Investigation into the mechanism of arachidonic acid increased fluconazole susceptibility in *Candida albicans* biofilms and application to drug repurposing

by

Oluwasegun Olalekan Kuloyo

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Promoter: Prof. C.H. Pohl – Albertyn

Co- Promoter: Prof. J. Albertyn

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Oluwasegun Kuloyo
Department of Microbial, Biochemical and Food Biotechnology,
University of the Free State,
South Africa.

DEDICATION

To friends and family who kept cheering while the mile lasted

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This thesis is written in the form of five standalone articles, of which the first is a review paper, and the others are independent research articles. As a result, there is some overlap of information and references that could not be avoided.

LITERATURE REVIEW

A Review on *Candida albicans* Biofilms: Infection and Treatment

1.0. Motivation for Study

The *Candida* genus consists of a heterogeneous group of organisms with more than 200 species (Eggimann et al., 2003; Brandt and Lockhart, 2012; Sardi et al., 2013). However, only approximately 15% of these species are associated with human infections, which include thrush, vulvovaginitis, chronic mucocutaneous candidiasis and chronic atrophic stomatitis (Eggimann et al., 2003; Pfaller and Diekema, 2007; Pfaller et al., 2010; Brandt and Lockhart, 2012) Amongst the pathogenic *Candida* species, *C*. *albicans* is the most common, and it is followed closely by *C*. *glabrata*, *C*. *tropicalis* and *C*. *parapsilosis* (Miceli et al., 2011; Pfaller et al., 2019). *Candida albicans* is a natural commensal of the human microbiome, which can be transmitted vertically from mother to child during birth (Miranda et al., 2009; Ward et al., 2017). However, it can become pathogenic, causing mild to severe infections as an opportunistic pathogen, especially to immune-compromised individuals (Biswas et al., 2007). Its is also the fourth most common cause of nosocomial bloodstream infections (Fenn, 2007; Shareck and Belhumeur, 2011).

An essential virulence trait of *C. albicans* is its ability to form biofilms. These biofilms grow on the surface of catheters, dentures and mucosal cells in infected individuals (Mayer et al., 2013; Sardi et al., 2013). Biofilms are characterised by high antifungal resistance, which makes eradicating the infection challenging (Sanglard et al., 2003; Spampinato and Leonardi, 2013). The use of modern medical procedures, coupled with the high antifungal resistance, has made *C*. *albicans* biofilm of noteworthy concern, necessitating alternative treatment options (Costa et al., 2015).

Several alternative treatment options, including polyunsaturated fatty acids (PUFAs) (e.g. arachidonic acid, stearidonic acid and eicosapentaenoic acid) have been investigated. Polyunsaturated fatty acids have been reported to inhibit the growth of *C*. *albicans* biofilms, possibly by the impediment of mitochondrial function, which leads to apoptosis and oxidative stress (Thibane et al., 2010, 2012). Also, a combination of arachidonic acid, together with the azole, clotrimazole, was shown to increase the susceptibility in *C. albicans* biofilms (Ells et al., 2008). Although this combination

treatment is promising, an understanding of the underlying mechanism of this increased susceptibility is essential to harness its potential as a treatment option for *C. albicans* biofilms infections. Gaining such an understanding is therefore the aim of the present study.

2.0. Introduction

Candida albicans is a polymorphic yeast, which is a human commensal in the gastrointestinal, genitourinary and oral mucosal tracts. It is also a potentially deadly pathogen to immune-compromised individuals, such as organ transplant recipients, HIV infected, chemotherapy and diabetes patients (Biswas et al., 2007; Cleary et al., 2010; Gonçalves et al., 2016). The advent of modern medical procedures, such as the use of potent antibiotics, medical implants, chemotherapy, major abdominal surgery and cytotoxic drugs, contributed to *C*. *albicans* becoming an important nosocomial pathogen (Douglas, 2003; Fox and Nobile, 2012; Spampinato and Leonardi, 2013). Virulence factors such as morphological transition, adhesin expression, thigmotropism, secretion of tissue-damaging hydrolytic enzymes and biofilm formation, contribute to its pathogenicity (Mayer et al., 2013; Sardi et al., 2013).

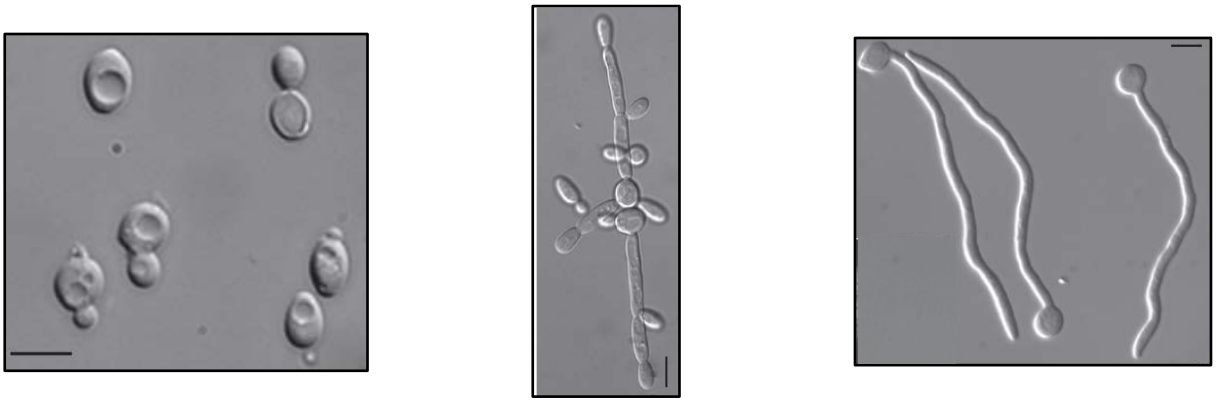
Candida albicans infections, commonly known as candidiasis, can either be superficial or systemic. Superficial candidiasis is generally not life-threatening and occurs on the skin, as well as oral, vaginal, gastrointestinal and conjunctival tissues (Molero et al., 1998; Fidel and Wozniak, 2010). In affected individuals, the manifestation of superficial candidiasis can cause a substantial deterioration to the quality of life (Bondaryk et al., 2013). Oral candidiasis affects approximately 90% of HIV infected patients, premature infants, denture wearers and older adults (Flevari et al., 2013; Dantas et al., 2015). Also, about 75% of women within the childbearing age, will experience vulvovaginal candidiasis, while approximately 50% of those infected will suffer recurrent infections even after treatment (Sobel, 2007; Achkar and Fries, 2010; Mayer et al., 2013). In contrast, systemic candidiasis is a life-threatening condition, associated with deep-seated infections and candidaemia (bloodstream infections) (Ben-Ami, 2018; Pappas et al., 2018). The incidence of systemic candidiasis is high, with more than 700,000 cases reported annually globally, and a crude mortality rate between 30-50% of infected patients (Sudbery, 2011; Dantas et al., 2015; Bongomin et al., 2017).

Candida albicans can grow either as yeasts, hyphae or pseudohyphae (Figure 1.1) depending on the environmental condition (Berman and Sudbery, 2002; Berman, 2006). The yeast form is usually white, smooth with a round to ovoid shape in appearance (Sudbery et al., 2004; Berman, 2006). Conversely, the pseudohyphal form appears as an ellipsoid, chain-forming elongated yeast cell, while the hyphae appear long and parallel without constrictions at the septa (Berman and Sudbery, 2002; Berman, 2006). The switch between the different morphologies is an essential virulence trait associated with host colonisation during infection (Jacobsen et al., 2012). The yeast form of *C*. *albicans* is more suited for dissemination within the host bloodstream while hyphae and pseudohyphae are more suited for the invasion of tissues and organs (Berman and Sudbery, 2002; Mayer et al., 2013). The switch between the *C. albicans* yeast and hyphal forms can be induced by several environmental signals, including serum, 37^{o}C temperature, *N*-acetyl-D-glucosamine (GlcNAc), neutral pH, 5% CO_2 and starvation. Also, growth in synthetic media such as Lee's media (which contains a mixture of amino acids), Spider media, (which has mannitol as its carbon source), and M199, (a mammalian tissue culture media), induce the yeast to hyphal transition (Berman and Sudbery, 2002; Biswas et al., 2007; Sudbery, 2011).

The regulation of *C*. *albicans* yeast to hyphal transition is carried out by several transcription factors, including Efg1p (Stoldt et al., 1997), Cph1p (Liu et al., 1994), Cph2p (Lane et al., 2001), Tec1p (Schweizer et al., 2000), Flo8p (Cao et al., 2006), Czf1p (Brown Jr et al., 1999), Rim101p (Davis et al., 2000), Ndt80p (Sellam et al., 2010) and Ume6p (Banerjee et al., 2008). Efg1p, which is considered the master regulator for *C*. *albicans* morphogenesis, is regulated by the cyclic AMP-Protein Kinase A (cAMP-PKA), possibly through direct phosphorylation in response to environmental signals from serum, CO_2, pH and GlcNAc (Lo et al., 1997; Stoldt et al., 1997; Bockmüh and Ernst, 2001). In contrast, Cph1p is activated by Mitogen-Activated Protein Kinase (MAPK) in response to serum (Lo et al., 1997). Most of the mentioned transcription factors are necessary for hyphal morphogenesis under certain conditions; however, their ectopic expression is not adequate to induce true hyphal morphogenesis except for Ume6p (Kornitzer, 2019).

Candida albicans biofilms comprise of the yeast, hyphae and pseudohyphae morphologies wrapped in a dense layer of a carbohydrate-rich extracellular matrix

(Chandra et al., 2001; Douglas, 2002; Fanning and Mitchell, 2012). These biofilms have a significant impact on healthcare because they colonise the surfaces of the teeth, mucosal and medical implants (Kojic and Darouiche, 2004) and have a higher antifungal resistance than planktonic cells (Mathé and Van Dijck, 2013; Taff et al., 2013). Infections of *C*. *albicans* are controlled with the administration of either fungicidal or fungistatic drugs belonging to the azole, polyene, echinocandin, allylamine and flucytosine classes (Mathé and Van Dijck, 2013; Spampinato and Leonardi, 2013). The effectiveness of these antifungals are hampered by resistance via mechanisms which include reduced accumulation of drug, decreased affinity of drug targets and counteraction of the effect of the drug (Vandeputte et al., 2012; Spampinato and Leonardi, 2013).



Yeast Pseudohyphae Hyphae

Figure 1.1. *Candida albicans* morphology as a yeast, hyphae and pseudohyphae (Sudbery, 2011).

3.0. *Candida albicans* Biofilms

Biofilms are microbial cells encased with a dense layer of an extracellularly produced matrix that can attach to a wide variety of surfaces from living and non-living surfaces (Donlan, 2002). The developmental process of *C*. *albicans* biofilm has been studied both *in vivo* and *in vitro* (Chandra et al., 2001; Andes et al., 2004). The formation of *C*. *albicans* biofilm *in vitro,* as shown in Figure 1.2, involves four sequential stages, beginning with the adhesion of the yeast cells followed by proliferation of yeast cells to form a basal layer. After that, the growth of hyphae and pseudohyphae and the production of extracellular matrix takes place. Finally, the biofilm matures, while non-adherent cells disperse to colonise new niches (Chandra et al., 2001; Douglas, 2003; Nobile et al., 2006b).

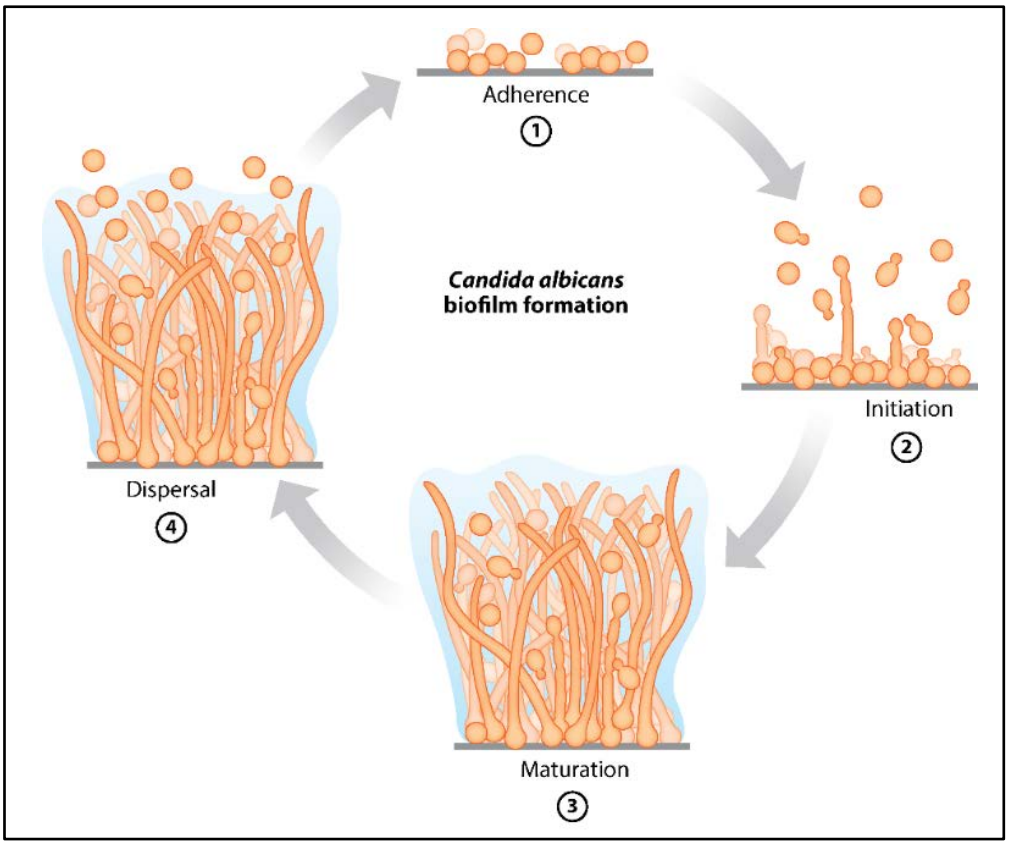


Figure 1.2. Stages of *C. albicans* biofilm formation. (1) Adherence of yeast cells to a surface. (2) Initiation of cell proliferation, forming a basal layer of anchoring cells. (3) Maturation, including the growth of hyphae concomitant with the production of the extracellular matrix material. (4) Dispersal of yeast cells from the biofilm to colonise new sites (Nobile and Johnson, 2015).

The formation of a mature *C. albicans* biofilm *in vitro* takes approximately 72 h. The early phase is approximately 11 h. Yeast cells begin to adhere to the surface within 1-2 h, while distinct microcolonies appear within 3-4 h with a compact fungal growth by 11 h. After that, the intermediate phase (between 12-30 h) is characterised by the formation of a bilayer comprising the yeast and hyphae, together with the production of the extracellular polymeric substances. The biofilm matures between 38-72 h, with a thick layer of extracellular material which entirely encases the yeast and hyphae in a dense network (Samaranayake et al., 1995; Chandra et al., 2001; Seneviratne et al., 2008b; Chandra and Mukherjee, 2015).

Biofilm formation *in vivo* is similar to the *in vitro* process. However, it appears to be faster with a shortened period of early biofilm formation, as indicated by Andes and co-workers (2004), using a rat central venous catheter. With biofilm formation *in vivo*, several layers of cells, surrounded by the extracellular matrix, were observed at 8 h

compared to only a few adherent cells seen *in vitro* after 8 h (Andes et al., 2004). Furthermore, maturation of biofilm could be seen after 24 h in comparison with 38-72 h observed *in vitro* (Chandra et al., 2001; Andes et al., 2004).

3.1. Regulation of biofilm formation in *Candida albicans*

The process of *C*. *albicans* biofilm formation is under the regulation of several transcription factors. However, six transcriptional regulators namely Efg1p, Tec1p, Bcr1p, Ndt80p, Rob1p and Brg1p have been identified as master regulators for biofilm formation both *in vivo* and *in vitro* (Fox and Nobile, 2012; Nobile et al., 2012). These transcriptional regulators are in a complex transcriptional circuit, where they regulate each other's expression (Figure 1.3) and the expression of approximately 1000 target genes, of which 23 are bound by all six regulators (Nobile et al., 2012). These genes play a role in processes which are essential to biofilm formation, such as hyphal formation, adhesion, drug resistance, and matrix production. However, most of the identified genes have not been characterised and also show no similarity to genes in other organisms (Fox and Nobile, 2012). Furthermore, an additional 44 transcriptional regulators, which bind directly to at least one of the six master regulators, have been identified. It was observed that deleting any of these regulators affects biofilm formation in *C*. *albicans* (Fox and Nobile, 2012; Nobile and Johnson, 2015).

Additional to the six reported master regulators, three other regulators, Flo8p, Gal4p and Rfx2p, play unique roles in biofilm formation both *in vivo* and *in vitro* (Fox et al., 2015). Flo8p is known to contribute to hyphal growth. However, its homozygous mutant is highly defective in biofilm formation. Also, its role as part of the biofilm regulatory network is indicated with its binding to existing master regulators Ndt80p, Brg1p and Efg1p, as shown with chromatin immunoprecipitation studies (Cao et al., 2006; Fox et al., 2015). In addition, Gal4p (a glycolysis and carbohydrate metabolism regulator) and Rfx2p (which plays a role in hyphal formation) have a negative regulatory effect on biofilm formation (Askew et al., 2009; Hao et al., 2009). The filament specific transcriptional regulator, Ume6p, has been shown to determine *C*. *albicans* morphology in a dose-dependent manner (Carlisle et al., 2009). The transcription factors associated with hyphae induction also play essential roles in biofilm formation. The importance of hyphae to *C*. *albicans* biofilm formation is demonstrated with the formation of a defective biofilm in *efg1*Δ/Δ and double deletion

(*efg1*Δ/Δ/*cph1*Δ/Δ) strains, which grow entirely as yeast (Ramage et al., 2002c; Nobile and Mitchell, 2005).

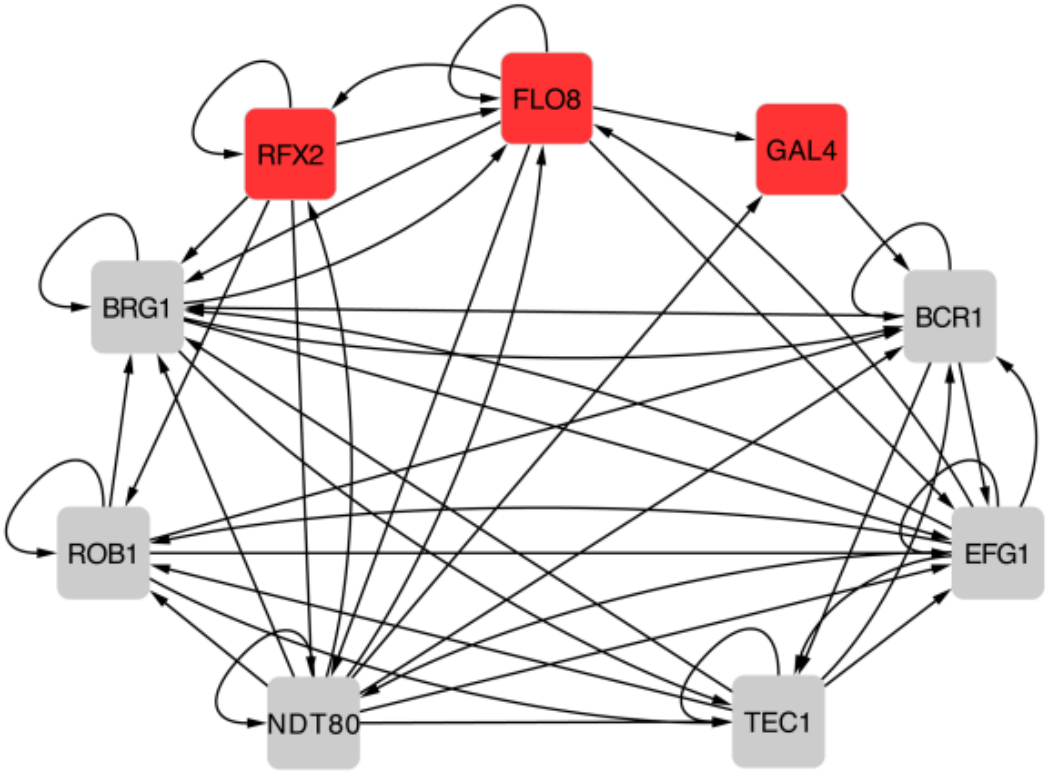


Figure 1.3. Transcriptional network for *C. albicans* biofilm formation. The initial identified six transcriptional regulators required for standard biofilm formation are highlighted with the grey boxes while the new regulators are highlighted with the red boxes. The black arrows indicate target genes whose promoters are bound by each regulator (Fox et al., 2015).

3.1.1. Regulation of adhesion in *Candida albicans* biofilm formation

Adhesion between *C. albicans* cells and the host or material surface is the first crucial step to biofilm formation (Cavalheiro and Teixeira, 2018). Attachment of *C. albicans* cells to surfaces is facilitated by nonspecific interactions (electrostatic and hydrophobic forces) and specific adhesin-ligand bonds (Chaffin et al., 1998; Jeng et al., 2005; Ramage et al., 2005). In *C. albicans*, the glycophosphatidylinositol-dependent cell wall proteins (GPI-CWPs), which are characterised by an N-terminal signal peptide and C-terminal sequence containing a GPI anchor, mediate biofilm attachments (Chaffin, 2008). The GPI-CWP adhesins in *C. albicans* include the ALS (Agglutinin Like Sequence) family, Hwp1 (Hyphal Wall Protein 1) and Eap1 (Enhanced Adherence to

Polystyrene 1). These proteins are downstream targets of the Bcr1p transcription factor, and they mediate *C*. *albicans* attachment to non-living and living surfaces, including human endothelial and epithelial cells (Ramage et al., 2005; Nobile et al., 2006b, 2006a).

The ALS family comprise eight members (Als 1-7, 9) (Hoyer, 2001; Hoyer et al., 2008; Hoyer and Cota, 2016). Amongst the different *C. albicans* adhesin genes, *ALS1*, which is expressed both in the yeast and hyphal phase, has the highest differential regulation during biofilm formation (Garcıa-Sanchez et al., 2004; Coleman et al., 2010). Als1p shares a high similarity in sequence, function and regulation with Als3p and both adhesins interact with Hwp1p to facilitate cell-to-cell adhesion (Sheppard et al., 2004; Nobile et al., 2008). *Candida albicans ALS3*, which is expressed only on the hyphae and germ tubes in abundance, plays a vital role in biofilm adhesion (Zhao et al., 2006; Coleman et al., 2009). The formation of defective biofilms by *als1*Δ/Δ/*als3*Δ/Δ double mutants both *in vivo* and *in vitro* and scanty, disorganised biofilms of *als3*Δ/Δ produced *in vitro*, indicates their importance to *C. albicans* biofilm formation (Nobile et al., 2006a, 2008; Zhao et al., 2006; Finkel and Mitchell, 2011).

EAP1, which is expressed both in yeasts and hyphae, is also essential for adhesion and biofilm formation in *C. albicans* (Li and Palecek, 2003). The adhesion of a nonadherent *Saccharomyces cerevisiae* to a polystyrene surface due to the expression of *C. albicans EAP1* demonstrates the importance of Eap1p in adhesion (Li and Palecek, 2003). Also, a significant reduction in adherence and a defect in biofilm formation was observed *in vitro* and *in vivo* in *eap1*Δ/Δ mutants (Li et al., 2007; Finkel and Mitchell, 2011). The initial adhesin required for *in vivo* biofilm formation is the cell surface protein Hwp1p, which is expressed only in the hyphae (Sundstrom, 2002; Nobile et al., 2006b). It is a substrate for host transglutaminases which cross-link covalently with epithelial cell surfaces (Staab et al., 1999). Homozygous deletion of *HWP1* (*hwp1*Δ/Δ) results in a slight defect in biofilm formation *in vitro*, which mainly comprises of yeast cells. However, the deletion is more detrimental *in vivo* with a failure to produce biofilm (Nobile et al., 2006b, 2006a).

