis of the RNA-Seq data found in this study. Here, a subset of genes found to be up-regulated in LBF and HBF were identified however, the roles of many of these genes are unknown. Therefore, further in depth analysis of such genes could provide crucial information for explaining the differences in *C. albicans* biofilm formation. Although data in this thesis was based around a limited number of isolates, it gives a solid foundation for investigating these phenotypic, biochemical and transcriptional differences. In fact, our group are currently investigating these findings but on a larger scale collaboratively, where all candidaemia cases within Scotland between February 2012 to January 2013 will be screened for their biological characteristics. The advantage of this follow up study is that there is clinical data to match each candidaemia isolate, allowing for biofilm formation to be related back to a number of clinical parameters. Future work will also continue to examine the impact strain variation has on the host to gain a further understanding to how these isolates behave. It is acknowledged that the kidneys are one on the main organs affected by systemic candidiasis *in vivo* (MacCallum, 2009, Jacobsen et al., 2014), therefore assessing the impact of isolates with LBF and HBF on a recently developed renal cell line (Szabo and MacCallum, 2014) would seem a reasonable next step before moving on to *in vivo* studies.

As antifungal resistance is an increasing problem, the development of novel agents for the treatment of *C. albicans* infections is of great importance. This study has evaluated the antimicrobial and immunomodulatory properties of the natural compound CHD-FA. Although it was shown to have excellent anti-biofilm activity against fungi and bacteria, its inability to disrupt biofilms is a disadvantage. However, further studies plan to investigate the activity of CHD-FA with a known disruptive agent such as DNase or lauroyl glucose to further increase its activity against biofilms. CHD-FA has also shown potential anti-inflammatory properties that are of particular interest for an oral hygiene product as many oral diseases are associated with inflammation. One mediator that was reduced in the presence of CHD-FA was NF- $\kappa$ B. Further investigations are required to fully understand the mechanisms behind this and to whether it is a direct effect on NF- $\kappa$ B that results in the reduced expression of other pro-inflammatory mediators or whether CHD-FA is to inhibit toll-like receptors. Finally, as CHD-FA was used at various pHs in this piece of work, studies need to now focus on an appropriate pH for the product to be used within the clinical setting. Currently, formulations are being prepared at pH 5.8-6.0 for its use as a mouthwash and a denture cleanser, however further studies *in vitro* and *in vivo* are required to evaluate the activity of these.

## 6.6 Summary

The key findings of the research presented in this thesis are as follows

- *C. albicans* biofilm formation is differential
  - Clinical isolates have varied levels of biomass relating to virulence and antifungal therapy
  - Chitinases have been shown to be involved in the release of eDNA, contributing to the *C. albicans* biofilm formation and stability
  - Various transcriptional targets have been identified as potential biomarkers to be utilised as a diagnostic tool for *C. albicans* biofilm-related disease

- CHD-FA as an alternative antimicrobial agent
  - CHD-FA has broad spectrum activity against bacterial and fungal biomass, to similar levels as CHX
  - The agent acts non-specifically against cells membranes and is not impacted by known *C. albicans* resistance mechanisms
  - CHD-FA is able to inhibit inflammatory mediators that are up-regulated upon bacterial and fungal stimulation

Overall, these findings have increased the understanding of *C. albicans* biofilm differentiation within clinical isolates and evaluated the antimicrobial activity of the novel agent CHD-FA. Together these allow for the development of diagnostic tools and alternative therapies for biofilms infections.

## 1 Appendix I - List of *C. albicans* clinical isolates

| <b>LBF</b> | <b>HBF</b> | <b>Remaining isolates</b> |       |
|------------|------------|---------------------------|-------|
| 27915      | 2623       | 42341                     | 2972  |
| 42340      | 28158      | 20626                     | 11689 |
| 76369      | 68518      | 82942                     | 26839 |
| 88301      | 77013      | 78462                     | 20705 |
| 65867      | 9629       | 69299                     | 62054 |
| 74174      | 34106      | 40144                     | 72419 |
| 50348      | 58535      | 91696                     | 62585 |
| 77014      | 74503      | 3022                      | 79214 |
| 92228      | 71114      | 2974                      | 54821 |
| 31903      | 39286      | 29659                     | 91694 |
|            |            | 19032                     | 83889 |

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