3.1.2. Regulation of *Candida albicans* biofilm initiation and filamentation

After adhesion, the yeast cells multiply and form a population of hyphal and pseudohyphal, cells which constitute the basal layer (Costa et al., 2015). Hyphae are

an essential component to the biofilm as a structural support framework for the yeast cells, thus allowing for biofilm development and preservation (Nobile and Johnson, 2015). Many of the regulators at this stage are also involved in the regulation of hyphae formation. Efg1p mainly facilitates proliferation and morphological transition. However, other regulators, including Tec1p, Ndt80p, and Rob1p are also involved in this process (Nobile et al., 2006a; Ramage et al., 2009; Nobile and Johnson, 2015). The Bcr1p transcriptional master regulator is not required in this stage. However, its expression induces cell-surface proteins such as Als3p and Hwp1p, which allows the hyphae to adhere to one another (Nobile and Mitchell, 2005; Finkel and Mitchell, 2011; Nobile and Johnson, 2015).

3.1.3. Regulation of *Candida albicans* biofilm maturation and production of extracellular matrix

Maturation of *C. albicans* biofilm comprises growth, coupled with the accumulation and enclosure of cells with extracellular matrix (ECM) (Finkel and Mitchell, 2011). The ECM comprises of a mixture of carbohydrates (most of which are α-mannan, β-1,6 glucan, and a small portion of β-1,3 glucan), lipids, hexosamine, phosphorus, uronic acid and nucleic acids (Al-Fattani and Douglas, 2006; Zarnowski et al., 2014). Two transcriptional regulators, Zap1p (Csr1p) and Rlm1p are implicated in the regulation of ECM production in *C*. *albicans* (Nobile and Johnson, 2015; Lohse et al., 2018). The zinc-response transcription factor, Zap1p, is a negative regulator of ECM formation. Zap1p activates the expression of Csh1p and Ifd6p, which repress ECM production and also inhibit expression of *GCA1*, *GCA2*, and *ADH5,* which are positive regulators of ECM production (Nobile et al., 2009; Costa et al., 2015). The transcription factor, Rlm1p, has been shown to play an essential role in cell wall maintenance (Delgado-Silva et al., 2014) and deletion of Rlm1p reduces ECM accumulation (Nett et al., 2011).

3.1.4. Regulation of *Candida albicans* dispersal

The dissemination of unadhered cells indicates the final stages of the biofilm formation process. However, it has been reported that the dissemination of cells is continuous during the entire biofilm formation process, while higher numbers are dispersed at biofilm maturity (Uppuluri et al., 2010a; Lohse et al., 2018). The dispersed cells can colonise new sites for the development of new biofilms or disseminate within the host to initiate infection (Lohse et al., 2018). Transcriptional regulators, Ume6p, Pes1p and

Nrg1p, are associated with the dispersal of cells from *C. albicans* biofilms. Overexpression of Pes1p and Nrg1p increase the number of dispersed cells from the biofilm, while the overexpression of Ume6p has a repressive effect on cell release, and reduces cell dispersal (Uppuluri et al., 2010b, 2010a).

3.2. Infections caused by *Candida albicans* biofilms

Candida albicans is an opportunistic pathogen which can invade any site within its human host, and it is also commonly found in nosocomial infections (Perlroth et al., 2007). Amongst the *Candida* spp, *C. albicans* is a leading cause of infections, commonly known as candidiasis, which can either be superficial or systemic (Kabir et al., 2012; Spampinato and Leonardi, 2013). However, most of the infections caused by *C. albicans* are associated with the formation of biofilms (Ramage et al., 2005; Nett and Andes, 2006; Mathé and Van Dijck, 2013). The disruption of the host-microbial balance, probably due to the excessive use of antibiotics, alteration of the environment (pH or nutrition) and changes to the immune system (immunocompromised), can induce *C. albicans* growth and possibly biofilm formation (Douglas, 2003; Gulati and Nobile, 2016). Infections caused by *C. albicans* biofilms can either be on a host tissue or mucosal surface (oral and vaginal cavities) or medical implant, such as the vascular catheters, dentures, pacemakers and prosthetics (Figure 1.4) (Kojic and Darouiche, 2004; Tsui et al., 2016). The oral cavity is a popular niche of *C. albicans* biofilm, causing oral candidiasis, commonly referred to as thrush. It appears as creamy white lesions on the palate, buccal mucosa, tongue and sometimes the pharynx (Fidel, 2011; Williams and Lewis, 2011). Oral candidiasis affects approximately 90% of HIV patients, 5% of newborn babies and 10% of elderly patients (Samaranayake et al., 2009). Infections on the vaginal mucosal surfaces are also caused by the adherence of *C. albicans* biofilms (Harriott et al., 2010). It is estimated that 75% of healthy females of childbearing age will experience at least an episode of vulvovaginal candidiasis while between 8-10% will encounter a recurrent episode (Sobel et al., 1998; Sobel, 2007). Infections of *C. albicans* biofilms associated with medical implants are a challenge because they result in a high mortality rate which ranges between 30-50% (Sudbery, 2011; Dantas et al., 2015). Once the biofilms are formed, they act as a reservoir from which they can potentiate a systemic infection (Gulati and Nobile, 2016).

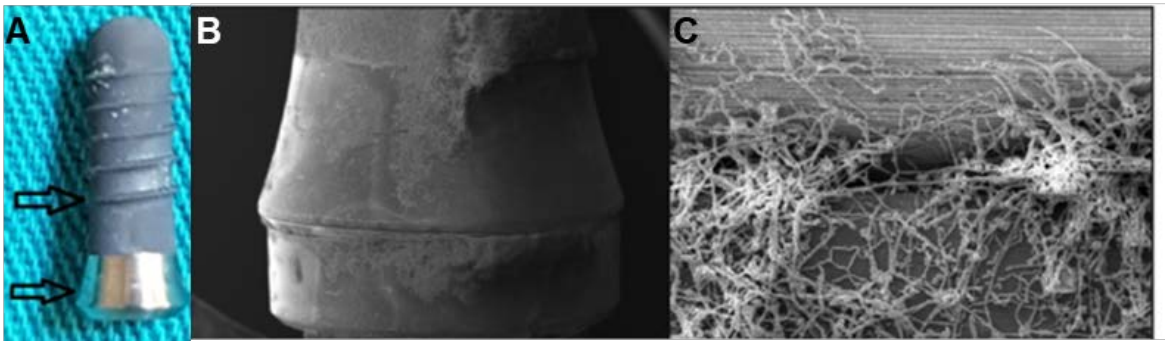


Figure 1.4. A scanning electron micrograph of *C. albicans* biofilm growth on the surface of a tissue level implant. (A) Presence of *C. albicans* biofilm growing on the surface of the tissue implant as indicated with the arrow. (B) *Candida albicans* biofilm on an implant surface viewed with a scanning electron microscope. (C) A micrograph of *C. albicans* biofilm extracted from an implant (Gökmenoglu et al., 2018).

3.3. Treatment of *Candida albicans* biofilms infections

The infections caused by *C. albicans* biofilm may be treated with antifungals or the removal of the infected device by a surgical process. However, the biofilms are highly resistant, and the surgical process is expensive and challenging for the patient, either because of an underlying condition or its anatomical position (Walsh and Rex, 2002; Cauda, 2009; Spampinato and Leonardi, 2013).

Several classes of antifungals, including polyenes, azoles, pyrimidine analogues and echinocandins, are used in the treatment of *C. albicans* infections. The different antifungal classes target different sites within the yeast to initiate growth inhibition and death (Mathé and Van Dijck, 2013; Spampinato and Leonardi, 2013).

3.3.1. Polyenes

The polyene antifungal class, which include nystatin and amphotericin B, is produced by fermentation of a complex medium with *Streptomyces nodosus*. Polyenes, which are fungicidal, are amphipathic molecules (Monji and Mechlinski, 1976; Resat et al., 2000; Ng et al., 2003). The drugs bind to ergosterol in the cell membrane, creating drug-lipid complexes which incorporate into the membrane to form transmembrane channels. The channel allows the leakage of cytosolic components, such as potassium ions, resulting in disruption of the proton gradient and cell death (Ghannoum and Rice, 1999; Denning and Hope, 2010; Ostrosky-Zeichner et al., 2010). Although amphotericin B is effective against *C. albicans* (Ramage et al., 2002b; Uppuluri et al.,

2011), its use in treatment is limited, due to the induction of nephrotoxicity as a side effect (Fanos and Cataldi, 2000; Ng et al., 2003). However, drug delivery systems of amphotericin B using liposomal formulations have been developed to considerably mitigate the challenge posed by the toxicity (Segarra et al., 2002; Groll et al., 2003).

3.3.2. Azoles

The azoles, comprising of imidazoles, and triazoles, are generally fungistatic, and they are the most commonly administered antifungal class (Shapiro et al., 2011; Spampinato and Leonardi, 2013) They are heterocyclic compounds with a characteristic five-member nitrogen-containing ring. Although several azole drugs are available, only fluconazole, itraconazole, voriconazole, and posaconazole are licenced for use clinically in the treatment of invasive fungal infections (Ostrosky-Zeichner et al., 2010; Roemer and Krysan, 2014). Azoles are imported into the cell via facilitated diffusion (Mansfield et al., 2010), and the mode of action is to inhibit lanosterol 14-α demethylase, an enzyme of the ergosterol biosynthesis pathway (Tobudic et al., 2012). Azoles inhibit this enzyme by binding to the heme in the active site, via an unhindered nitrogen atom from the azole ring, to prevent oxygen activation, which is required for lanosterol demethylation (White et al., 1998; Odds et al., 2003). Azole antifungal effects are enhanced by doxycycline, possibly due to the depletion of heme-associated iron (Fiori and Van Dijck, 2012). The inhibition of the lanosterol 14-α demethylase also results in the accumulation of a toxic sterol 14-methyl 3,6 diol, which is produced by a 5,6 desaturase enzyme. This causes severe membrane stress (Shapiro et al., 2011).

In addition, fluconazole has been reported to induce oxidative and nitrosative stress in *C. albicans* (Kobayashi et al., 2002; Wang et al., 2009; Arana et al., 2010). Glutathione, which is the most predominant cellular thiol, is essential for maintaining redox homeostasis and protecting biomolecules from oxidative damage (Circu and Yee Aw, 2008). However, the treatment of *C. albicans* with fluconazole reduces intracellular glutathione levels, which induces oxidative stress due to an imbalance between antioxidant activity and the production of reactive oxygen species (ROS) (Lee and Lee, 2018). Oxidative and nitrosative stress can cause irreversible damage to proteins, lipids and nucleic acids, and also induce programmed cell death (Phillips et al., 2003; Halliwell, 2007; Brown et al., 2009; Delattin et al., 2014).

3.3.3. Pyrimidine analogue

Flucytosine is an example of the pyrimidine analogue antifungal class, and it is transported into the cell by a cytosine permease and metabolised via the pyrimidine salvage pathway. However, it only acts as an antifungal inside the yeast after conversion to 5-fluoro-uridine triphosphate, and 5-fluoro-2-deoxyuridylate (White et al., 1998; Hope et al., 2004). 5-Fluoro-uridine triphosphate is a toxic product which interferes with and halts RNA synthesis, while 5-fluoro-2-deoxyuridylate inhibits thymidylate synthetase, an enzyme essential for DNA synthesis (Hope et al., 2004; Spampinato and Leonardi, 2013; Taff et al., 2013). Flucytosine is commonly administered in combination with other antifungals, such as amphotericin B, due to the ease with which *C. albicans* acquires secondary resistance if it is administered alone (Pappas et al., 2009). Also, flucytosine causes bone marrow suppression with peak plasma concentrations higher than 100 mg ml^{-1} (Denning and Hope, 2010).

3.3.4. Echinocandins

The echinocandins, which includes caspofungin, micafungin, and anidulafungin, are large semi-synthetic cyclic lipohexapeptides with a molecular weight of approximately 1200 Da (Denning, 2003; Denning and Hope, 2010). They act as a non-competitive inhibitor of β-1,3 glucan synthase, an essential enzyme for cell wall formation (Denning, 2003; Shapiro et al., 2011). The interference of echinocandins with the cell wall integrity compromises the viability of the cell, resulting in cell death (Taff et al., 2013).

3.3.5. Allylamines

Allylamines, which include terbinafine and naftifine, disrupts the cell membrane by inhibiting squalene-epoxidase, which is involved in ergosterol biosynthesis (Petranyi et al., 1984; Ghannoum and Rice, 1999). The use of allylamine for the treatment of invasive candidiasis is limited due to reduced efficacy and adverse effects. However, it is the drug of choice for dermatophyte infections (Gupta et al., 2008; Vandeputte et al., 2012).

3.4. Resistance mechanisms of *Candida albicans* biofilms

An attribute of *C. albicans* biofilms is the exhibition of inherent high-level resistance to antifungals, which makes infections challenging to combat (Kumamoto, 2002;

Douglas, 2003). The antifungal resistance demonstrated by *C. albicans* biofilms is associated with a different mechanism, including the upregulation of drug efflux pumps, modification of drug target, high cell density and presence of extracellular matrix (Ramage et al., 2012; Mathé and Van Dijck, 2013; Taff et al., 2013; Silva et al., 2017).

3.4.1. Upregulation of drug efflux pumps

The upregulation of genes encoding efflux pumps, which facilitates the transport of antifungals out of the cell to reduce intracellular accumulation, is a well-described resistance mechanism of *C. albicans* biofilms (White, 1997; Ramage et al., 2002a; Mukherjee et al., 2003). The two major efflux pump classes involved in drug exportation in *C. albicans* are the ATP binding cassette (ABC) transporter superfamily (Cdr1 and Cdr2), which is transcriptionally regulated by Tac1p, and the major facilitator superfamily (MFS) transporter (Mdr1), which is controlled transcriptionally by Mrr1p (Prasad et al., 1995; Sanglard et al., 1996; White, 1997; Ramage et al., 2002a; Coste et al., 2004; Morschhäuser et al., 2007). Both superfamilies comprise of proteins with membrane-spanning domains; however, they differ in their source of energy (Prasad et al., 2019). While ABC transporters are primary transporters, which utilise energy obtained from ATP hydrolysis to drive drug efflux (Higgins, 1992), the MFS transporter are secondary transporters, which depend on an electrochemical gradient (proton-motive force) for energy (Cannon et al., 2009; Redhu et al., 2016).

Another difference between the two superfamilies is the substrates transported. The Cdr1p ABC transporter is highly promiscuous and accepts a wide variety of substrates, including azoles, lipids and rhodamine 6G, while the MFS transporter has a limited substrate range, which includes fluconazole (Sanglard and Odds, 2002; Prasad et al., 2015). Efflux pumps are upregulated in *C. albicans* planktonic cells in response to the presence of antifungals. However, when *C. albicans* grows as a biofilm, efflux pumps are significantly upregulated during the early stages of biofilm development upon attachment to a surface, even in the absence of an antifungal (Mukherjee et al., 2003; Mateus et al., 2004; Nett et al., 2009; Nobile and Johnson, 2015). Moreover, efflux pumps have been shown not to contribute significantly to resistance in matured biofilms (Ramage et al., 2002a).

3.4.2. Modification or overexpression of drug target

Another resistance mechanism employed by *C. albicans* biofilms is the modification of drug target, either by a mutation or overexpression, thus reducing the effectiveness of the drug in eliminating the pathogen (White et al., 1998). This phenomenon has been observed with the azole antifungal target, lanosterol 14-α demethylase (Erg11p) (Cowen et al., 2015). Amino acid substitutions due to point mutations are common in this enzyme. Although more than 140 substitutions have been identified, the most common are R467K and G464S, which are near the binding site (Casalinuovo et al., 2004; Morio et al., 2010; Cowen et al., 2015). The analyses of these substitutions have shown that they decrease the binding affinity of azoles *in vitro* (Lamb et al., 2000). The overexpression of *ERG11* the drug target is also commonly observed in azole-resistant *C. albicans* (Franz et al., 1998; White et al., 1998). The transcriptional regulator, Upc2p controls the expression of *C. albicans ERG11*, and it becomes upregulated to increase ergosterol production in response to azole, thus contributing to azole resistance (Silver et al., 2004). Alternatively, point mutations in the Upc2p transcription factor also contribute to *ERG11* overexpression for azole resistance (Dunkel et al., 2008; Heilmann et al., 2010; Flowers et al., 2012). Also, overexpression of *ERG11* can be induced by the duplication of chromosome five or the occurrence of a chr5L isochromosome (Selmecki et al., 2006).

3.4.3. High cell density

High cell density also contributes to antifungal resistance in *C. albicans* biofilms due to the concentration of a large number of cells in a small area (Mathé and Van Dijck, 2013; Tsui et al., 2016). In an investigation conducted by Perumal and co-workers (2007), the influence of cell density on antifungal resistance was compared between planktonic cells and *C. albicans* biofilms with similar cell density. They found that, at high cell densities, planktonic cells had a distinctive reduction in antifungal susceptibility. The observation was concurrent with what was seen in *C. albicans* biofilms with similar cell density. However, the observed reduced susceptibility was not associated with efflux pumps because strain lacking *CDR1*, *CDR2* and *MDR1* also showed an identical trend. Similarly, Seneviratne and co-workers (2008b) reported identical observations, although only for ketoconazole and 5-flucytosine. Therefore, it appears that high cell density may not necessarily be a resistance mechanism associated with *C. albicans* biofilms since the same pattern was observed with the

planktonic cells (Seneviratne et al., 2008a; Mathé and Van Dijck, 2013; Tsui et al., 2016).

3.4.4. Extracellular matrix

The extracellular matrix (ECM), which acts as a network, binding the biofilm architecture to provide structure, also serves as a resistance mechanism to repel drugs from penetrating the biofilm (Nett et al., 2010b). A component of the matrix which contributes to drug resistance is the β-1,3-glucan polysaccharide (Nett et al., 2007, 2010a). The importance of β-1,3-glucan as a resistance mechanism was demonstrated with an increase in biofilm fluconazole susceptibility after β-1,3-glucanase treatment. Also, the addition of exogenous β-1,3-glucans increased fluconazole tolerance in planktonic cells (Nett et al., 2007; Martins et al., 2010). Another component of the ECM is the extracellular DNA, which has a predominant role in maintaining the structural integrity of biofilm (Martins et al., 2010). The presence of extracellular DNA contributes to resistance by reducing antifungal diffusion as demonstrated with the increase in antifungal susceptibility after biofilm treatment with DNAse (Martins et al., 2012).

3.5. Drug Discovery for *Candida albicans* Biofilm Infections

Due to these resistance mechanisms, there are currently no specific drugs available for the complete eradication of biofilm-based infection, hence the development of alternative options is urgently needed (De Cremer et al., 2015; Gulati and Nobile, 2016; Gebreyohannes et al., 2019). Combination therapy is considered as one such potential alternative treatment of *C. albicans* biofilms infections (Mukherjee et al., 2005; Mortale and Karuppayil, 2018). This approach is advantageous because it improves the efficiency of the drug and also reduces the possible toxic effects due to lower dose administration. It also reduces the likelihood of resistance, since the drugs in combination may utilise a different mode of action (Lewis and Kontoyiannis, 2001; Johnson and Perfect, 2010; Belanger et al., 2015; Shrestha et al., 2015). One example of such an approach is the combination of fluconazole with the tetracycline derivative, minocycline. According to the investigation, minocycline enhances fluconazole penetration into *C. albicans* biofilms and also interferes with intracellular calcium homeostasis (Shi et al., 2010). Another tetracycline derivative, doxycycline, is also synergistic with fluconazole by reducing the expression of *CDR1*, *CDR2* and *MDR1*

efflux pump genes and increasing the intracellular calcium concentration (Miceli et al., 2009; Gao et al., 2013, 2014).

Besides the use of combination therapy, another option is to prospect within existing drugs for possible antifungal drugs, a strategy branded drug repurposing (Ashburn and Thor, 2004; Xue et al., 2018). The application of drug repurposing to identify probable inhibitors of *C*. *albicans* biofilm was investigated by Siles and co-workers (2013) with the discovery of 38 compounds with inhibitory properties against *C*. *albicans* biofilm from a list of 1200 off-patent drugs from the Prestwick Library. Also, drugs such as lorazepam, diazepam, midazolam, and phenobarbitone, belonging to the γ-aminobutyric acid (GABA) receptor agonist class, for the treatment of antiepileptic disorders, have been shown to have antibiofilm properties against *C*. *albicans* (Kathwate et al., 2015). Furthermore, statins such as simvastatin, which are administered to reduce the level of lipoprotein cholesterol in the blood, as well as propranolol, which is an antiarrhythmic, administered for the treatment of myocardial infarction, high blood pressure and arrhythmia, were also reported to have antibiofilm properties against *C*. *albicans* (Derengowski et al., 2009; Liu et al., 2009). Besides the above mentioned, several other drugs classes, such as non-steroidal anti-inflammatory drugs (NSAIDs) (Alem and Douglas, 2004; Moraes and Ferreira-Pereira, 2019) and miltefosine were also reported to have antibiofilm properties against *C*. *albicans* (Vila et al., 2015, 2016). Although there has been much-reported progress on drug discovery for *C*. *albicans* biofilms treatment, however, there is still a need to correlate these discoveries with *in vivo* observations.

3.6. *Candida albicans* Biofilms and Polyunsaturated Fatty acids

Exogenous polyunsaturated fatty acids (PUFAs) can be incorporated into the membrane of yeast cells (Black and DiRusso, 2003). Interestingly, the PUFAs stearidonic acid (C18:4 n-3), eicosapentaenoic acid (EPA) (C20:5 n-3), arachidonic acid (AA) (C20:4 n–6), and docosapentaenoic acid (22:5 n-3) have been shown to have antifungal activity against *C. albicans* biofilms, reducing biomass accumulation as well as metabolic activity (Thibane et al., 2010). The inhibition of *C. albicans* biofilms by these PUFAs is possibly by inhibiting mitochondrial function, leading to oxidative stress and apoptosis (Thibane et al., 2010, 2012; Pohl et al., 2011). Furthermore, PUFAs have also been shown to enhance the inhibitory properties of antifungals.

According to an investigation by Ells and co-workers (2009), the presence of 1 mM AA increased the susceptibility of *C. albicans* and *C. dubliniensis* biofilms to both clotrimazole and amphotericin B. However, Mishra and co-workers (2014) conducted a similar investigation with half the fluconazole MIC and 500 µM AA on biofilms of *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*. The study showed that 500 µM AA could not increase the susceptibility of *C. albicans* and *C. tropicalis* to fluconazole. Although there was a reduction in the metabolic activity of *C. glabrata* and *C. parapsilosis*. Therefore, a high concentration of AA is essential to the observed increase in *C. albicans* biofilms susceptibility to azoles. Although the AA concentration used is higher than the concentration in the human body, which is estimated to be between 1.2-8.6 µM in the plasma (Abdelmagid et al., 2015), understanding the mechanism involved would contribute towards indicating and developing targets for therapies.

4.0. Aim of the Study

Based on this background, the study aims to characterise the role of PUFAs in the increased susceptibility of *C*. *albicans* biofilms to fluconazole. The specific objectives include an investigation of *C*. *albicans* biofilm transcriptomes in the presence of AA and fluconazole (Chapter 3), the influence of AA on *C*. *albicans* biofilm resistance mechanisms (Chapter 4) and the application of this knowledge to attempt the repurposing of potential drugs against *C*. *albicans* biofilms (Chapter 5).

5.0. References

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CHAPTER 2

Influence of Exogenous Polyunsaturated Fatty Acids on Azole Sensitivity in *Candida albicans*

Abstract

Candida albicans is a commensal yeast within the human microbiota with significant medical importance because of its pathogenic potential, which is facilitated by an immunocompromised system. The yeast also produces biofilms which are highly resistant to available antifungals, including the commonly administered azole class. The presence of polyunsaturated fatty acids, such as arachidonic acid, has been reported to increase the susceptibility of *C*. *albicans* biofilms to azoles. However, the underlying mechanism is unknown. To unravel this mechanism, a preliminary investigation to affirm the reported increased susceptibility of *C*. *albicans* biofilms to azoles in the presence of polyunsaturated fatty acids was conducted. The influence of 1 mM arachidonic acid and eicosapentaenoic acid on the susceptibility of *C*. *albicans* biofilms to clotrimazole and fluconazole was tested. The investigation established that the presence of these polyunsaturated fatty acids does increase *C*. *albicans* biofilms susceptibility to azoles, as evident with a significant reduction in metabolic activity even with a drug concentration of 1 mg L^{-1}. A feasible treatment option could be developed from an understanding of the mechanism involved in the increased susceptibility.

2.1. Introduction

The yeast *Candida albicans* is a human commensal which colonises the gastrointestinal, genitourinary, and oral cavities in healthy individuals (Biswas et al., 2007). However, it can also be responsible for widespread infections, commonly known as candidiasis, which can either be superficial or systemic; with the systemic infection being life-threatening (Berkow and Lockhart, 2017). Several antifungal classes such as the azoles, polyenes, echinocandins, and allylamines have been identified to treat *C. albicans* infections. However, azoles, which are fungistatic, are the most commonly administered class due to their oral bioavailability and reduced toxicity (Hoffman et al., 2000). Azoles are transported into yeast cells by facilitated diffusion (Mansfield et al., 2010). They bind to a free nitrogen atom on the heme in the

active site of lanosterol 14-α demethylase (Erg11p) to inhibit ergosterol synthesis. This results in the inhibition of cell growth due to the production of a toxic 14 alpha methylated sterol, which cannot replace ergosterol (White et al., 1998; Shapiro et al., 2011).

Candida albicans can produce highly resistant biofilms which consist of the yeast, hyphae and pseudohyphae morphologies surrounded by an extracellular matrix (Douglas, 2002; Mayer et al., 2013). The high antifungal resistance of *C. albicans* biofilm is due to the presence of different mechanisms such as the mutation and overexpression of drug targets, reduction in ergosterol content and the increased expression of efflux pumps (Ramage et al., 2012; Spampinato and Leonardi, 2013). The observed failure of existing drugs to eradicate *C*. *albicans* biofilm infections necessitates the need to develop alternative treatment options (Costa et al., 2015).

Polyunsaturated fatty acids (PUFAs), such as stearidonic acid (C18:4 n-3), arachidonic acid (AA) (C20:4 n–6), eicosapentaenoic acid (EPA), (C20:5 n-3) and docosapentaenoic acid (22:5 n-3) (Figure 2.1) have been reported to inhibit *C*. *albicans* biofilms, possibly by inhibiting mitochondrial function, leading to oxidative stress and apoptosis (Thibane et al., 2010, 2012). Also, investigations by Ells and co-workers (2008) as well as Thibane and coworkers (2012), reported increased antifungal susceptibility of *C*. *albicans* biofilms grown in the presence of polyunsaturated fatty acids. However, the mechanism behind the reported increase in susceptibility is unknown. As a starting point to investigate this further, this chapter deals with the influence of the PUFAs, AA and EPA, on the susceptibility of *C*. *albicans* biofilms to azole antifungals.

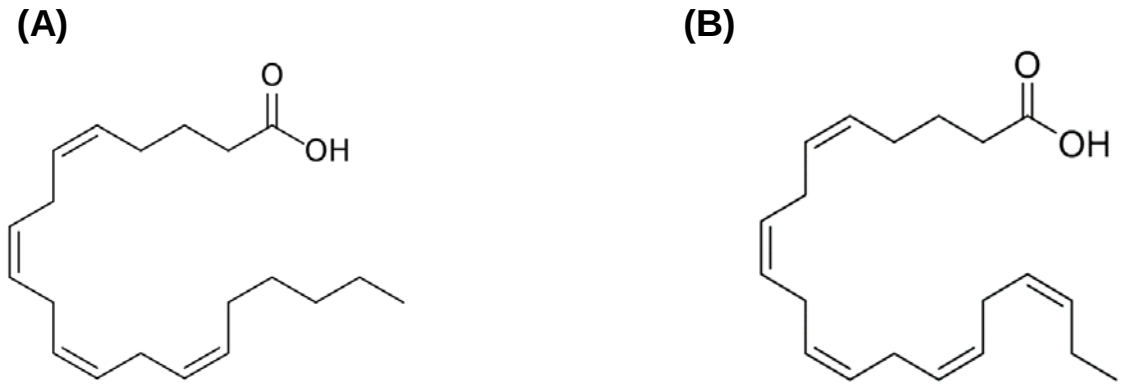


Figure 2.1. Structure of arachidonic acid (AA) and eicosapentaenoic acid (EPA)

2.2. Materials and Methods

2.2.1. Antifungal stock solutions

Stock solutions of antifungals [clotrimazole and fluconazole (Sigma-Aldrich, USA)] and fatty acids [AA and EPA (Sigma-Aldrich, USA)] were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) and ethanol (Merck, RSA), respectively. The media was prepared with the antifungal working solution ensuring that the solvent concentration was below 1%.

2.2.2 Yeast strains and cultivation

Candida albicans CBS 8758 (SC5314) was obtained from the UNESCO MIRCEN culture collection at the University of the Free State and stored in 15% glycerol (Merck, RSA) solution at -80^{o}C until use. The yeast was revived on Yeast Malt extract (YM) agar (10 g.L^{-1} glucose, 5 g.L^{-1} peptone, 3 g.L^{-1} yeast extract, and 3 g.L^{-1} malt extract 16 g.L^{-1} agar) at 30^{o}C for 24 h.

2.2.3. Biofilm formation

Candida albicans was inoculated in 10 mL Yeast Nitrogen Base (YNB) broth (6.7 g.L^{-1} YNB, 10 g.L^{-1} glucose) and incubated at 30^{o}C for 24 h. The cells were harvested by centrifugation at 3080 *g* for 5 min and washed thrice with sterile Phosphate Buffer Saline (PBS) (Sigma-Aldrich, USA). The cell count was done with a hemocytometer, and the cells diluted to 1 x 10^{6} cells.mL^{-1} in sodium bicarbonate containing filter sterilised RPMI 1640 medium (Sigma-Aldrich, USA). Biofilm growth was initiated with the transfer of 100 µl of the standardised cells to a flat bottom 96 well cell culture plate (Corning Incorporated, Costar®) and incubated without agitation at 37^{o}C for 90 min. After that, the wells were washed thrice with PBS to remove the non-adherent cells. The wells containing adhered cells were then replenished with 200 µl of filter sterilised RPMI 1640 medium and incubated at 37^{o}C for 48 h without agitation to produce biofilms.

2.2.4. Effect of polyunsaturated fatty acids on antifungal susceptibility of *C. albicans*

Upon initiation of biofilm formation, the culture medium was supplemented with different concentrations (0.5, 1, 2, 4, 8, 16, 32 µg.mL^{-1}) of clotrimazole or fluconazole.

These were performed in the presence and absence of 1 mM of either AA or EPA, as described by Ells and coworkers (2008). In all experiments, an equivalent volume of the drug vehicles (DMSO and/or ethanol) was included as appropriate controls.

2.2.5. XTT assay

Metabolic activity in the biofilms was measured by assessing formazan formation through the reduction of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) by mitochondrial dehydrogenases as described by Kuhn and co-workers (2003). Briefly, matured biofilms, in flat bottom 96 well microtitre plates, were washed twice with sterile PBS. After that, 50 µl of 1 $g.L^{-1}$ XTT (Sigma-Aldrich, USA) solution, dissolved in PBS, together with 4 µl of 1 mM menadione solution, dissolved in acetone, was added to each well. Afterwards, the plates were incubated in the dark at 37^{o}C for 3 h. The formazan product produced was measured with a microtiter plate reader at an absorbance of 492 nm.

2.2.6 Statistical analyses

All experiments were conducted in biological triplicates, each with at least three technical replicates, and the averages and standard deviations calculated. The t-test was performed in order to assess the significance of differences. *P* values of at least 0.05 were considered to be significant.

2.3. Results and Discussion

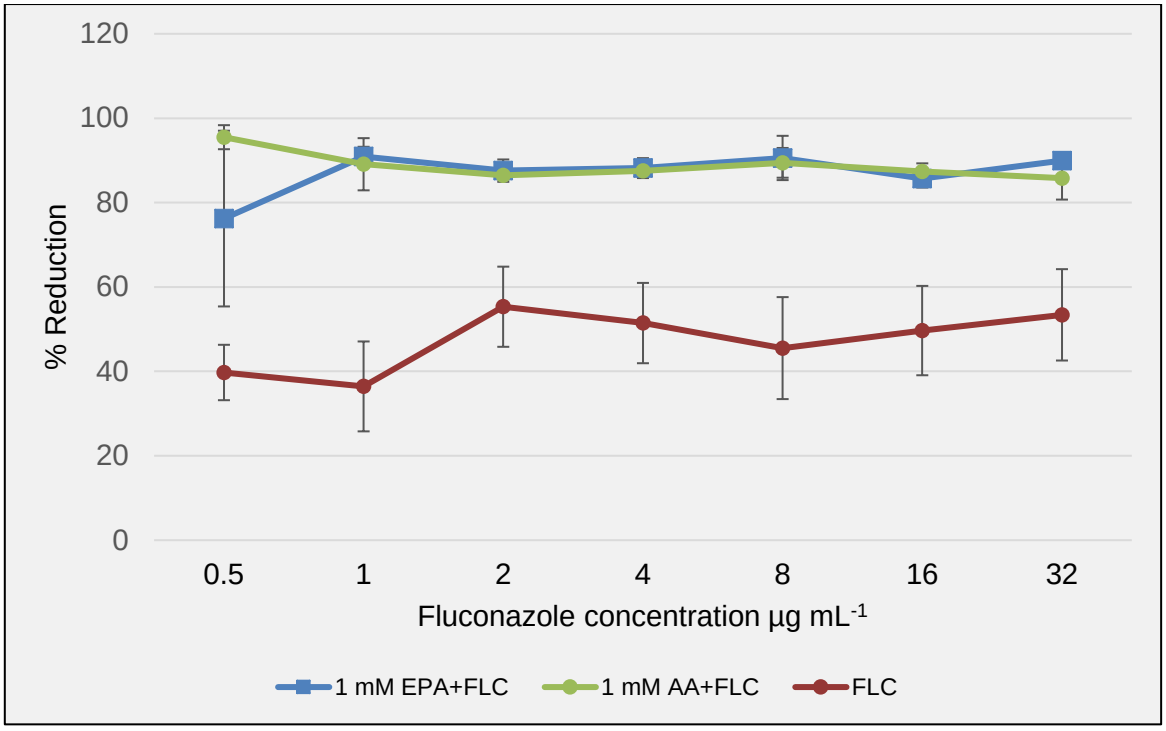
2.3.1. Polyunsaturated fatty acids increase azole susceptibility of *Candida albicans* biofilms

Azoles are the most commonly administered antifungals in the treatment of *C*. *albicans* infection (Hoffman et al., 2000). Although they can easily cross the *C*. *albicans* cells membrane via facilitated diffusion, their effectiveness is hampered by the high resistance of the biofilms towards azoles (Chandra et al., 2001; Mansfield et al., 2010). In this investigation, *C*. *albicans* biofilms were grown in the presence of either fluconazole or clotrimazole, which are the commonly clinically administered azole antifungals, together with either AA or EPA. The use of 1 mM of the PUFAs was based on the report of Ells and co-workers (2008), which indicated increased susceptibility to clotrimazole and amphotericin B in the presence of 1 mM AA. The effect was

determined using XTT assay which allows for the measurement of biofilm metabolic activity with the formation of a water-soluble formazan product quantifiable by spectrophotometry without the disruption of the biofilms (Kuhn et al., 2003).

In this study, there was an increase in biofilm susceptibility to fluconazole with the addition of 1 mM AA or EPA (Figure 2.2). The most significant ($P < 0.05$) increase in susceptibility was observed when 1 $mg.L^{-1}$ of fluconazole was combined with 1 mM of either PUFA. The observed increase was approximately 144% and 149% for AA and EPA, respectively compared to biofilms grown in the presence of fluconazole only. Similarly, there was also an observed increase in the biofilm susceptibility to clotrimazole in the presence of both PUFAs tested. The most significant increase was observed at 1 $mg.L^{-1}$ of clotrimazole in combination with 1 mM AA or EPA. An increase of 155% and 154% was observed with the addition of AA and EPA, respectively. These observations were similar to the report of Ells and co-workers (2008) where the susceptibility of *C*. *albicans* biofilms to 1.25 $mg.L^{-1}$ clotrimazole increased by approximately 160% in the presence of 1 mM AA, in comparison with biofilms without AA. In a similar investigation by Mishra and co-workers (2014), with a combination of 2 mg L^{-1} fluconazole and 500 µM AA, there was no significant increase in fluconazole susceptibility. This observation indicates that the presence of high AA concentration is vital to the observed increase in fluconazole susceptibility. Although the presence of both PUFAs increased the susceptibility of *C*. *albicans* biofilms to both azoles, the results obtained for clotrimazole were more inconsistent; therefore the combination of 1 $mg.L^{-1}$ of fluconazole with 1 mM AA was selected for all further experimentation.

(A)



(B)

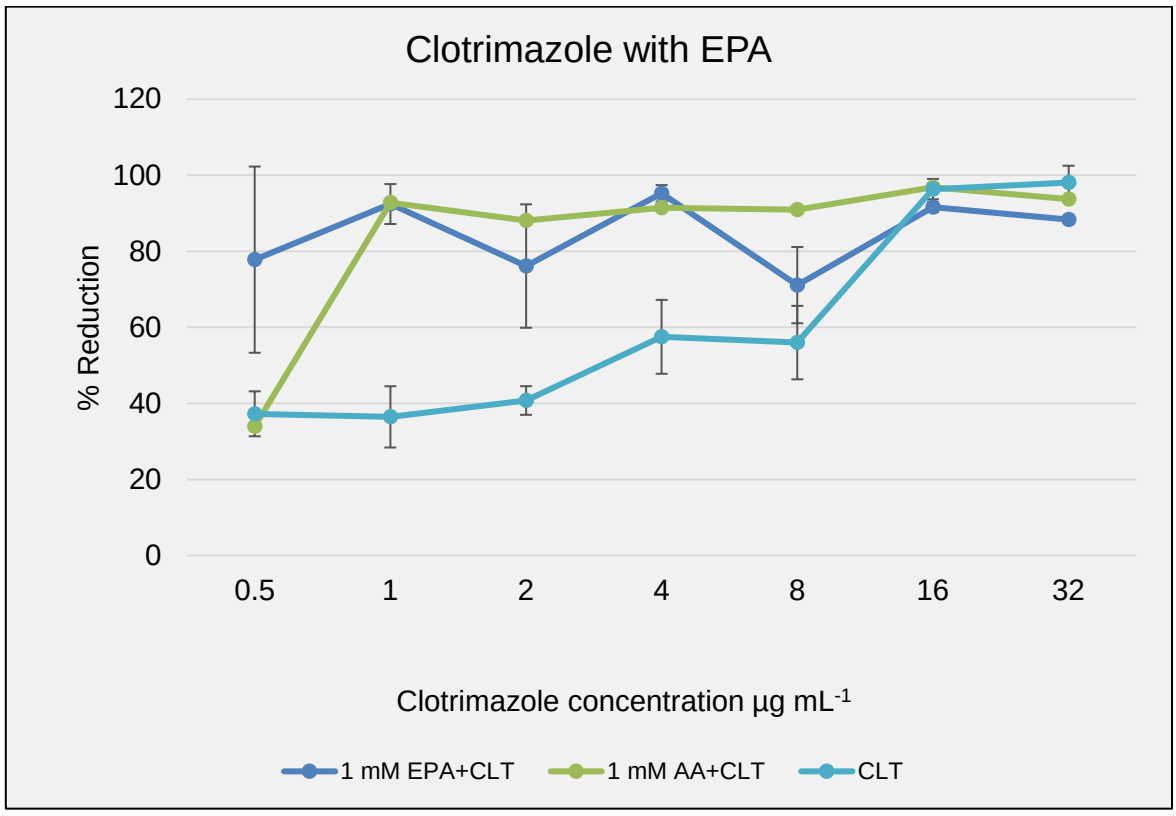


Figure 2.2. Influence of arachidonic acid (AA) and eicosapentaenoic acid (EPA) on the percentage reduction in metabolic activity of *C. albicans* biofilm grown in the presence of (A) fluconazole (FLC) and (B) clotrimazole (CLT) after 48 hours of incubation. Values are the mean of three independent experiments, and the standard deviation is indicated by the error bars.

2.5. Conclusions

Candida albicans infections are responsible for the deaths of several patients, especially amongst the immunocompromised individuals. The high resistance of its biofilms has resulted in the need to investigate alternative treatment options. The addition of AA and EPA increase *C. albicans* biofilms susceptibility to the azoles, fluconazole and clotrimazole, thereby reducing resistance potential. This contributes to the previous reports on the effect of PUFAs on the susceptibility of *C. albicans* to other antifungals. Investigation of the molecular mechanisms by which this increase in susceptibility is facilitated may identify possible novel drug targets for further study and may proffer a possible solution towards mitigating the challenge of *C. albicans* biofilms resistance.

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CHAPTER 3

Transcriptome Analyses of *Candida albicans* Biofilms Exposed to Arachidonic Acid and Azoles

Abstract

Biofilm formation is a critical factor in the infection caused by *C*. *albicans*. The high level of antifungal resistance exhibited by *C*. *albicans* biofilms using different mechanisms has resulted in the need for alternative treatment models to mitigate the challenge. Polyunsaturated fatty acids such as arachidonic acid have been reported to increase azole susceptibility in *C. albicans* biofilm significantly; however; the mechanism remains unknown. An investigation to unravel the mechanism behind this phenomenon was conducted using RNA sequencing of biofilms grown in four different combinations of arachidonic acid and fluconazole. The Protein ANalysis THrough Evolutionary Relationships (PANTHER) classification analysis of the differentially expressed genes, obtained revealed the influence of arachidonic acid and fluconazole on the respective biological processes which are associated with ATP generation such as AMP biosynthetic processes, fumarate metabolic process, ATP synthesis coupled with proton transport and fatty acid oxidation processes. The differential regulation of selected genes obtained from RNA sequencing was confirmed with the NanoString nCounter platform, and it showed there was consistency in the fold change value between the two platforms.

3.1. Introduction

Candida albicans is a leading cause in bloodstream infections to immunocompromised individuals, due to their ability to form biofilms both on living tissues and medically implanted devices. Infections caused by *C*. *albicans* biofilms are often life-threatening with a mortality rate between 30 and 50% of affected individuals (Douglas, 2003; Cleary et al., 2010; Mayer et al., 2013). Over the years, several types of antifungals such as polyenes, pyrimidine analogues, azoles, allylamines and echinocandins have been developed to combat *C*. *albicans* infections (Mathé and Van Dijck, 2013). However, from these classes, azoles, polyenes, and echinocandins are commonly administered with the azoles being the most popular in the treatment of candidiasis (Denning and Hope, 2010; Ostrosky-Zeichner et al., 2010). According to an

investigation on the dispensing pattern of antifungal drugs in South Africa, the azole, fluconazole, was the most commonly prescribed antifungal (Truter and Graz, 2014).

The frequent use of azoles in the treatment of *C*. *albicans* infections stems from its excellent bioavailability and reduced toxicity (Meis and Verweij, 2001; Denning and Hope, 2010; Ben-Ami, 2018). However, the use of fluconazole in the treatment of *C*. *albicans* biofilm infections has become a challenge due to the high antifungal resistance. The known resistance mechanisms employed by *C*. *albicans* biofilm against azoles include the reduction of ergosterol and the increased expression of drug efflux pumps (Chandra et al., 2001; Shapiro et al., 2011; Taff et al., 2013). Therefore, the treatment of *C*. *albicans* biofilm infections requires alternative and improved treatment options.

Saturated and unsaturated fatty acids have been documented to have antifungal properties. These compounds can naturally insert themselves into the lipid bi-layer to cause membrane disruption and increase membrane fluidity, which ultimately results in the release of intracellular components (Pohl et al., 2011). The polyunsaturated fatty acids (PUFAs), arachidonic acid (AA) (C20:4 n-6) and eicosapentaenoic acid (EPA) (C20:5 n-3) have been reported to inhibit *C*. *albicans* biofilms (Thibane et al., 2010, 2012). Arachidonic acid and EPA also increases the susceptibility of *C*. *albicans* biofilm to azole antifungals (Ells et al., 2009, Chapter 2 of this study). However, the molecular mechanism involved remains unknown.

In a bid to unravel the mechanism involved, RNA sequencing (RNA-seq), which is frequently used to determine differentially expressed genes across two or more conditions, is a useful tool (Wang et al., 2009; McDermaid et al., 2018). In this chapter, transcriptome analysis was conducted on *C*. *albicans* biofilms grown in the presence of AA; fluconazole or a combination of the two, to identify differentially expressed genes in the different conditions.

3.2. Materials and Methods

3.2.1. Antifungal stock solutions

Stock solutions of fluconazole (Sigma-Aldrich, USA) and the fatty acid, AA (Sigma-Aldrich, USA) were dissolved with dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) and ethanol (EtOH) (Merck, RSA), respectively. Working concentrations was added to

the media, ensuring that the solvent concentration was below 1%. Fluconazole was added to a final concentration of 1 $mg.L^{-1}$ while a concentration of 1 mM was used for AA.

3.2.2. Yeast strain and cultivation

Candida albicans CBS 8758 (SC5314), was revived from glycerol stocks kept in liquid nitrogen, on Yeast malt (YM) extract agar (10 $g.L^{-1}$ glucose, 5 $g.L^{-1}$ peptone, 3 $g.L^{-1}$ yeast extract, 3 $g.L^{-1}$ malt extract and 16 $g.L^{-1}$ agar bacteriological) at 30°C for 24 h.

3.2.3. Biofilm formation and storage

Candida albicans SC5314 was inoculated into 10 mL Yeast Nitrogen Base (YNB) broth (6.7 $g.L^{-1}$ YNB, 10 $g.L^{-1}$ glucose) and incubated on a shaker at 30°C for 24 h. The cells were harvested by centrifugation at 3080 *g* for 5 min and washed thrice with sterile phosphate buffer saline (PBS) (Sigma-Aldrich, USA). Cell count was determined with a hemocytometer, and the cells diluted to 1 x 10^6 $cells.mL^{-1}$ in 20 mL sodium bicarbonate containing sterile RPMI 1640 media (Sigma-Aldrich, USA). Biofilm was initiated in a polystyrene Petri dish and incubated without agitation at 37°C for 90 min and washed with PBS to remove non-adherent cells. The medium was replenished with 20 mL RPMI medium containing the desired concentration of fluconazole and/or AA, or an equivalent volume of the drug vehicles, which was below 1% (Ramage et al., 2001; Mishra et al., 2014). The investigated conditions include **A**A (dissolved in EtOH) and **D**MSO (DA), **f**luconazole (dissolved in DMSO) and **E**tOH (FE) as well as **f**luconazole (dissolved in DMSO) and **A**A (dissolved in EtOH) (FA). The control contained both solvents, **D**MSO and **E**tOH (DE). A total of 5 biofilms were prepared in triplicate for each condition. The plates were covered with parafilm before incubation for 6 h at 37°C to allow for biofilm formation. The biofilms were scraped off the surface of the plate using a cell scraper and centrifuged at 1971 *g* for 3 min at 4°C. The supernatant was aspirated and replaced with 2 mL of Qiagen RNA*later*® (Qiagen) to prevent RNA degradation before storage at -80°C.

3.2.4. Total RNA extraction and sequencing

Stored biofilms samples were placed on ice to thaw and centrifuged at 4000 *g* for 5 min. RNA*later*® was aspirated, and total RNA extraction was carried out using the RNeasy protect mini kit (Qiagen), while the DNA present was removed with RNase-

Free DNase Set (Qiagen), according to the manufacturer's instructions. The quality of RNA in each sample was determined at the Centre for Proteomic and Genomic Research (CPGR) before sequencing. Quality tests carried out include checking for contaminants with the NanoDrop ND1000, absolute concentration using the Qubit® RNA HS Assay Kit, and an Agilent Bioanalyzer Nano Assay to evaluate the sample integrity. Samples having contaminations were cleaned up using an Agencourt RNAClean XP kit (Beckman Coulter) and the quality confirmed. Ribosomal RNA was removed from 1 µg of RNA per sample, using the Illumina Ribo-zero rRNA removal kit, and purified with an Agencourt RNAClean XP kit. Indexed libraries were prepared using the ScriptSeq™ v2 RNA-Seq Library Preparation Kit and ScriptSeq™ Index PCR Primers-Set 1 (Illumina). The library size was profiled with the Bioanalyzer High Sensitivity Assay Kit (Agilent) and quantified with the Qubit HS DNA Assay Kit. The samples were diluted and spiked with a Phix control library (Illumina). Sequencing was completed on a Nextseq 500 (Illumina) using the Nextseq 500 High Output (150 cycles) kit which yielded paired-end reads with 96% and 97% of total data having bases having a Phred score % ≥ Q30.

3.2.5. Sequence analysis for differentially regulated genes

Fastq files, obtained from the sequencing, were analysed for quality using FastQC v0.11.5; (Andrews, 2010) and the reads with low quality (<Q30) were discarded, using PRINSEQ-lite v0.20.4 (Schmieder and Edwards, 2011). The sequences were aligned to the *C*. *albicans* SC5314 genome assembly 21 (Skrzypek et al., 2017) using TopHat2 (Trapnell et al., 2012; Kim et al., 2013) with the fr-secondstrand, -r 250 mate-std- I 10000 -G option, which gave a much better alignment rate (Dutton et al., 2016). Subsequently, the aligned files were merged with SAMtools (Li et al., 2009). Gene expression count tables were built from BAM files, created with TopHat2 using the BEDTools multicov command (Quinlan and Hall, 2010). These counts were used to evaluate the differential expression of genes with DESeq2 (Love et al., 2014). Cuffdiff (Trapnell et al., 2012) was also used to generate differential expression data using aligned reads obtained from TopHat2 (Trapnell et al., 2012; Kim et al., 2013) which were assembled with Cufflinks and merged with Cuffmerge (Trapnell et al., 2012). Differentially expressed genes identified from DESeq2 and Cuffdiff with a $P \leq 0.05$ were considered significant. Heat maps, volcano plots and principal component analysis were done as described by Love and co-workers (2014) with a modification

of the script as described on github.com/stephenturner. The Protein ANalysis THrough Evolutionary Relationships (PANTHER) classification system was used for enrichment analysis of biological processes of the significant differentially regulated genes in the different data sets (Ashburner et al., 2000; Thomas, 2003; Mi et al., 2017; The Gene Ontology Consortium, 2019). The sequencing and analysis data are deposited in the gene expression omnibus database under the accession number GSE137423. The password to the records is attached in the additional documents.

3.2.6. NanoString analysis

The extracted RNA from the different treatment conditions [DA= AA (dissolved in EtOH) and DMSO, FE = fluconazole (dissolved in DMSO) and EtOH, FA = fluconazole (dissolved in DMSO), AA (dissolved in EtOH), and DE = the solvents, DMSO and EtOH] were analysed with the NanoString nCounter analysis system (Geiss et al., 2008), using a gene expression TagSet that targets 36 genes including three housekeeping genes (*orf19.1191*, *SLF1*, *orf19.2184*). Custom-designed TagSets, which comprise a fluorescent-labelled reporter tag and a biotinylated universal capture tag, were designed for each gene. Both the reporter tag and capture tag (referred together to as CodeSet) was hybridised with 100 ng of total RNA at 67^{o}C in a preheated thermocycler for 16 h. Subsequently, the hybridised samples were transferred into a nCounter *SPRINT* Cartridge and loaded into a nCounter *SPRINT* Profiler to quantify the transcripts. The nCounter raw expression data file (.RCC) obtained was uploaded into the nSolver Analysis Software 4.0 for review of quality control metrics. The data were normalised by subtracting the geometric mean of the negative control of each sample from the raw counts. The data were grouped between the experiments and control, and their expression ratio was determined. The Log_2 fold change obtained with nSolver was compared to the values obtained with RNA-seq analysis.

3.3. Results and Discussion

3.3.1. Different conditions produced a unique transcriptome profile

RNA sequencing has become an important technique to understand genomic functions (Hrdlickova et al., 2017). The most common application of RNA-seq is for the identification of differentially regulated genes between two or more conditions

(McDermaid et al., 2018). In this experiment, RNA-seq was applied to identify differentially regulated genes in *C*. *albicans* biofilms grown in fluconazole, AA or the combination of the two, compared to the drug vehicles as controls after 6 h of incubation. A time of 6 h was chosen in order to obtain the transcriptome information from the biofilms before they experience glucose exhaustion.

The independent Illumina sequencing runs of *C*. *albicans* biofilms biological replicates produced a total of 325 million raw reads, with 92.4% having a Phred score % ≥ Q30. Principal component analysis between the different replicates obtained showed a close correlation between the number of reads obtained for each run, as shown by the close clustering of the spots, which represents individual runs (Figure 3.1). The variation between the different test conditions (FA, DA, and FE) and the control (DE) was minimal as represented by the distance between the spots. The high reproducibility amongst of the data sets was demonstrated with a heat map which showed close clustering between identical replicates, which can be seen with the corresponding colour intensity (Figure 3.1). Volcano plots to show the number of statistically significant differentially regulated genes are shown in Figure 3.2. As shown, there were more differentially regulated genes in FA and FE than in DA.

The data obtained after RNA-Seq were initially analysed with Cuffdiff to identify differentially expressed genes from different conditions. The biofilms which were grown in the presence of DE served as the control and was compared with the other three experimental conditions of DA, FA and FE to identify differentially expressed genes. The open reading frames were identified with pipelines by aligning the reads to the *C*. *albicans* genome haploid assembly 21 from the *Candida* genome database (CGD) (Skrzypek et al., 2017). Generally, Cuffdiff converts aligned read counts to FPKM (Fragments Per Kilobase of exon per Million fragments mapped), then calculates the observed change in expression and the statistical significance. However, the program assumes that the number of reads produced by each transcript is proportional to its abundance (Trapnell et al., 2012). An observation made using Cuffdiff was genes which are close to a particular locus were assigned the same fold change value. Therefore, to resolve this challenge, the data were analysed with DESeq2, which uses the count matrix (Love et al., 2014).

According to the analysis, the number of significantly regulated ($P \leq 0.05$) genes in each of the tested conditions differed between Cuffdiff and DESeq2, with DESeq2 identifying more differentially regulated genes than Cuffdiff. Studies to compare the different software packages for differential expression analysis have also reported the presence of more genes using DESeq2 (Nookaew et al., 2012; Seyednasrollah et al., 2015). An evaluation of the performance of different software packages for differential expression analysis conducted by Zhang and coworkers (2014), showed that the performance of DESeq and Cuffdiff2 was similar with the analysis of sequencing dept ≥ 20 million reads. The investigation suggested that if the number of false positives is a concern, differentially regulated genes shared between the two programs should preferably be used for further analysis. Therefore, in this study, only the significantly differentially regulated genes ($P \leq 0.05$), which intersect between Cuffdiff and DESeq2 were considered for further investigation. In the different conditions tested, the number of genes intersecting between the two programs are indicated in Table 3.1. Genes with a Log_2 fold change more than one ($Log_2 \geq 1$) were identified from the gene list in each of the experimental conditions and for further analysis (Table 3.1).

Table 3.1. Number of significant differentially expressed genes identified from different conditions. DA (**D**MSO and **A**rachidonic acid), FA (**F**luconazole and **A**rachidonic acid), and FE (**F**luconazole and **E**tOH).

Conditions	**Significant regulation**		**Genes common to Cuffdiff and DESeq2**	**Genes with fold change ($Log_2 \geq 1$)**	
	Cuffdiff	DESeq2		Downregulated	Upregulated
DA	1200	2124	783	183	244
FA	1790	2853	1253	343	407
FE	1089	1954	803	229	312
Total	4079	6931			

The distribution of the differentially regulated genes between the different test conditions was investigated. The Venn diagram, comprising the genes of all the tested conditions, which were either upregulated or and downregulated is shown in Figure 3.3. According to the distribution, most genes present are unique to a treatment condition, while others are shared between two treatment conditions with only a few being shared between all the treatment conditions. These suggest that all the different treatment conditions may have induced unique processes or proteins.

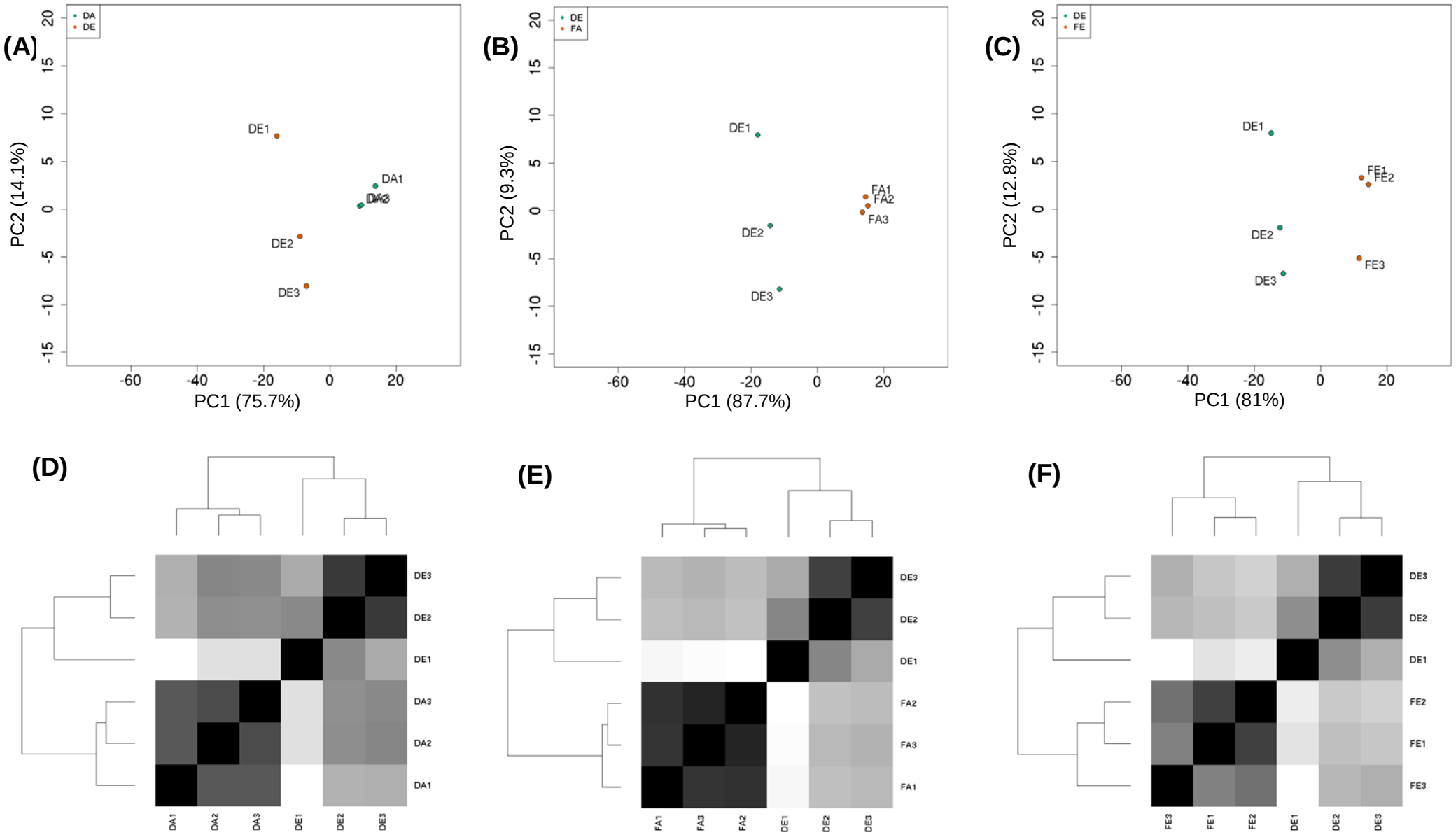

Figure 3.1. Biplot showing variation distance between replicates of the different conditions tested. The further the distances between the points, the higher the variation. (A) DE vs DA (B) DE vs FA (C) DE Vs FE. Heat maps and dendrograms showing the relationships between the samples (D) DE vs DA (E) DE vs FA (F) DE vs FE. The darker the box the more closely associated the condition. DE (**D**MSO and **E**tOH), DA (**D**MSO and **A**rachidonic acid), FA (**F**luconazole and **A**rachidonic acid), FE (**F**luconazole and **E**tOH).

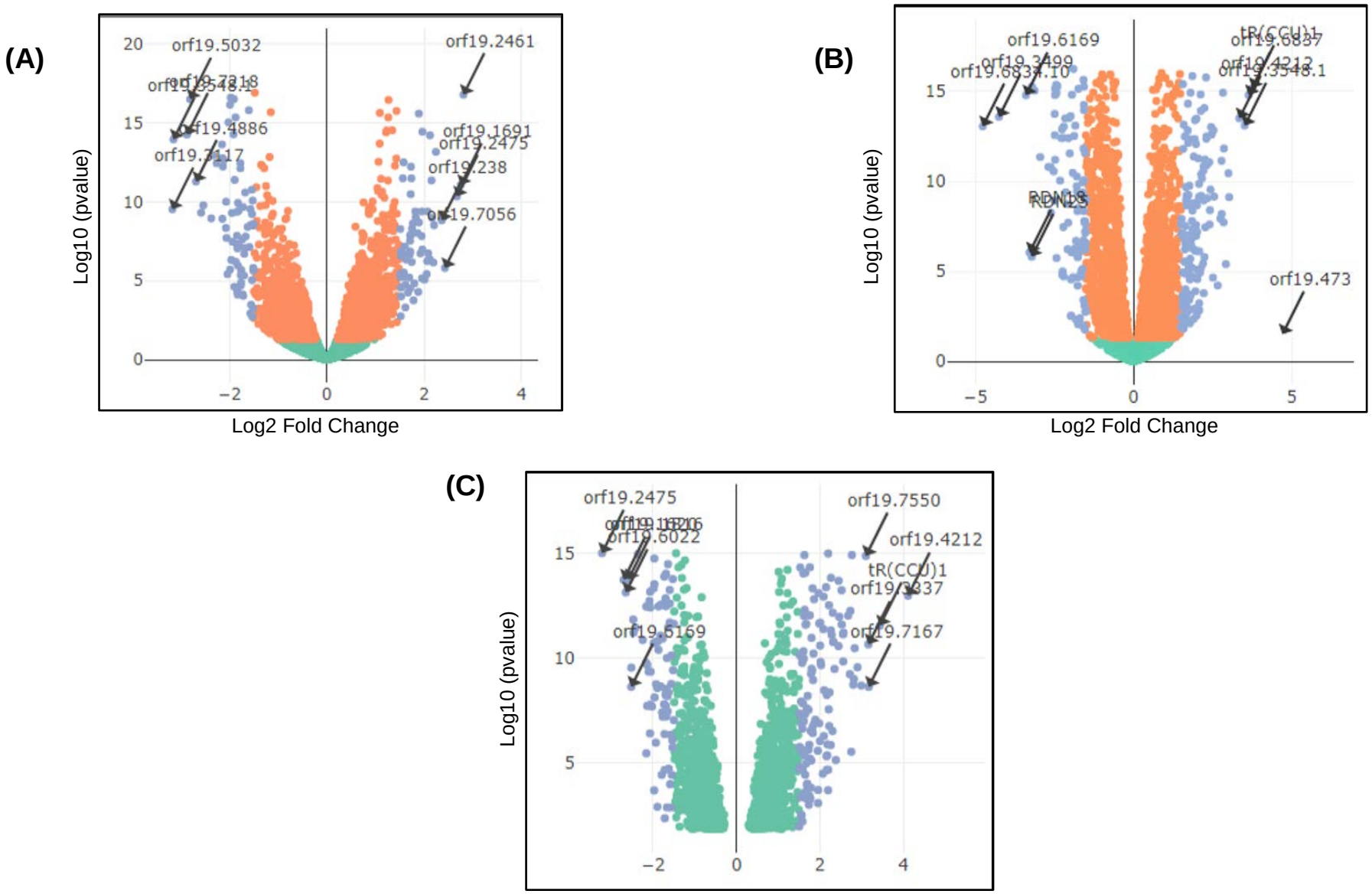

Figure 3.2. Volcano plot showing the statistical significance versus magnitiude of change (A) DE vs DA (B) DE vs FA (C) DE Vs FE. .DA (**D**MSO and **A**rachidonic acid), FA (**F**luconazole and **A**rachidonic acid), and FE (**F**luconazole and **E**tOH).

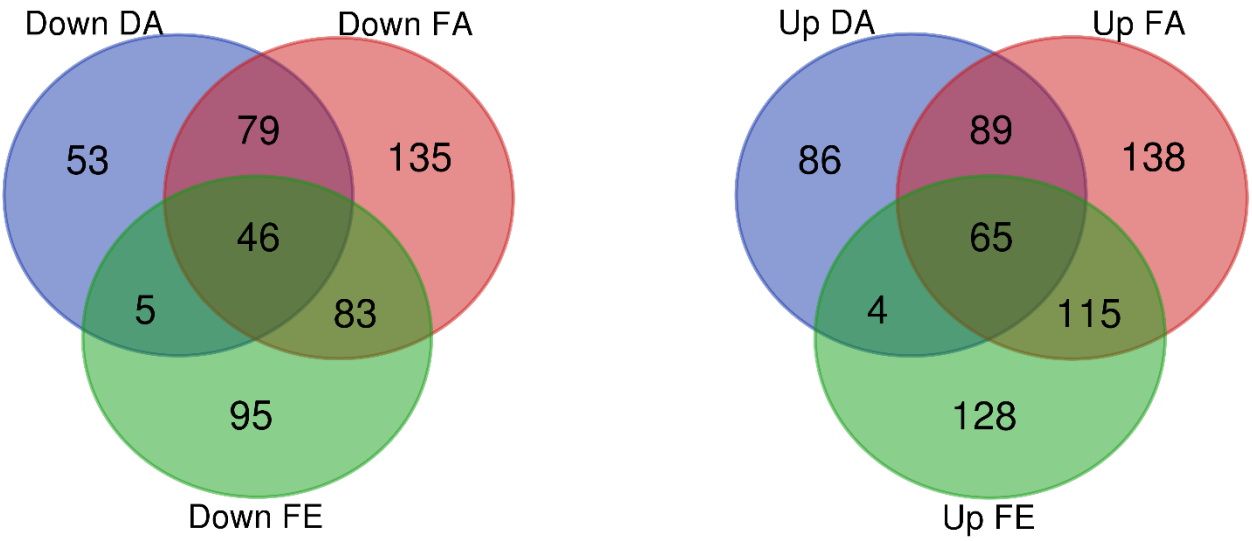

Figure 3.3. Relationship between differentially regulated genes with a fold change greater than one ($Log_2 \geq 1$) in all tested conditions. (A) Downregulated genes (B) Upregulated genes. DA (**D**MSO and **A**rachidonic acid), FA (**F**luconazole and **A**rachidonic acid) and FE (**F**luconazole and **E**tOH)

3.3.2. Enrichment analysis of differentially regulated genes

Biological process enrichment analysis was conducted to assign gene ontology (GO) terms to the genes obtained from different conditions, using Protein ANalysis THrough Evolutionary Relationships (PANTHER) Gene Ontology (GO) (Ashburner et al., 2000; Mi et al., 2017; The Gene Ontology Consortium, 2019). Different clusters shown on the Venn diagram for both upregulated and downregulated genes were individually analysed. The downregulated and upregulated genes shared by all the tested conditions were not enriched for specific GO terms, possibly because most of the genes present in this section are yet to be characterised. However, a search of the possible role of the downregulated genes indicates they are associated with filamentous growth, cell wall, and membrane transport processes. With the upregulated genes linked to roles in transcriptional regulation, secretory functions, and transport.

3.3.3. Processes enriched by growth with arachidonic acid

The unique downregulated genes associated with biofilms grown in the presence of AA (DA) were enriched for four GO terms, namely, valine catabolic process, aerobic respiration process, drug metabolic processes and oxidation-reduction (Table 3.2). In the valine catabolic process ontology, *HPD1* (which encodes hydroxypropionate dehydrogenase) and *ALD6* (which encodes malonate aldehyde dehydrogenase) were identified. In *C*. *albicans*, these genes are involved in the degradation of propionyl-CoA via a modified *β*-oxidation pathway to produce acetyl-CoA (Otzen et al., 2014). Propionyl-CoA is a toxic intermediate produced by yeast cells from the breakdown of cholesterol, valine, odd-chain fatty acids, methionine, isoleucine and threonine (Brock and Buckel, 2004; Zhang et al., 2004). Although *C*. *albicans* can use propionyl-CoA as a sole carbon source, glucose is still the preferred carbon source for *C*. *albicans* (Otzen et al., 2014). Therefore, the downregulation of propionyl-CoA degrading genes in biofilms grown in the presence of AA could imply either the sufficient availability of glucose or the absence of propionyl-CoA.

Aerobic respiration GO term was associated with *NDH51* and *ACO1*, genes which encode a flavoprotein subunit of the NADH dehydrogenase complex I (CI) and aconitase, respectively. NADH dehydrogenase complex I is a primary component of *C. albicans* mitochondrial electron transport chain which is responsible for NADH

binding to CI (Nosek and Fukuhara, 1994). Besides, it is required for growth on nonfermentable carbon sources such as ethanol and acetate. It is also required for filamentation (McDonough et al., 2002). Aconitase, an iron-sulphur containing protein, catalyses the conversion of citrate to isocitrate in the tricarboxylic acid cycle (Gangloff et al., 1990). Expression of *ACO1* gene is regulated by catabolite repression in the presence of glucose and glutamate (Gangloff et al., 1990; Flores et al., 2000). Glucose availability to the cell also influences the expression of NDH51 (McDonough et al., 2002; Vellucci et al., 2007).

The *CAT1* gene which encodes catalase enzyme was assigned to the drug metabolic process GO term. Catalase converts toxic hydrogen peroxide produced during oxidative phosphorylation to oxygen and water (Wysong et al., 1998). Glucose availability has been reported to repress the expression of catalase in *C*. *albicans*, even when treated with hydrogen peroxide (Nakagawa et al., 1999). The downregulation of the genes associated with these processes indicates that the presence of AA may be interfering with glucose uptake and metabolism.

The enriched upregulated genes, which were unique to biofilms grown in the presence of AA, were associated with four GO terms, namely, ribosomal large subunit biogenesis, rRNA processing, single-species submerged biofilm formation, translation and cellular response to drugs (Table 3.3). Ribosomal large subunit biogenesis was linked to *DBP3*, *NOC2*, *DRS1*, *RPL8B*, *RPL14*, *ERB1*, *RPP0,* and *DBP7*. The ribosome is a part of the essential translation machinery which converts messenger RNA into the respective protein. The process of ribosomal biogenesis entails highly coordinated procedures with intense energy demands for the synthesis, processing, transport, and assembly ribosomes (Thomson et al., 2013). *DRS1*, *DBP3*, and *DBP7* are DEAD-box proteins, also known as RNA helicases. These proteins are involved in RNA metabolism processes, which include the unwinding of double-stranded RNA molecules (Linder, 2006). However, *DRS1*, *DBP3*, and *DBP7* are involved in the maturation of ribosomal RNA (Issi et al., 2017). *Candida albicans* ribosome biogenesis processes are downregulated due to the high energy required when the cell undergoes stress due to reactive oxygen species (ROS) and nutrient depletion (Loar et al., 2004; Kos-Braun and Koš, 2017). The upregulation of genes associated with ribosomal biogenesis indicates the presence of acceptable energy levels in the cells when grown

in the presence DA. Also, hyphae inducers such as serum and *N*-acetyl-D-glucosamine causes a reduction in ribosomal RNA (Fleischmann and Rocha, 2015) and the fact that AA inhibits yeast to hyphae transition in *C. albicans* (Clément et al., 2007; Shareck and Belhumeur, 2011), may explain the opposite effect observed here.

Genes which play essential roles in biofilm formation in *C. albicans* such as *CSH1*, *GCA1*, *NDT80*, *ADH1*, *TEC1,* and *TYE7* were assigned to the single-species submerged biofilm formation GO term which was enriched in the presence of AA. The *CSH1* gene which encodes cell surface hydrophobicity is a vital virulence factor in *C. albicans* (Antley and Hazen, 1988). It is also involved in the irreversible adhesion of cells during the early biofilm formation, with adhesion being critical to biofilm formation (Chandra et al., 2001; Garcıa-Sanchez et al., 2004; Bujdáková et al., 2013). *GCA1* is a plasma membrane-associated glucoamylase enzyme with a role in the formation of *C. albicans* biofilm matrix (Nobile et al., 2009). Also, it has been reported to assist with starch degradation and energy generation (Maicas et al., 2016). Alcohol dehydrogenase encoding *ADH1* catalyses the reversible conversion of aldehyde to ethanol (Bertram et al., 1996) and it has been reported to be downregulated in *C. albicans* biofilms (Mukherjee et al., 2006). Transcriptional regulators *NDT80*, *TEC1* and *TYE7* were also associated with this ontology, while *NDT80* and *TEC1* are amongst the six *C. albicans* biofilm master circuit transcriptional regulators (Nobile et al., 2012), *TYE7* is involved in the activation of the glycolytic pathway (Askew et al., 2009). Transcriptional factors *NDT80* and *TEC1* are required for hyphal growth, although *NDT80* is not always required in all growth condition such as during the use of glycerol as a carbon source (Daniels et al., 2015; Min et al., 2018).

The upregulation of genes associated with the biofilm formation ontology may be a possible attempt by the yeast to compensate for low biofilm formation after treatment with DA. The presence of low biofilm formation may be associated with the upregulation of *ADH1,* which restricts biofilm growth in *C. albicans*.

Table 3.2. Gene ontology terms obtained from the PANTHER classification system for downregulated genes unique to biofilms grown in the presence of arachidonic acid (DA)

Analysis Type: PANTHER Overrepresentation Test (Released 20171205)							
Annotation Version and Release Date: GO Ontology database Released 2018-05-21							
Analyzed List: upload_1 (*Candida albicans*)							
Reference List: *Candida albicans* (all genes in database)							
Test Type: FISHER							
			upload_1				
GO biological process complete	**Candida albicans (REF)**	**#**	**expected**	**fold enrichment**	**over (+)/under (-)**	**raw *P*-value**	**FDR**
valine catabolic process (GO:0006574)	3	2	0	> 100	+	1.11E-05	1.84E-02
aerobic respiration (GO:0009060)	59	3	0.07	44.69	+	3.30E-05	3.30E-02
drug metabolic process (GO:0017144)	222	4	0.25	15.84	+	5.66E-05	4.72E-02
oxidation-reduction process (GO:0055114)	449	7	0.51	13.7	+	1.17E-08	5.84E-05

Table 3.3. Gene ontology terms obtained from the PANTHER classification system for upregulated genes unique to biofilms grown in the presence of arachidonic acid (DA)

Analysis Type: PANTHER Overrepresentation Test (Released 20171205)							
Annotation Version and Release Date: GO Ontology database Released 2018-05-21							
Analyzed List: upload_1 (*Candida albicans*)							
Reference List: *Candida albicans* (all genes in database)							
Test Type: FISHER							
			upload_1				
GO biological process complete	**Candida albicans (REF)**	**#**	**expected**	**fold enrichment**	**over (+)/under (-)**	**raw *P*-value**	**FDR**
Ribosomal large subunit biogenesis (GO:0042273)	78	8	0.01	0.75	+	1.21E-06	8.66E-04
rRNA processing (GO:0006364)	156	12	0.13	1.5	+	3.84E-08	3.20E-05
Single-species submerged biofilm formation (GO:0090609)	122	7	0.22	1.17	+	1.97E-04	3.07E-02
Translation (GO:0006412)	267	12	0.39	2.56	+	8.96E-06	3.45E-03
Cellular response to drug (GO:0035690)	291	11	0.89	2.79	+	1.03E-04	1.98E-02

3.3.4. Processes enriched by growth with fluconazole

Gene ontologies enriched in downregulated genes which are unique to biofilms grown in the presence of fluconazole (FE) include DNA methylation or demethylation, negative regulation of helicase activity, DNA replication checkpoint, DNA strand elongation involved in DNA replication and DNA-dependent DNA replication maintenance of fidelity (Table 3.4). DNA methylation and demethylation processes are epigenetic modification which represses transcription and transposon control (Finnegan et al., 2000; Martienssen and Colot, 2001). In *C*. *albicans*, this process is associated with the repression of genes involved with phenotypic switching, structural genes, and gene regulation. Additionally, the presence of environmental stressors can also induce *C*. *albicans* DNA methylation (Mishra et al., 2011). Therefore, the downregulation of DNA methylation or demethylation process may indicate a reduction in morphological transition, which may lead to reduced biofilm formation.

The histone H3 isoform encoding gene, *HHT21*, was associated with the DNA methylation or demethylation ontology. According to an investigation by Zacchi and coworkers (2010), transcription and chromosome segregations are affected with a change in the number of *C*. *albicans* histones, and this results in growth defects and aneuploid formation. Fluconazole exposure to *C*. *albicans* cells has been shown to induce the formation of a significant number of aneuploid cells. These cells initially appear as trimeras, then tetraploids, before forming aneuploids (Harrison et al., 2014). This phenomenon results in abnormal cell cycle progression, inducing chromosome changes such as loss of heterozygosity in *C*. *albicans* (Selmecki et al., 2006, 2008). The presence of azoles such as fluconazole has been associated with stresses which have been explicitly identified as the primary cause for the formation of trimera in *C*. *albicans* (Harrison et al., 2014). Also, this could be a resistance response to fluconazole, as the alteration of histone amounts have been previously linked with antifungal resistance (Selmecki et al., 2006). Therefore, the presence of a low fluconazole concentration may initiate a response with the downregulation of several DNA associated processes.

Upregulated genes unique to biofilms grown in the presence of fluconazole, were enriched for GO terms which include farnesyl diphosphate metabolic process, ergosterol biosynthetic process, cell wall chitin biosynthetic process and localisation

within the membrane (Table 3.5). Farnesyl diphosphate is a precursor in the isoprenoid pathway for the synthesis of ergosterol, which is essential for the survival of yeast cells. As depicted in Figure 3.4, farnesyl diphosphate is the substrate for squalene synthase (*ERG9*) to produce squalene, which is further metabolised after several enzymatic steps to produce ergosterol (Kuranda et al., 2010). Ergosterol is essential in yeast because it assists with the maintenance of membrane fluidity, permeability, and integrity (Lv et al., 2016). Fluconazole inhibits ergosterol synthesis by binding to lanosterol demethylase enzyme encoded by *ERG11* (Bondaryk et al., 2013). Several genes associated with ergosterol synthesis including *ERG3*, *ERG1*, *ERG11*, *ERG9,* and *CYB5* were linked with this ontology term. The upregulation of ergosterol synthesis genes is one of the known resistance mechanism of *C. albicans* biofilms against fluconazole treatment (Sanglard et al., 2003; Tsui et al., 2016). In the presence of fluconazole, *ERG3* and *ERG1* which are up and downstream of the drug target (*ERG11)* have been reported to be upregulated (Henry et al., 2000; Nailis et al., 2010). The upregulation of processes associated with ergosterol synthesis in the presence of fluconazole indicates a resistance response to compensate for the presence of the antifungal (Bondaryk et al., 2013).

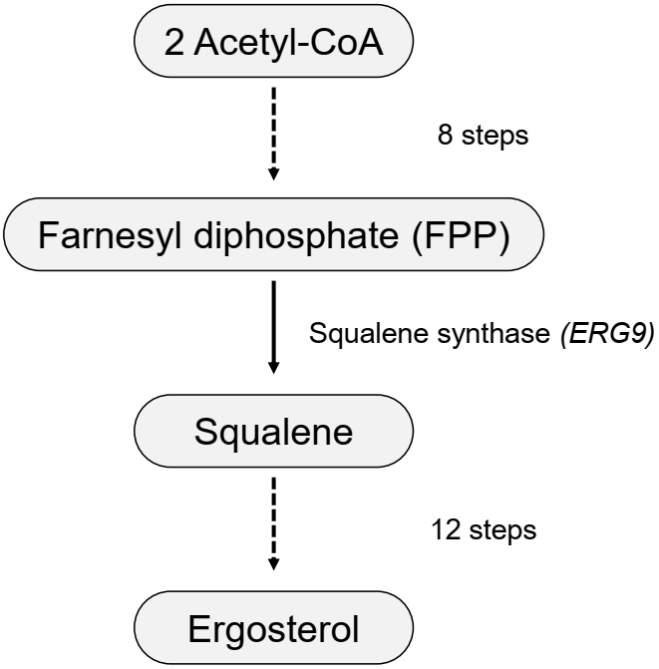

Figure 3.4. An abridged isoprenoid pathway showing the link between farnesyl diphosphate and ergosterol synthesis. Broken arrows indicate the presence of multiple enzymatic steps. (adapted from Kuranda et al., 2010 and Lv et al., 2016).

Cell wall chitin biosynthetic process was also associated with genes upregulated in biofilms grown in the presence of fluconazole. Chitin is an essential component of the yeast cell wall (Lipke and Ovalle, 1998; Lenardon et al., 2010) and comprises up to 2% of the cell wall dry weight (Orlean, 2012). Upregulation of the chitin process in *C. albicans* can be linked with the presence of fluconazole. Fluconazole induces membrane and cell wall stress, by sterol disruption and increasing the fluidity of the plasma membrane (Abe et al., 2009; Klis and Brul, 2015). In response to cell wall stress, *C. albicans* increases the amount of chitin and expression of cell wall repair proteins (Munro et al., 2007; Sorgo et al., 2011). Under this condition, the upregulation of glucosamine-6-phosphate synthase (*GFA1*), which is the first enzyme of the hexosamine pathway was also observed (Smith et al., 1996). The hexosamine pathway produces UDP-N-acetylglucosamine, a potent precursor for the chitin synthesis (Lipke and Ovalle, 1998).

3.3.5. Processes enriched by growth with both fluconazole and arachidonic acid

The downregulated genes which were unique to biofilms grown in the presence of both fluconazole and AA (FA) was not enriched for any GO term. Most of the genes in this cluster are uncharacterised. However, those with known functions have roles in zinc homeostasis, oxidative stress and copper and zinc transport.

Upregulated genes, unique to cells grown in the presence of fluconazole and AA (FA), are enriched for AMP biosynthetic processes, fumarate metabolic process, ATP synthesis coupled proton transport, fatty acid oxidation, tricarboxylic acid process and acyl-CoA metabolic process (Table 3.6). Purine nucleotides are required for the synthesis of essential cellular components which include genetic material (DNA and RNA), cofactors and energy transporters for the proper functioning of the cell (Rolfes, 2006; Chitty and Fraser, 2017). Purines synthesis in *C. albicans* occurs via the *de novo* biosynthesis pathway and the salvage pathways (Rolfes, 2006) which produces adenosine monophosphate (AMP) as an intermediate product (Chitty and Fraser, 2017). Adenylosuccinate synthase (encoded by *ADE12*) and adenosine kinase (encoded by *ADO1*), which catalyses different steps of the purine synthesis pathway, were assigned to the AMP biosynthetic process ontology. Ade12p catalyses the conversion of inosine monophosphate (IMP) to adenylosuccinate which is further

catalysed to AMP by Ade13p (Chitty and Fraser, 2017) while Ado1p is involved in the recycling of adenosine to AMP (Figure 3.5).

The energy demand for purine biosynthesis is high; therefore, the regulation of this process is via feedback inhibition by the ATP and ADP end products and the presence of excess purine in the cell-extracellular environment (Rébora et al., 2001; Rolfes, 2006). The upregulation of genes associated with purine synthesis in biofilms grown in the presence of fluconazole and AA indicates an attempt to increase ATP generation under these conditions.

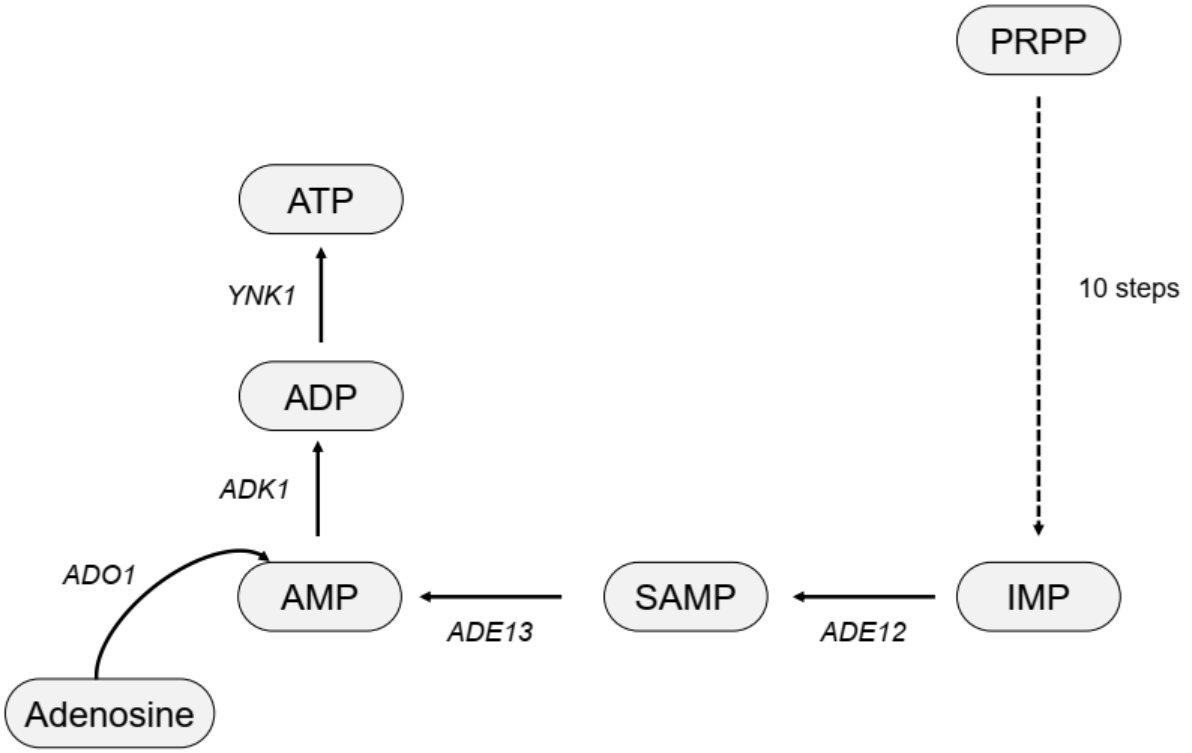

Figure 3.5. Abridged purine de novo metabolism pathway. Abbreviations: PRPP, 5-phosphoribosyl-α-1-pyrophosphate; IMP, inosine monophosphate; SAMP, adenylosuccinate; AMP, adenosine monophosphate; ADP, adenosine diphosphate. The broken arrow indicates the presence of multiple enzymatic steps (adapted from Lecoq et al., 2001; Rébora et al., 2001).

Table 3.4. Gene ontology terms obtained from the PANTHER classification system for downregulated genes unique to biofilms grown in the presence of fluconazole (FE)

Analysis Type: PANTHER Overrepresentation Test (Released 20190308)							
Annotation Version and Release Date: GO Ontology database Released 2019-01-01							
Analyzed List: upload_1 (*Candida albicans*)							
Reference List: *Candida albicans* (all genes in database)							
Test Type: FISHER							
		upload_1					
GO biological process complete	**Candida albicans (REF)**	**#**	**expected**	**fold enrichment**	**over (+)/under (-)**	**raw *P*-value**	**FDR**
DNA methylation or demethylation (GO:0044728)	3	2	0.03	63.86	+	1.03E-03	4.28E-02
Negative regulation of helicase activity (GO:0051097)	3	2	0.03	63.86	+	1.03E-03	4.19E-02
DNA replication checkpoint (GO:0000076)	12	4	0.13	31.93	+	1.72E-05	1.64E-03
DNA strand elongation involved in DNA replication (GO:0006271)	13	4	0.14	29.47	+	2.23E-05	2.05E-03
DNA-dependent DNA replication maintenance of fidelity (GO:0045005)	19	5	0.2	25.21	+	3.66E-06	4.50E-04

Table 3.5. Gene ontology terms obtained from the PANTHER classification system for upregulated genes unique to biofilms grown in the presence of fluconazole (FE)

Analysis Type: PANTHER Overrepresentation Test (Released 20171205)							
Annotation Version and Release Date: GO Ontology database Released 2018-05-21							
Analyzed List: upload_1 (*Candida albicans*)							
Reference List: *Candida albicans* (all genes in database)							
Test Type: FISHER							
		upload_1					
GO biological process complete	**Candida albicans (REF)**	**#**	**expected**	**fold enrichment**	**over (+)/under (-)**	**raw *P*-value**	**FDR**
Farnesyl diphosphate metabolic process (GO:0045338)	4	2	0.01	> 100	+	5.19E-05	0.00811
Cell wall chitin metabolic process (GO:0006037)	15	4	0.03	> 100	+	3.14E-08	8.73E-06
Ergosterol biosynthetic process (GO:0006696)	25	6	0.05	> 100	+	8.76E-12	4.38E-08
Localization within membrane (GO:0051668)	11	2	0.02	93.23	+	0.000268	0.0312

ATP synthesis coupled proton transport GO term was represented by ATP synthase alpha subunit (encoded by *ATP1*), F1F0-ATP synthase beta subunit (encoded by *ATP2*), oligomycin-sensitive conferring protein (OSCP) (encoded by *ATP5*) and F1F0 ATP synthase subunit h (encoded by *ATP14*), these proteins constitute different components of the mitochondrial ATP synthase (Arnold et al., 1998). Mitochondrial ATP synthase is an essential enzyme which catalyses the phosphorylation of ADP to ATP using the energy provided by the electrochemical gradient (Xu et al., 2015). The absence of *ATP1* in *C*. *albicans* cells have been reported to result in the cells being avirulent, unable to grow on non-glucose carbon sources, defective in filamentation, biofilm formation and a reduction in ATP (Li et al., 2017). The role of *ATP2* in *C*. *albicans* can be seen with the homozygous mutants, which are avirulent with a decline in ATP and growth on non-fermentable carbon sources (Li et al., 2018). Lack of growth in media containing glycerol and lactate as a carbon source was observed in *Saccharomyces cerevisiae ATP14* homozygous mutant (Arselin et al., 1996). This also supports the possible need for ATP generation in biofilms grown in the presence of both fluconazole and AA.

Fumarate metabolic process and tricarboxylic acid process are represented by genes encoding enzymes within the tricarboxylic acid (TCA) cycle. Fumarate hydratase (fumarase) (encoded by *FUM12*) was assigned to both ontologies while adenylosuccinate synthase (encoded by *ADE12*) was assigned to the fumarate metabolic process and malate dehydrogenase (encoded by *MDH1-1*) and succinate-CoA ligase (encoded by *LSC1*) to the tricarboxylic acid process. Fumarase is present in the cytoplasm and mitochondria of eukaryotic cells. The mitochondrial fumarase catalyses the reversible hydration of fumarate to L-malate in the TCA cycle (Woods et al., 1988), while the cytosolic fumarase is synthesised in response to DNA damage (Yogev et al., 2010). The L-malate product of mitochondrial fumarase can be further oxidised to oxaloacetate by malate dehydrogenase via the NAD/NADH coenzyme system for ATP production in the TCA cycle or gluconeogenesis through phosphoenolpyruvate (Saayman and Viljoen-Bloom, 2017). Energy can also be generated from malate via the shuttle of malate or aspartate across the mitochondrial matrix (Minárik et al., 2002; Tylicki et al., 2008) while succinate-CoA ligase catalyses the conversion of succinyl-CoA to succinate in the TCA cycle (Przybyla-Zawislak et al., 1998). The upregulation of genes associated with the TCA cycle in the presence

of both fluconazole and AA may also be as a result of the need for an increase in ATP generation.

Putative pyruvate dehydrogenase (encoded by *PDB1*), carnitine acetyltransferase (encoded by *CAT2*) and succinate-CoA ligase (encoded by *LSC1*) were associated with the GO term acyl-CoA metabolic process. The functions of *CAT2* and *LSC1* in the cell have been discussed earlier, but *PDB1* which encodes a β–subunit of the pyruvate dehydrogenase complex, catalyses the conversion of pyruvate to acetyl-CoA and CO_2 (Vellucci et al., 2007). The GO terms enriched in the upregulated genes of *C*. *albicans* cells grown in the presence of fluconazole and AA are mostly associated with energy generation. This suggests that under this treatment condition, the cell attempts to increase its energy output, and this indicates that there is a demand for energy.

3.3.6. Overlapping processes enriched by growth with either arachidonic acid or fluconazole

The upregulated and downregulated genes shared between biofilms grown in the presence of either AA (DA) or fluconazole (FE) were not enriched for any specific GO terms. The reported descriptions for the downregulated genes include phosphatase complex subunit, SIN3-binding protein, a heme oxygenase, and a high-affinity iron permease, while the upregulated genes are associated with the cell wall and DNA synthesis.

3.3.7. Overlapping processes enriched by growth with either arachidonic acid or a combination of fluconazole and arachidonic acid

The downregulated genes which were shared between biofilms grown in the presence of either AA (DA) and a combination of fluconazole and AA (FA) were enriched for GO terms which include hydrogen sulphide biosynthetic process, sulfate assimilation and sulfur amino acid biosynthetic process (Table 3.7). The different processes mentioned above are represented by ATP sulfurylase (encoded by *MET3*), putative adenylylsulfate kinase (encoded by *MET14*), putative sulphite reductase beta subunit (encoded by *ECM17*) and O-acetylhomoserine O-acetylserine sulfhydrylase (encoded by *MET15*) which are genes in the *C*. *albicans* methionine/cysteine biosynthesis pathway. Methionine/cysteine synthesis is essential for biofilm formation in *C*. *albicans*, as indicated by the transcriptional analysis which showed significant

upregulation of genes associated with methionine and cysteine biosynthesis from as early as the adhesion phase (García-Sánchez et al., 2004; Murillo et al., 2005). A failure in the formation of proper biofilm diminished adhesion, and a decrease in filamentation was observed in homozygous *ecm17*Δ/Δ *C*. *albicans* strains and with the absence of methionine or cysteine (Li et al., 2013). *MET3*, which is an activator of the methionine/cysteine pathway for sulphur assimilation (Figure 3.6) is one of the most highly upregulated genes during biofilm formation in *C*. *albicans* (Murillo et al., 2005). However, the presence of methionine or cystine at a concentration higher than 1 mM represses the expression of *MET3* (Care et al., 1999). The concentrations of methionine and cysteine in RPMI media are 0.43 mM and 0.062 mM, respectively. Hence, the downregulation in methionine/cystine processes may not be due to catabolite repression but rather the presence of AA in both these conditions, which may repress the methionine/cysteine pathway. The repression could result in low biomass in the biofilm.

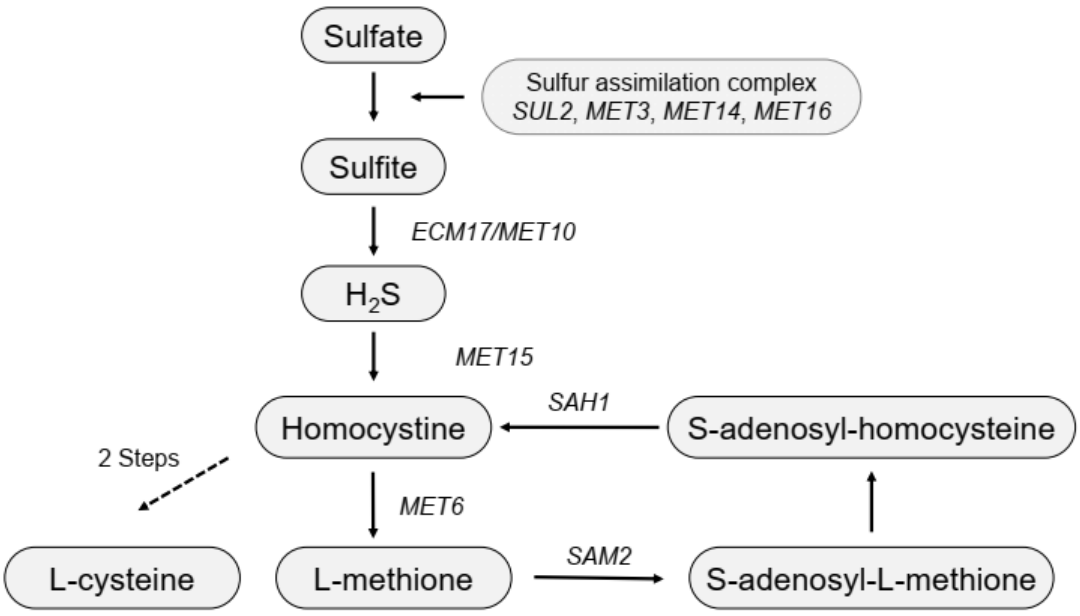

Figure 3.6. *Candida albicans* methionine/cysteine biosynthesis pathway and the associated genes via sulphur assimilation. The broken arrow indicates the presence of multiple enzymatic steps (adapted from Li et al., 2013).

Table 3.6. Gene ontology terms obtained from the PANTHER classification system for upregulated genes unique to biofilms grown in the presence of both fluconazole and arachidonic acid (FA)

Analysis Type: PANTHER Overrepresentation Test (Released 20171205)							
Annotation Version and Release Date: GO Ontology database Released 2018-05-21							
Analyzed List: upload_1 (*Candida albicans*)							
Reference List: *Candida albicans* (all genes in database)							
Test Type: FISHER							
				upload_1			
GO biological process complete	**Candida albicans (REF)**	**#**	**expected**	**fold enrichment**	**over (+)/under (-)**	**raw *P*-value**	**FDR**
AMP biosynthetic process (GO:0006167)	4	2	0.03	73.25	+	6.62E-04	4.24E-02
Fumarate metabolic process (GO:0006106)	4	2	0.03	73.25	+	6.62E-04	4.19E-02
ATP synthesis coupled proton transport (GO:0015986)	21	5	0.14	34.88	+	6.64E-04	1.23E-04
Fatty acid oxidation (GO:0019395)	16	3	0.11	27.42	+	2.60E-04	1.83E-02
Tricarboxylic acid cycle (GO:0006099)	24	4	0.16	24.42	+	3.32E-05	3.46E-03
Acyl-CoA metabolic process (GO:0006637)	19	3	0.13	23.13	+	4.08E-04	2.76E-02

Table 3.7. Gene ontology obtained from the PANTHER classification system for downregulated genes unique to biofilms grown in the presence of both arachidonic acid (DA) and a combination of fluconaziole and arachidonic acid (FA)

Analysis Type: PANTHER Overrepresentation Test (Released 20171205)							
Annotation Version and Release Date: GO Ontology database Released 2018-05-21							
Analyzed List: upload_1 (*Candida albicans*)							
Reference List: *Candida albicans* (all genes in database)							
Test Type: FISHER							
				upload_1			
GO biological process complete	**Candida albicans (REF)**	**#**	**expected**	**fold enrichment**	**over (+)/under (-)**	**raw *P*-value**	**FDR**
Hydrogen sulfide biosynthetic process (GO:0070814)	2	2	0	> 100	+	3.16E-06	3.16E-03
Sulfate assimilation (GO:0000103)	7	2	0.01	> 100	+	1.89E-05	1.18E-02
Sulfur amino acid biosynthetic process (GO:0000097)	26	3	0.02	> 100	+	9.33E-07	1.56E-03

The GO terms enriched in upregulated genes shared between biofilms grown in the presence of AA (DA) and a combination of fluconazole and AA (FA) include xenobiotic transport, spermine transport, and spermidine transport (Table 3.8). These GO terms were represented by an ATP-binding cassette (ABC) transporter (encoded by *YOR1*) as well as *FLU1*, which encodes for fluconazole resistance 1 protein, an efflux pump belonging to the major facilitator superfamily (MFS) class of transporters involved with drug efflux (Cannon et al., 2009). However, contrary to expectations of its role in fluconazole efflux, the disruption of *FLU1* in *C. albicans* has a negligible effect on its fluconazole susceptibility, although its deletion causes susceptibility to mycophenolic acid, indicating that mycophenolic acid is a more suitable substrate than fluconazole (Calabrese et al., 2000).

The *C. albicans YOR1* is similar to the *S. cerevisiae YOR1*, which is an ATP-binding cassette (ABC) transporter, which acts as an oligomycin resistance permease (Gaur et al., 2005). However, *YOR1* in *C. albicans* effluxes beauvericin, a compound which enhances azole susceptibility, from the cell (Shekhar-guturja et al., 2016). Although the upregulated genes associated with this GO term in the presence of AA and a combination of fluconazole and AA are involved in transport, their role in the transport of either fluconazole or AA has not been identified.

Table 3.8. Gene ontology terms obtained from the PANTHER classification system for upregulated genes shared by biofilms grown in the presence of both arachidonic acid (DA) and a combination of fluconazole and arachidonic acid (FA)

Analysis Type: PANTHER Overrepresentation Test (Released 20171205)							
Annotation Version and Release Date: GO Ontology database Released 2018-05-21							
Analyzed List: upload_1 (*Candida albicans*)							
Reference List: *Candida albicans* (all genes in database)							
Test Type: FISHER							
		upload_1					
GO biological process complete	**Candida albicans (REF)**	**#**	**expected**	**fold enrichment**	**over (+)/under (-)**	**raw *P*-value**	**FDR**
Xenobiotic transport (GO:0042908)	4	2	0.01	> 100	+	0.000094	0.0392
Spermine transport (GO:0000296)	4	2	0.01	> 100	+	0.000094	0.0362
Spermidine transport (GO:0015848)	5	2	0.01	> 100	+	0.000131	0.047

Table 3.9. Gene ontology terms obtained from the PANTHER classification system for downregulated genes shared by biofilms grown in the presence of both fluconazole (FE) and a combination of fluconazole and arachidonic acid (FA)

Analysis Type: PANTHER Overrepresentation Test (Released 20171205)							
Annotation Version and Release Date: GO Ontology database Released 2018-05-21							
Analyzed List: upload_1 (*Candida albicans*)							
Reference List: *Candida albicans* (all genes in database)							
Test Type: FISHER							
		upload_1					
GO biological process complete	**Candida albicans (REF)**	**#**	**expected**	**fold enrichment**	**over (+)/under (-)**	**raw *P*-value**	**FDR**
Filamentous growth (GO:0030447)	554	19	2.07	9.17	+	1.32E-16	5.67E-13
Cytoskeleton-dependent cytokinesis (GO:0061640)	35	3	0.13	22.93	+	3.50E-04	4.49E-02
Biological adhesion (GO:0022610)	104	5	0.39	12.86	+	4.08E-05	8.16E-03
Cellular response to starvation (GO:0009267)	238	8	0.89	8.99	+	1.65E-06	8.25E-04

3.3.8. Overlapping processes enriched by growth with either fluconazole or a combination of fluconazole and arachidonic acid

The GO terms enriched for downregulated genes shared between biofilms grown in the presence of either fluconazole (FE) and a combination of fluconazole and AA (FA) include filamentous growth regulation, cytoskeleton-dependent cytokinesis, biological adhesion, and cell response to starvation (Table 3.9). The genes linked with the filamentous growth ontology include Zn(II)2Cys6 transcription factors (encoded by *ROB1*, *AHR1* and *UME6*) as well as a G-protein alpha subunit (encoded by *GPA2*). Biofilm formation in *C. albicans* is under the control of six master circuit transcriptional factors, including Rob1p. These transcriptional factors regulate the expression of several target genes which are associated with biofilm formation and also regulate the expression of each other (Nobile et al., 2012). The importance of *ROB1* to *C. albicans* biofilm formation is demonstrated with the failure of *rob1*Δ/Δ mutants to form biofilms and the haploinsufficiency of the heterozygous mutant (Nobile et al., 2012; Glazier et al., 2017). The zinc cluster transcription factor, Ahr1p, activates adhesion and hyphal regulatory genes (Askew et al., 2011). Cell adhesion to host surface and hyphae formation are essential processes of *C. albicans* biofilms formation and also contributes to the biofilm structure (Chandra et al., 2001; Finkel and Mitchell, 2011). *UME6* is an essential regulator for hyphal extension and virulence in *C. albicans* (Banerjee et al., 2008). Banerjee and co-workers (2013), reported a significant increase in biofilm growth with the increased expression of *UME6*. The downregulation of critical transcriptional regulatory genes in biofilms grown in the presence of fluconazole and a combination of fluconazole and AA may indicate the possible presence of poorly formed biofilms under these conditions.

This is also seen in the enrichment of the biological adhesion GO term, which was represented by agglutinin-like protein 3 (encoded by *ALS3*), Zn(II)2Cys6 transcription factor (encoded by *UME6*), and the zinc cluster transcription factor (encoded by *AHR1*). *Candida albicans* agglutinin-like protein 3 (Als3p) is known to function in adhesion, biofilm formation, and iron acquisition processes (Almeida et al., 2008; Liu and Filler, 2011). However, its expression is only observed in the hyphae and pseudohyphae (Hoyer et al., 1998). The deletion of *ALS3* in *C. albicans* resulted in the formation of thin and disorganised biofilms *in vitro* (Nobile et al., 2006).

The cytoskeleton-dependent cytokinesis GO term was represented by septin (encoded by *CDC10*), formin-binding protein (encoded by *HOF1*), and Ras GTPase-activating protein (encoded by *IQG1*). Septins are filament-forming proteins which comprise Cdc3p, Cdc11p, Cdc12p and Cdc10p. (Longtine et al., 1996; Frazier et al., 1998). These proteins function in septum formation between mother and daughter cells, and they also act as a barricade against protein diffusion across the cell cortex (Warenda and Konopka, 2002; Sudbery, 2007). Although *C*. *albicans* viability or growth rate is not affected by a mutation of *CDC10*, the observed defects in *C*. *albicans* *CDC10* mutants include a failed cytokinesis due to abnormal cell morphology of elongation and enlargement of cells without separation as well as defects in chitin localisation (Warenda and Konopka, 2002).

The *HOF1* encodes an F-box protein involved in cytokinesis and the formation of a ring around the bud neck of the mother and daughter cells. In the absence of *HOF1*, *C. albicans* cells suffer severe cytokinesis and separation defects which is evident in the formation of chains of cells (Li et al., 2008; Jonkers and Rep, 2009). *IQG1* also localises to the bud neck contributing to neck formation with the assembly and contraction of the actomyosin ring. In the absence of *IQG1*, cells necks are swollen and broaden (Li et al., 2008). The downregulation of processes associated with cytokinesis process in biofilms grown in the presence of fluconazole as well as a combination of fluconazole and AA may be due to the presence of fluconazole as it has been shown to induce trimeras as discussed previously (Harrison et al., 2014).

The GO term enriched in the upregulated genes shared by biofilms grown in the presence of fluconazole (FE) and a combination of fluconazole and AA (FA) include the glyoxylate cycle, acetate catabolic process, carbon utilisation, and fatty acid catabolic process (Table 3.10). All these processes were represented by the isocitrate lyase (encoded by *ICL1*), malate synthase (encoded by *MLS1*) and a multifunctional enzyme of the β-oxidation pathway (encoded by *FOX2*). *ICL1* and *MLS1* are the first two genes of the glyoxylate cycle, an alternative pathway which allows the conversion of two-carbon acetate into a four-carbon dicarboxylic acid, which replenishes the TCA cycle or serves as a precursor for either amino acid or carbohydrate synthesis (Kunze et al., 2006).

Conversely, Fox2p catalyses both enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities in the β-oxidation pathway (Hiltunen et al., 2003). Alternative energy-generating pathways such as the β-oxidation pathway and the glyoxylate pathway allow the yeast to grow on alternative carbon sources such as fatty acids, ethanol, and acetate (Kunze and Hartig, 2013). Treatment of *C*. *albicans* cells with fluconazole has been shown to increase metabolites connected with the central carbon metabolic pathway (Katragkou et al., 2016). Therefore, the upregulation of glyoxylate pathway genes and *FOX2* from the β-oxidation pathway may indicate an increase in the energy requirements of the biofilms under these conditions.

3.3.9. Confirmation of RNA-Seq data with NanoString

Genes of interest with significant differential expression were selected for confirmation on the NanoString nCounter platform. The foldchange obtained with NanoString compared with RNA-Seq are shown in Table 3.11. There was consistency in the expression data between the two platforms.

Table 3.10. Gene ontology terms obtained from the PANTHER classification system for upregulated genes shared by biofilms grown in the presence of arachidonic acid (DA) and a combination of fluconazole and arachidonic acid (FA)

Analysis Type: PANTHER Overrepresentation Test (Released 20190417)							
Annotation Version and Release Date: GO Ontology database Released 2019-02-02							
Analyzed List: upload_1 (*Candida albicans*)							
Reference List: *Candida albicans* (all genes in database)							
Test Type: FISHER							
		upload_1					
GO biological process complete	**Candida albicans (REF)**	**#**	**expected**	**fold enrichment**	**over (+)/under (-)**	**raw *P*-value**	**FDR**
Glyoxylate cycle (GO:0006097)	3	3	0	> 100	+	2.18E-09	1.22E-05
Acetate catabolic process (GO:0045733)	6	2	0	> 100	+	9.20E-06	5.71E-03
Carbon utilization (GO:0015976)	12	2	0.01	> 100	+	2.99E-05	9.81E-03
Fatty acid catabolic process (GO:0009062)	28	3	0.02	> 100	+	4.88E-07	6.82E-04

Table 3.11. Comparison between RNA-Seq and NanoString data for genes of interest. DA (**D**MSO and **A**rachidonic Acid), FA (**F**luconazole and **A**rachidonic acid), FE (**F**luconazole and **E**tOH).

	Log$_2$								
	Cuffdiff			**DESeq2**			**NanoString**		
Gene	**DA**	**FA**	**FE**	**DA**	**FA**	**FE**	**DA**	**FA**	**FE**
ACT1	1.12	1.06	1.30	0.86	0.82	1.02	0.51	-0.18	0.38
CDR1	2.77	2.70	1.56	2.66	2.68	1.62	2.83	2.39	1.39
CDR2	-0.06	-0.09	0.20	2.92	3.21	1.74	4.28	4.97	2.02
CSR1	-0.82	-2.27	-0.78	-0.78	-2.18	-0.79	-0.61	-2.83	-0.89
DAL8	0.41	1.07	0.88	0.06	0.53	0.95	1.24	0.87	0.91
ECM17	-1.92	-1.68	0.59	-1.70	-1.71	0.50	-2.14	-2.18	-0.06
EHT1	3.66	3.61	0.52	3.39	3.59	0.61	4.32	3.6	0.95
ERG11	-1.14	0.39	1.29	-1.10	0.41	1.28	-1.38	-0.09	1.2
FCR1	1.56	1.47	-0.44	1.52	1.47	-0.43	1.92	1.9	-0.55
FLU1	2.03	1.93	0.28	1.85	1.77	0.30	2.88	1.95	0.73
HSP12	1.06	-0.30	0.96	1.05	0.16	0.75	1.54	0.01	0.97
IPT1	1.25	1.09	-0.12	1.48	1.33	0.13	0.72	0.67	-0.55
LSC2	0.27	0.58	-0.11	1.00	1.19	0.42	0.84	0.58	0.11
MDR1	0.11	0.57	1.18	0.08	0.47	1.22	1.77	1.38	2.84
MET4	-1.34	-1.41	0.14	-1.10	-1.32	0.06	-1.82	-1.86	-0.57
NRG1	1.66	1.98	1.65	1.87	2.05	1.62	2.04	1.83	1.28
orf19.1191	0.08	0.02	0.05	0.03	0.01	0.04	0.1	-0.28	-0.05
orf19.2184	0.08	0.08	0.09	-0.02	0.11	0.10	-0.1	0.28	0.05
PDR16	1.56	1.60	-0.49	1.73	1.71	-0.43	1.52	2.1	-0.65
PGA13	-2.04	-2.27	1.16	-0.98	-1.76	1.48	-2.72	-3.91	0.07
PST1	-0.99	-1.67	-0.51	-1.29	-2.44	-0.83	-1.07	-1.63	-0.01
RTA3	5.19	5.52	4.38	4.24	4.80	3.77	5.83	6.03	4.88
SAP6	0.18	0.44	0.52	-0.68	1.34	1.54	-0.78	0.88	1.73
SET3	-0.25	0.93	1.67	-1.01	1.14	1.97	-1.28	1.06	2.11
SFL1	1.21	1.43	0.45	1.37	1.55	0.37	2.44	1.91	0.23
SMI1	0.41	0.35	0.21	-0.44	-0.26	-0.18	-0.03	-0.69	-0.19
SUL2	-2.24	-2.48	-0.98	-1.92	-2.67	0.17	-3.59	-3.8	-0.92
SUT1	2.03	2.50	-0.63	1.84	2.30	-0.69	2.59	3.41	-0.32
TAC1	1.01	1.58	1.42	1.09	1.79	1.47	1.28	1.76	1.1
TDH3	0.55	-1.25	-0.53	0.76	-0.09	-0.25	0.27	-0.73	-0.53
TYE7	1.04	0.41	0.30	1.08	0.51	0.25	2.28	0.49	0.88
UPC2	-0.58	1.01	2.40	-0.46	0.99	2.40	-0.55	0.87	2.01
WOR1	-0.05	-0.78	-0.22	0.02	-0.58	-0.22	-0.81	-1.3	-1.48
YOR1	1.62	1.40	0.57	1.27	1.37	0.57	2.37	1.94	0.83
YWP1	2.53	2.17	1.31	3.07	2.16	1.25	2.22	0.43	0.7
ZNC1	0.01	0.53	0.46	-0.06	0.50	0.50	0.54	0.61	0.55

3.4. Conclusions

Differentially regulated genes were obtained in all the tested conditions with the most differentially regulated genes obtained with the combination of fluconazole and AA (FA). Analysis of the biological processes indicated that in the presence of AA (DA), energy generation processes were downregulated, which may be due to an inhibition of glucose uptake. The low energy generation in the presence of AA may also be associated with the possible upregulation of biofilm formation processes to compensate for low biomass. As expected, the presence of fluconazole enriched for processes involved in ergosterol synthesis and an increase in chitin content. Also, processes associated with abnormal cell cycle such as aneuploidy was enriched in the fluconazole treated cells. In cells grown with the combination of fluconazole and AA, several processes associated with ATP synthesis was enriched, indicating the need to produce ATP via alternative processes. Also, methionine/cysteine synthesis processes which are required for biofilm formation were downregulated in the presence of AA. It can be suggested that the presence of AA interferes with the energy generation and methionine synthesis processes as a mechanism for increased susceptibility. However, more investigations are required to establish if these processes are affected when *C. albicans* biofilms are treated with a combination of both compounds.

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CHAPTER 4

Investigating the Effect of Arachidonic Acid on Known Fluconazole Resistance Mechanism of *C. albicans* Biofilms

Abstract

Candida albicans is a human commensal, which causes opportunistic infections with high morbidity and mortality and is characterised by the production of highly resistant biofilms. The addition of polyunsaturated fatty acids, such as arachidonic acid, to azoles, has been shown to increase azole susceptibility of *C*. *albicans* biofilms significantly. However, the underlying mechanism is not known. A resistance mechanism associated the increased antifungal resistance is the overexpression of ABC efflux pumps such as Cdr1p. Transcriptome analyses of biofilms exposed to sub-inhibitory concentrations of arachidonic acid alone, fluconazole alone or a combination of fluconazole and arachidonic acid indicated a significant increase in *CDR1* expression during early biofilm formation in the presence of arachidonic acid. The increased expression of *CDR1* was confirmed with intense fluorescence of *CDR1-GFP* tagged cells after arachidonic acid treatment. However, efflux activity in this protein was severely diminished in the presence of arachidonic acid, which indicates the loss of function of the membrane-associated protein, possibly due to membrane disruption. Also, the presence of arachidonic acid may be interfering with the synthesis of ATP required for Cdr1p efflux. Another mechanism employed in resistance to azoles is the overexpression of *ERG11* or the increase of ergosterol content. High expression of *ERG11* was observed in biofilms treated with fluconazole. However, the observed upregulation of *ERG11* was reversed in the presence of arachidonic acid combined with fluconazole and arachidonic acid alone. Furthermore, oxidative stress, induced by the presence of fluconazole and arachidonic acid, was identified as a possible mechanism for the increased azole susceptibility. The investigation demonstrates that multiple mechanisms are involved in the increased susceptibility and also that the differential expression of genes does not always correspond to differences in functionality.

4.1. Introduction

The yeast *C*. *albicans* is the primary cause of the life-threatening systemic candidiasis in immunocompromised individuals, transplant recipients and patients undergoing chemotherapy (Shareck and Belhumeur, 2011; Costa et al., 2015). An essential virulence factor of *C*. *albicans* is the formation of biofilms on abiotic and biotic surfaces (Mayer et al., 2013; Sardi et al., 2013). *Candida albicans* biofilms are notorious for their high antifungal resistance, and they also serve as a potential source of systemic infection (Gulati and Nobile, 2016; Tsui et al., 2016). Although several antifungal classes have been identified as treatment options for *C*. *albicans* infections, the azoles, particularly fluconazole, is the most administered, even amongst HIV patients, due to their excellent bioavailability, low cost and reduced toxicity (Denning and Hope, 2010; Patel et al., 2012; Lockhart and Berkow, 2017). Fluconazole acts by inhibiting the lanosterol 14-α demethylase (Erg11p) which catalyses the oxidative C14-demethylation of lanosterol during the synthesis of fungal-specific ergosterol (Shapiro et al., 2011; Tobudic et al., 2012). However, high resistance, especially in the treatment of biofilm-associated infections, hampers its efficiency (Taff et al., 2013; Fothergill et al., 2014; Whaley et al., 2017).

Several resistance mechanisms, including the modification of drug target, increasing expression of *ERG11*, overexpressing of drug efflux pumps and alteration of sterol synthesis is employed by *C*. *albicans* against fluconazole (Berkow and Lockhart, 2017; Whaley et al., 2017). The modification of drug target as a resistance mechanism is a common phenomenon, which involves the introduction of a point mutation causing amino acid substitution. Within Erg11p, the fluconazole target, more than 140 substitutions have been identified (Morio et al., 2010). The consequence of these substitutions includes the alteration of the protein structure and a reduction in fluconazole binding efficiency (Sanglard et al., 1998; Warrilow et al., 2010). Conversely, overexpression of *ERG11* is another resistance mechanism, initiated by a mutation in the *UPC2*, which encodes a zinc cluster transcriptional regulator, that controls upregulation of ergosterol synthesis genes (Macpherson et al., 2005; Vasicek et al., 2014). Within the Upc2p sequence, the following substitutions G648D, G648S, A643T, A643V, Y642F, W478C, and A646V have been

reported to induce an increase in *ERG11* expression and enhance fluconazole resistance in *C*. *albicans* (Heilmann et al., 2010; Flowers et al., 2012).

Alternatively, *C*. *albicans* can overexpress drug efflux pumps encoding genes, *CDR1* and *CDR2*, regulated by Tac1p, as well as *MDR1*, regulated by Mrr1p, as a resistance mechanism against fluconazole (Coste et al., 2004; Morschhäuser et al., 2007; Nobile and Johnson, 2015; Whaley et al., 2017). Cdr1p is highly promiscuous and effluxes several compounds, inducing fluconazole, across the membrane. The constitutive upregulation of *CDR1* and other efflux pump genes can facilitate fluconazole resistance (Cannon et al., 2009; Tsui et al., 2016; Berkow and Lockhart, 2017).

The alteration of sterol synthesis is an infrequent resistance mechanism initiated because of a loss of function mutation of *ERG3*. *ERG3* encodes a $\Delta^{5,6}$-sterol desaturase, and its inactivation in the presence of fluconazole permits a by-pass to the production of harmful sterol intermediate (Martel et al., 2010; Berkow and Lockhart, 2017; Whaley et al., 2017).

Besides the inhibition of enzymes involved in ergosterol synthesis, fluconazole also induces oxidative stress in *C. albicans* by the generation of reactive oxygen species (ROS) (Kobayashi et al., 2002; Wang et al., 2009; Arana et al., 2010). The generation and accumulation of ROS in yeast cells induce damage of proteins, lipids (including lipid peroxidation) as well as nucleic acids and stimulates apoptosis (Farrugia and Balzan, 2012). To counter the harmful effect of ROS accumulation, *C. albicans* induces the expression of antioxidant encoding genes such as *CAT1*, *TRX1*, *GRX2*, *SOD1*, *SOD5* (Dantas et al., 2015).

The earlier investigation has shown the increase in *C*. *albicans* biofilms fluconazole susceptibility, due to the presence of arachidonic acid (AA), and the influence of the increased susceptibility on *C*. *albicans* biofilms transcriptome was established. However, *C*. *albicans* biofilms have several resistance mechanisms against fluconazole treatment. Therefore, in this chapter, the influence of AA on different fluconazole resistance mechanisms employed by *C*. *albicans* biofilms was investigated.

4.2. Materials and Methods

4.2.1. Yeast strains used

Candida albicans CBS 8758 (SC5314), *Candida albicans* ASCa1 and *Candida albicans cdr1*Δ/Δ used for the experiments, were revived from glycerol stocks on Yeast Malt extract (YM) agar (10 $g.L^{-1}$ glucose, 5 $g.L^{-1}$ peptone, 3 $g.L^{-1}$ yeast extract, 3 $g.L^{-1}$ malt extract and 16 $g.L^{-1}$ agar bacteriological) at 30ºC for 24 h.

4.2.2. *CDR1* tagged *GFP* fluorescence

Candida albicans ASCa1, modified with the integration of a *CDR1-GFP-UR3* cassette into the *CDR1* chromosomal locus (Szczepaniak et al., 2015) was received as a gift from Professor Anna Krasowska. The cells were revived and grown in biofilm-forming conditions, as described in Chapter 2, sections 2.2.3. The cells were treated with either **A**A (dissolved in EtOH) and **D**MSO (DA), **f**luconazole (dissolved in DMSO) and **E**tOH (FE), **f**luconazole (dissolved in DMSO) and **A**A (dissolved in EtOH) (FA), and **D**MSO and **E**tOH (DE) and incubated for 6 h at 37ºC as described in Chapter 3, sections 3.2.3. Subsequently, the fluorescence of the GFP in the cells was observed with an inverted microscope (Olympus CKX53) combined with a 130 W high-pressure mercury lamp (Olympus HGLGPS). The cells were exposed to the same excitation intensity with identical exposure time to standardise the fluorescence. Also, the samples were observed in the dark at the same session to prevent photobleaching.

4.2.3. Rhodamine 6G efflux

Rhodamine 6G (Rh6G) uptake and glucose-induced efflux in *C. albicans* biofilms were conducted as previously described, with slight modifications (Maesaki et al., 1999; Ells et al., 2013; Szczepaniak et al., 2017). Briefly, the biofilms were grown in black flat-bottomed 96-well microtiter plates as described in Chapter 2, section 2.2.3 for 6 h at 37ºC. Afterwards, the media was removed, and the biofilms were washed twice with sterile phosphate-buffered saline (PBS). The biofilms were incubated in sterile PBS to de-energise for 1 h at 37ºC. The cells were incubated in 10 µM Rh6G dissolved in dimethyl sulfoxide (DMSO) at 37ºC for 1 h. Fluorescence measurement was taken at an excitation of 530 nm and an emission of 590 nm, every 10 min for 60 min to evaluate the uptake of

Rh6G. After Rh6G uptake measurements, the biofilms were washed with sterile PBS and treated with either DA, FE, FA and DE. Rh6G efflux from the cells was induced with the addition of 2 mM glucose dissolved in PBS. Fluorescence measurement was repeated to evaluate the efflux of Rh6G.

4.2.4. Influence of treatment condition on *Candida albicans cdr1*Δ/Δ

*Candida albicans cdr1*Δ/Δ homozygous mutant and the reintegrated add-back used for this investigation was previously engineered with the CRISPR-Cas9 homologous recombination system in our group. The strain was revived and grown in biofilm-forming conditions as described in Chapter 2, section 2.2.3 and treated with either DA, FE, FA and DE. The biofilms were incubated for 48 h at 37ºC, while the metabolic activity was measured with XTT assay.

4.2.5. Ergosterol quantification

Ergosterol was extracted from *C*. *albicans* biofilms as described, with slight modifications (Arthington-Skaggs et al., 1999; Khan et al., 2013; Zorić et al., 2017). Briefly, the cells were grown in biofilm-forming condition and treated with either DA, FE, FA or DE for 6 h at 37ºC. The biofilms were harvested by centrifugation at 2700 *g* for 5 min and washed with sterile distilled water. The biofilms were placed in a borosilicate tube while 3 mL of freshly prepared potassium hydroxide (25% m/v), dissolved with a solution of methanol, ethanol and water (700:315:15), was added. The solution was heated at 85ºC for 1 h in a water bath and allowed to cool to room temperature. Ergosterol was extracted with the addition of 3 ml of n-heptane, containing 10 µg ml^{-1} of ergosterol internal standard and 1 ml of water. The solution was mixed with a vortex and allowed to separate without disruption for 30 min. The n-heptane layer containing the ergosterol was collected into a new borosilicate tube and stored in -20ºC. Derivatisation was done by evaporating 1 ml of ergosterol solubilised with n-heptane to dryness under a stream of nitrogen gas. The pellet was derivatised with the addition of 50 µl of N,O-bis(trimethylsilyl)acetamide (BSTFA) containing 2% trimethylchlorosilane (TMCS) and incubated for 18 h at 80ºC. After incubation, the solution was evaporated to dryness under a stream of nitrogen gas and resuspended in 100 µl of N-decane before injecting into the GC/MS.

The GC/MS analysis was carried out on a Thermo Electron Trace 1310 Gas chromatograph connected to an ISQ 7000 single quadrupole mass spectrometer. The separation was effected with Agilent FactorFour VF-5 column with dimensions: length 60 m, inner diameter 0.32 mm and film thickness 0.25 µm. Helium was used as carrier gas at a flow rate of 5 ml min^{-1}. Injection port temperature was 175°C with a split ratio of 33:1. The initial oven temperature was 175ºC held for 1 minute, raised at 7ºC min^{-1} to 300ºC held for 10 min. Electron impact ionization was employed. MS source temperature was 250 ºC. Mass analysis was carried out in specific ion monitoring mode. Data was captured and analysed with Thermo Xcalibur 4.0 software employing the NIST 17 spectral library.

4.2.6. Determination of the role of oxidative stress

Candida albicans heterozygous mutants corresponding to antioxidant encoding genes, downregulated in the presence of FA, were selected from a double barcoded library of heterozygous *C. albicans* mutants (version 2013A) supplied by Merck Sharp & Dohme Corp. The mutants, together with wild type strain, *C. albicans* CA14, used in the construction of the heterozygous mutants, as well as *C. albicans* SC5314, were grown in biofilm-forming conditions as described in Chapter 2, section 2.2.3. The adhered cells were treated with fluconazole to a final concentration of 1 $mg.L^{-1}$ and incubated at 37ºC for 48 h. After that, metabolic activity was measured with XTT assay. A concentration of 0.045 mM butylated hydroxytoluene (BHT) was added to the biofilms of a selected mutant (*pst1Δ/PST1*) treated with fluconazole, to confirm the role of oxidative stress.

4.2.7 Statistical analyses

All experiments were conducted in biological triplicates, each with at least three technical replicates, and the averages and standard deviations calculated. The t-test was performed in order to assess the significance of differences. *P* values of at least 0.05 were considered to be significant.

4.3. Results and Discussion

4.3.1. *CDR1* expression in *Candida albicans* biofilms

Generally, *C. albicans* overexpress the *CDR1* efflux pump as a resistance mechanism against the treatment of fluconazole (Nobile and Johnson, 2015; Whaley et al., 2017). In *C. albicans* biofilms, the expression of *CDR1* begins from the early stage of biofilm formation, and the expression is maintained throughout the process of biofilm development (Mukherjee et al., 2003; Mateus et al., 2004; Lepak et al., 2006; Nett et al., 2009). However, it is reported to confer resistance during the early stages of biofilm formation (Ramage, 2002; Mukherjee et al., 2003; Ramage et al., 2012). Our investigation on *C. albicans* biofilms, at 6 h after treatment with AA alone or combined with fluconazole (DA and FA) was twice the level obtained in the presence of fluconazole only (FE). Counting the expression levels with the NanoString nCounter analysis system (described in Chapter 3), which digitally quantifies mRNA transcripts (Geiss et al., 2008; Eastel et al., 2019), gave similar results to what was obtained using RNA-seq (Table 4.1). A correlation between the two different approaches confirms the increased expression of *CDR1* both in the presence of AA and fluconazole during the early stage of biofilm formation. Subsequently, all the treatment conditions (DE, DA, FA and FE) were able to induce fluorescence, which indicates expression of Cdr1p in the *C. albicans CDR1-GFP* tagged strain (Figure 4.1). However, with the treatment conditions of AA alone, or combined with fluconazole (DA and FA), there was a higher fluorescence intensity, and it could be assumed to be due to the increased expression of *CDR1* in the presence of AA.

Table 4.1. *CDR1* expression levels under different test conditions. DA (**D**MSO and **A**rachidonic Acid), FA (**F**luconazole and **A**rachidonic acid), FE (**F**luconazole and **E**tOH).

	Log$_2$								
	Cuffdiff			**DESeq2**			**NanoString**		
	DA	**FA**	**FE**	**DA**	**FA**	**FE**	**DA**	**FA**	**FE**
CDR1	2.77	2.70	1.56	2.66	2.68	1.62	2.02	1.75	1.32

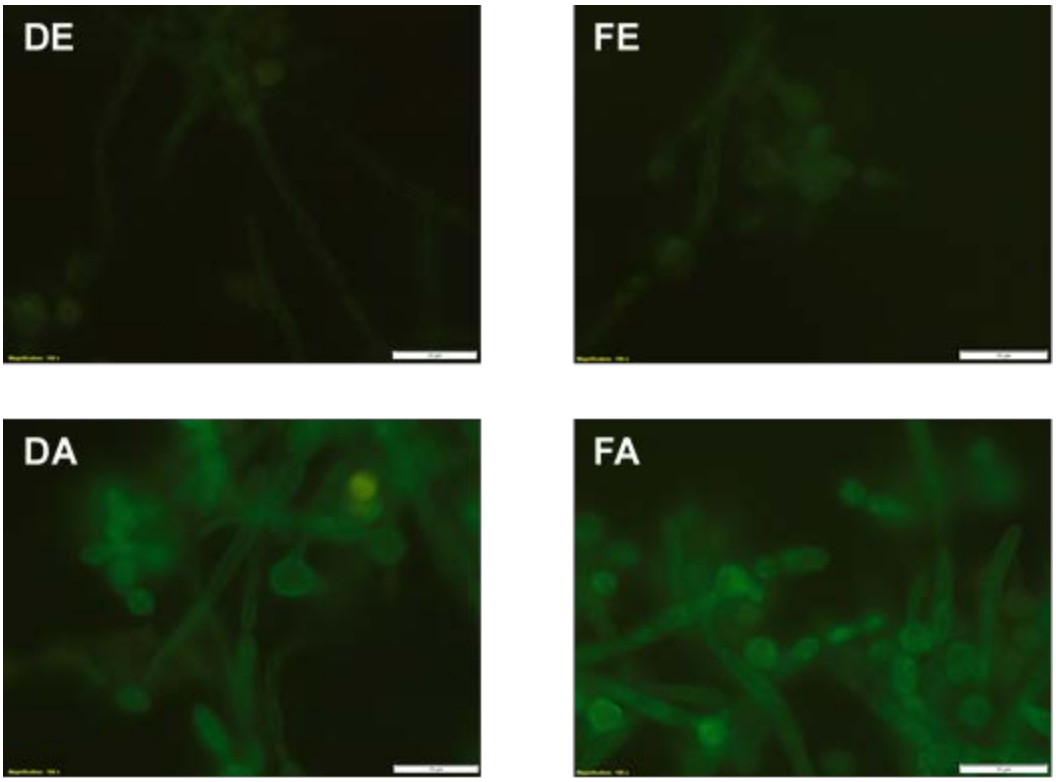

Figure 4.1. Fluorescence in *C. albicans* biofilms Cdr1p after different treatment conditions. DE (**D**MSO and **E**tOH), DA (**D**MSO and **A**rachidonic acid), FA (**F**luconazole and **A**rachidonic acid), FE (**F**luconazole and **E**tOH).

4.3.2. Rhodamine 6G efflux on biofilms

The Cdr1p ATP-binding cassette membrane transporter can transport multiple substrates, including xenobiotics, lipids, fluorescent dyes and drugs (Prasad et al., 2015). The fluorescent dye Rh6G is a known substrate of Cdr1p (Maesaki et al., 1999; Nakamura et al., 2001; Mukherjee et al., 2003). Glucose-induced Cdr1p efflux of Rh6G was investigated in biofilms of all the tested conditions (DE, DA, FA and FE) and untreated cells. During early biofilm formation, Rh6G efflux was observed in biofilms treated with fluconazole alone (FE) and solvent controls (DE). This efflux was identical to the untreated control biofilm (Figure 4.2). However, in the presence of AA alone or combined with fluconazole (DA and FA), efflux of Rh6G was severely diminished, even in the presence of fluconazole, indicating a possible inhibition of Cdr1p efflux activity by AA. The inhibition of Cdr1p efflux may be due to a disruption in membrane fluidity or lipid raft organisation because AA can intercalate between the phospholipids of yeast cells to alter

membrane fluidity and saturation (Kock and Ratledge, 1993; Ells et al., 2009; Mishra et al., 2014). A comparable observation was reported on conjugated linoleic acid (CLA) disrupting the membrane localisation of the membrane-bound Ras1p (Shareck et al., 2011). Increase in *C. albicans* membrane fluidity could lead to an incorrect localisation of Cdr1p, which could result in the absence of Cdr1p efflux activity (Krishnamurthy and Prasad, 1999; Kumar et al., 2015). Also, Cdr1p as an ABC protein requires ATP for its activity. As noted in the previous chapter, the presence of AA appears to interfere with ATP synthesis process, and this may be another reason for the absence of Cdr1p efflux in the presence of AA.

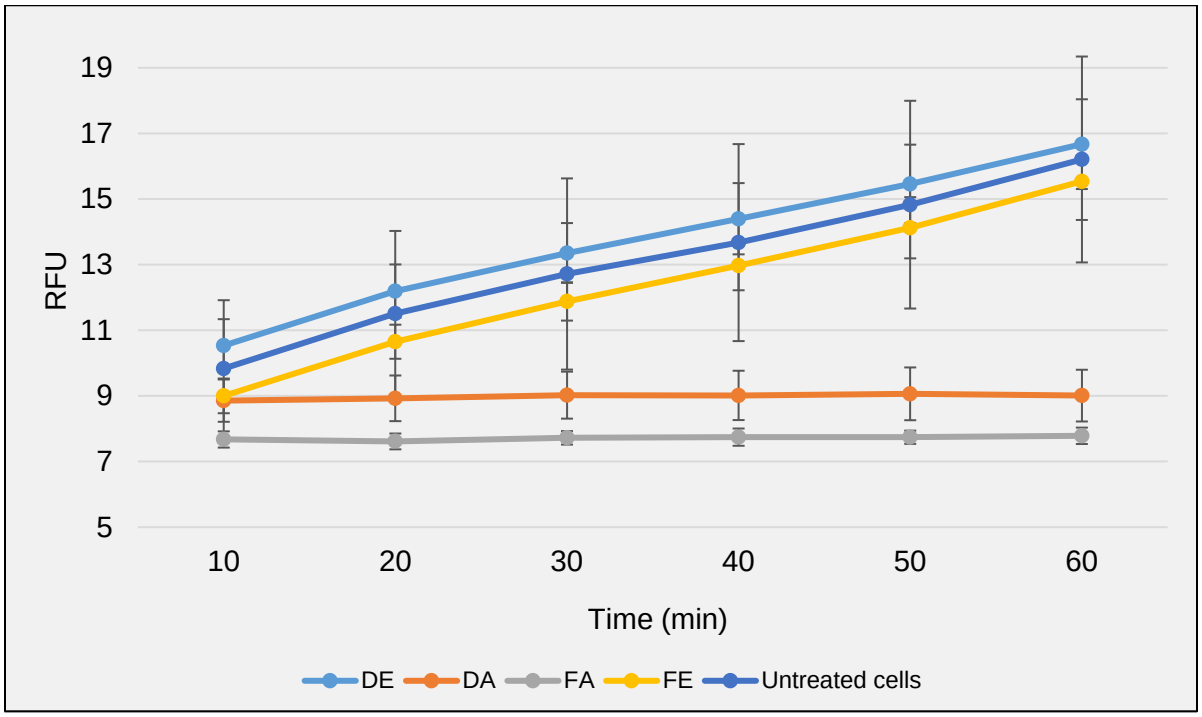

Figure 4.2. Glucose-induced R6G efflux in *C. albicans* biofilms after treatment with the different conditions. DE (**D**MSO and **E**tOH), DA (**D**MSO and **A**rachidonic acid), FA (**F**luconazole and **A**rachidonic acid), FE (**F**luconazole and **E**tOH). Values are the mean of three independent experiments, and the standard deviation is indicated by the error bars.

4.3.3. Effect of treatment conditions on *Candida albicans cdr1Δ/Δ*

The deletion of *CDR1* (*cdr1Δ/Δ*) significantly reduced metabolic activity in *C. albicans* biofilm treated with fluconazole combined with AA (FA) and fluconazole alone (FE)

(Figure 4.3). The reduction in the metabolic activity of *C. albicans* biofilm *cdr1*Δ/Δ in the presence of fluconazole is primarily due to the lack of *CDR1* as a resistance mechanism. Conversely, there was no significant difference in metabolic activity with the treatment of *C. albicans* biofilm *cdr1*Δ/Δ with AA only (DA) compared with the treatment with solvent only (DE) (*P* = 0.19). The observed reduction in metabolic activity after treatment with fluconazole combined with AA (FA) and fluconazole alone (FE) was reversed in the *CDR1* complemented strain.

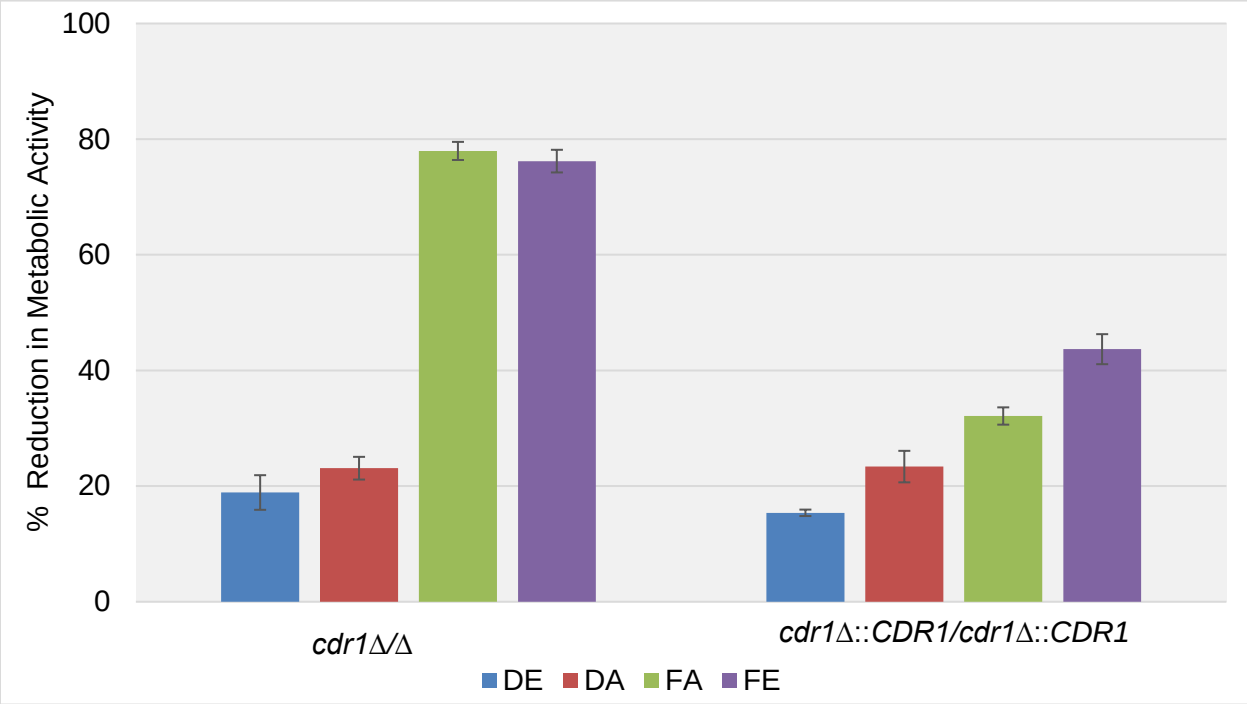

Figure 4.3. Effect of the different treatment conditions on *C. albicans* biofilm *cdr1*Δ/Δ metabolic activity. DE (**D**MSO and **E**tOH), DA (**D**MSO and **A**rachidonic acid), FA (**F**luconazole and **A**rachidonic acid), FE (**F**luconazole and **E**tOH). Values are the mean of three independent experiments, and the standard deviation is indicated by the error bars.

4.3.4. Effect of treatments on *Candida albicans* ergosterol content

In the presence of azoles, *C. albicans* biofilm overexpresses *ERG11* as a resistance mechanism (Mathé and Van Dijck, 2013; Lockhart and Berkow, 2017; Whaley et al., 2017). Ergosterol is an essential constituent of the yeast membrane lipids, which modulates fluidity, permeability, and integrity of the membrane (Lv et al., 2016). The

overexpression of *ERG11* increases the copy number of Erg11p to enhance ergosterol synthesis (Flowers et al., 2012; Alizadeh et al., 2017). The expression of *ERG11* in *C. albicans* biofilms treated with fluconazole alone (FE) increased slightly (below a Log_2 fold change of 2), and this level was obtained from both RNASeq and NanoString (Table 4.2). Interestingly, expression of *ERG11* was either downregulated or not differentially regulated in the presence of AA alone or in combination with fluconazole (DA and FA). This suggests that the presence of AA could alter increased *ERG11* expression, to override its effect as a resistance mechanism during the early stage of biofilm formation.

Table 4.2. Expression levels of *ERG11* under the different test conditions. DA (**D**MSO and **A**rachidonic acid), FA (**F**luconazole and **A**rachidonic acid), FE (**F**luconazole and **E**tOH).

	Log_2								
	Cuffdiff			**DESeq2**			**NanoString**		
	DA	**FA**	**FE**	**DA**	**FA**	**FE**	**DA**	**FA**	**FE**
ERG11	-1.14	0.39	1.29	-1.10	0.41	1.28	-2.19	-0.72	1.12

Ells and co-workers (2009) reported the increase in ergosterol content of *C. albicans* biofilms treated with AA. Similarly, the quantification of ergosterol in *C. albicans* biofilms treated with AA (DA) was higher than that in the solvent control biofilms (DE). However, as expected, with the treatment of the biofilms with fluconazole alone (FE) and even combined with AA (FA), there was a significant reduction in ergosterol. Reduced ergosterol content is a resistance mechanism of *C. albicans* biofilms at the intermediate and matured stages (Mukherjee et al., 2003; Ramage et al., 2012; Chandra and Mukherjee, 2015). The reduction in ergosterol content of biofilms treated with FE and FA may be a resistance mechanism response by *C. albicans* biofilms and may not necessarily be associated with the increased susceptibility observed with FA treatment.

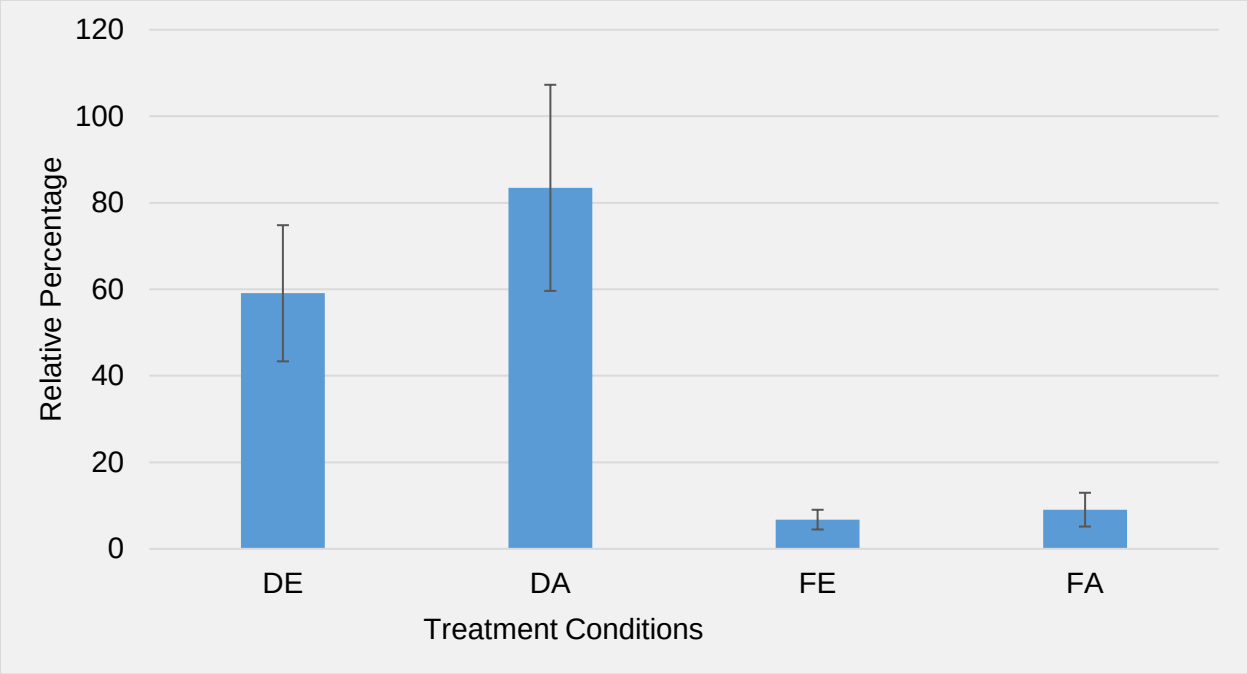

Figure 4.4. Effect of the different treatment conditions on the ergosterol levels of *C. albicans* biofilm. DE (**D**MSO and **E**tOH), DA (**D**MSO and **A**rachidonic Acid), FA (**F**luconazole and **A**rachidonic acid), FE (**F**luconazole and **E**tOH). Values are the mean of three independent experiments, and the standard deviation is indicated by the error bars.

4.3.5. The role of oxidative stress

One of the mechanisms employed by the host to protect itself from *C. albicans* invasion, is the generation of oxidative compounds such as H_2O_2 to induce reactive oxygen species (ROS) (Nicola et al., 2008; Brown, 2011). In addition, fluconazole has been shown to induce ROS in *C. albicans* (Arana et al., 2010). However, in response to the ROS attacks, *C. albicans* employs the expression of oxidative stress and antioxidant systems (Abegg et al., 2011; Komalapriya et al., 2015). In the RNA-Seq analysis of *C. albicans* biofilms treated with the combination of fluconazole and AA (FA), several oxidative stress encoding genes were downregulated, as shown in Table 4.3.

Table 4.3. Oxidative stress genes downregulated in the presence of FA (**F**luconazole and **A**rachidonic acid)

Genes	Description	Log_2	
		Cuffdiff	**DESeq**
GPX2	Glutathione peroxidases	-1.11	-2.63
PST1	Flavodoxin-like proteins	-1.67	-2.44
PRX1	Thioredoxin peroxidase	-0.89	-1.12
TPS1	Trehalose-6-phosphate synthase	-1.04	-1.07
ZWF1	Glucose-6-phosphate dehydrogenase	-0.96	-1.07
SOH1	RNA polymerase II mediator complex	-0.62	-1.01
AFT2	Transcription factor	-1.11	-0.97
SOD5	Superoxide dismutase	-0.93	-0.95
GPX1	Glutathione peroxidases	-1.11	-0.69
SSU81	MAP kinase activating adaptor protein	-0.65	-0.49
SOD4	Superoxide dismutase	-0.75	1.30

GPX1 and *GPX2* encode for glutathione peroxidases which detoxify H_2O_2 while expending glutathione as an electron donor. They are required by *C. albicans* to manage its oxidative stress by detoxifying peroxides (Miramón et al., 2014). The *C. albicans PST1* encodes for a member of flavodoxin-like proteins, which are induced by oxidative stress (Bruno et al., 2010) and contain sites for the transcription factor Cap1, which is induced by oxidative stress (Wang et al., 2006; Znaidi et al., 2009). *PRX1* encodes a thioredoxin peroxidase, localised in the cytoplasm and has a conserved Cys69, which is required for its peroxidase activity. Although *prx1*Δ/Δ was reported to show a weak response to H_2O_2 *in vitro*, it is a peroxidase for organic peroxides inside the cell (Srinivasa et al., 2012). *Candida albicans TPS1* encodes a trehalose-6-phosphate synthase, which is essential for the protection of the yeast against severe oxidative treatment. Its importance was observed with the rapid decrease of viable *C. albicans tps1*Δ/Δ after exposure to H_2O_2 (Alvarez-Peral et al., 2002; Zaragoza et al., 2003).

Candida albicans ZWF1 encodes glucose-6-phosphate dehydrogenase, an enzyme within the pentose phosphate pathway. Upon oxidative stress exposure, *ZWF1* is upregulated in a Cap1-dependent manner to regenerate NADPH as a reductant at the cost of glycolysis (Missall et al., 2004; Wang et al., 2006; Komalapriya et al., 2015). The *C. albicans SOH1* (*MED31*) encodes an RNA polymerase II mediator complex subunit associated with the regulation of genes involved in cell separation, adhesion,

biofilm formation and virulence (Uwamahoro et al., 2012). The mediator complex acts to bridge to transcriptional mediators and promoters RNA polymerase II transcription mechanism (Carlsten et al., 2013). *Candida albicans* mediator complex share similar roles with the *Saccharomyces cerevisiae* mediator complex which upon oxidative stress form amyloid-like protein aggregates (Zhu et al., 2015).

The *C. albicans AFT2* is a transcriptional regulator which has been identified to be involved in ferric reductase activity and virulence (Liang et al., 2010). However, it is also required for oxidative stress response in *C. albicans* as *aft2*Δ/Δ were severely defective in growth after exposure to high H_2O_2 exposure (Xu et al., 2013). The *C. albicans SSU81* (Sho1p) is an adaptor protein and upstream sensor of the Hog1 MAPK pathway. They have a distinct role in oxidative stress, and their mutants are sensitive to oxidative stress (Román et al., 2005; Biswas et al., 2007). *Candida albicans SOD4* and *SOD5* are cytoplasmic Cu, Zn-containing superoxide dismutases that converts superoxide radicals into H_2O_2 (Martchenko et al., 2004).

In this study, heterozygous mutants containing one copy of the selected gene were tested with 1 µg mL^{-1} fluconazole. All the genes displayed a significant reduction in metabolic activity (Figure 4.5). This suggests that these genes do indeed play a role in fluconazole resistance in *C. albicans* biofilms.

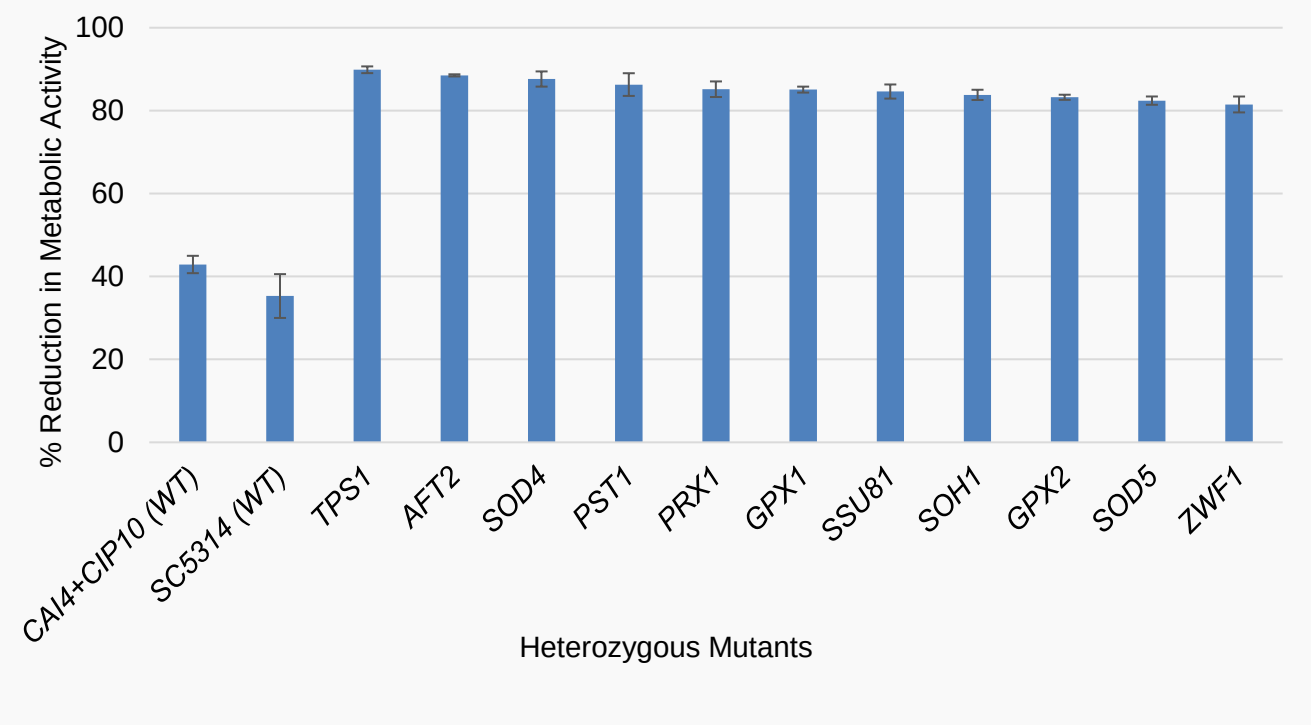

Figure 4.5. Effect of fluconazole on *C. albicans* biofilm oxidative stress encoding heterozygous mutants. Values are the mean of three independent experiments, and the standard deviation is indicated by the error bars.

In order to confirm this, the heterozygous *pst1Δ/PST1* mutant was selected as a representative to test for the reversal of oxidative stress in the presence of 0.045 mM butylated hydroxytoluene (BHT) a powerful antioxidant. The addition of BHT increased the metabolic activity of *pst1Δ/PST1* (Figure 4.6). Importantly, the results indicate that these genes that protect *C. albicans* from fluconazole induced oxidative stress are downregulated by AA and that this may be a contributing mechanism to the observed increased fluconazole susceptibility.

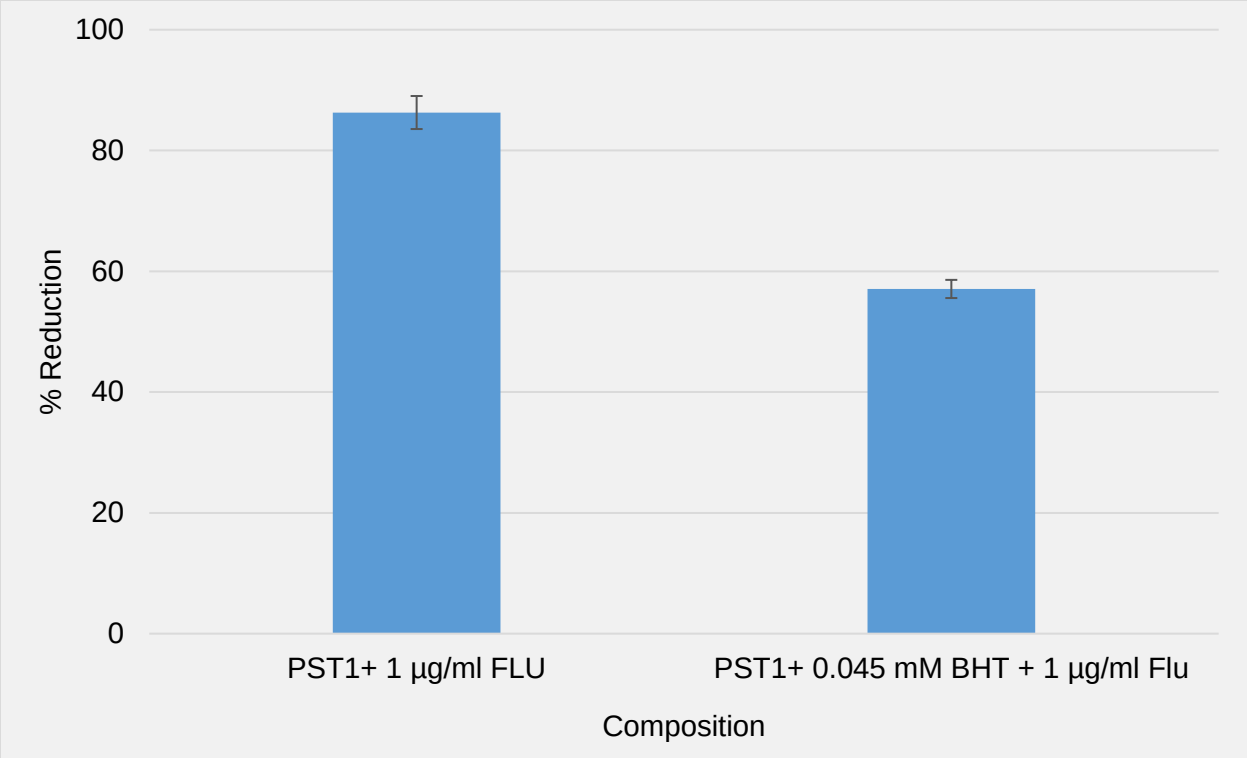

Figure 4.6. Effect of fluconazole on *PST1* heterozygous mutants and the reversals in the presence of butylated hydroxytoluene (BHT) Values are the mean of three independent experiments, and the standard deviation is indicated by the error bars.

4.4. Conclusions

Different resistance mechanism employed by *C. albicans* against the presence of fluconazole were investigated. In the presence of AA, the *CDR1* efflux pump was significantly upregulated while the *ERG11* expression was downregulated. Also, *CDR1* efflux was absent after AA treatment, although there was no significant difference in ergosterol content between FE and FA. The absence of *CDR1* efflux and downregulation of *ERG11* due to AA treatment are possible mechanisms involved in the observed increase in azole susceptibility. Also, the presence of AA may have contributed to increasing fluconazole susceptibility via oxidative stress as genes, involved in protecting *C. albicans* from fluconazole-induced oxidative stress are downregulated when AA is combined with fluconazole. These observations suggest that the presence of AA may be interfering with the resistance mechanism of *C. albicans* to increase its azole susceptibility.

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CHAPTER 5

Application of Transcriptome Data to Identify Existing Drugs for the Treatment of *Candida albicans* Biofilm

Abstract

The infections of *Candida albicans* biofilms are often associated with high morbidity and mortality. Although several antifungal drugs are currently available for the treatment of these infections, their effectiveness is diminishing rapidly due to high level of antifungal resistance. Therefore, new antifungals to combat the infections are required. However, the process of developing drugs *de novo* is expensive and time-consuming. Hence, alternatives such as the repurposing of existing drugs are currently being explored. In our investigation, transcriptome data was used to inform haploinsufficiency profiling assays of selected *C*. *albicans* mutants for potential drug targets. Possible inhibitors, which have been approved for use in humans, were identified in the BRaunschweig ENzyme Database (BRENDA) and tested at the reported maximum plasma concentration for inhibition of *C*. *albicans* biofilms. With the haploinsufficiency assay, several genes were identified to increase sensitivity to subinhibitory concentration of fluconazole. In the presence of the selected drugs cimetidine, vildagliptin, furosemide and warfarin, a minimal reduction was observed in *C*. *albicans* biofilms growth, when compared with the control. Contrary, the presence of nateglinide, roscovitine, bacitracin and gefitinib enhanced the growth of *C*. *albicans* biofilms. This investigation demonstrates the potential of this approach to identify possible drugs fit for repurposing and the associated effects of selected drugs on *C*. *albicans* biofilms.

5.1. Introduction

Candida albicans is the primary cause of most fungal infections. These infections range from superficial (oral thrush, vulvovaginal) to systemic (bloodstream infections) making it the fourth leading cause of nosocomial bloodstream infection (Brown et al., 2012; Lockhart, 2014; Suleyman and Alangaden, 2016; Tsui et al., 2016). The mortality associated with *C*. *albicans* is between 30 and 50%, with more than 400,000 cases

reported yearly (Brown et al., 2012). Infections of *C*. *albicans* are commonly treated with antifungals from the polyenes, azoles, and echinocandins classes (Denning and Hope, 2010; Ostrosky-Zeichner et al., 2010). However, the efficacy of these drugs is hampered by the highly resistant biofilms formed on host tissues and medically implanted devices. The incomplete elimination of *C*. *albicans* biofilms can make treatment even more challenging because they can serve as a source for recurrent infection (Ganguly and Mitchell, 2011; Sardi et al., 2013; Taff et al., 2013). The limited antifungal arsenal against biofilms and high resistance to the existing drugs necessitates the discovery of alternative treatment options (Sardi et al., 2011; Costa et al., 2015).

The process to develop a new drug is expensive, demanding and could take more than ten years. Therefore, repurposing the existing approved drugs has gained significant interest over the years as an alternative option, because of its reduced cost and high success rate and has been applied to identify alternative treatment options for bacterial and fungal infections (Katragkou et al., 2016; Miró-canturri et al., 2019). In the case of *C*. *albicans*, new inhibitors have also been identified using the drug repurposing approach. A list of 1581 FDA approved drugs from the Johns Hopkins Clinical Compound Library (JHCCL) version 1.0 was investigated by Kim and coworkers (2015) for inhibitors of *C*. *albicans*. The screen successfully identified mycophenolic acid, disulfiram, fluvastatin and octodrine, which have been used previously in treatment of non-infectious disease, as inhibitors for *C*. *albicans*. Siles and co-workers (2013) also identified 38 compounds from a list of 1200 off-patent drugs from the Prestwick Library with inhibitory properties against *C*. *albicans* biofilm.

Aside from drug screening to repurpose, yeast chemical genomic assays such as haploinsufficiency profiling (HIP) can also be used similarly to identify new drugs. Haploinsufficiency profiling involves growing heterozygous mutants of a diploid strain in the presence of the test compound. The observation with the HIP assay is that the mutant will be sensitive to the drug that targets the product of the locus. A drug target will be identified based on the sensitivity of a locus to a specific drug (Giaever et al., 1999; Smith et al., 2010). Haploinsufficiency profiling assay has been applied to the identification of well-characterised and novel drug compounds (Giaever et al., 1999; Ericson et al., 2008;

Smith et al., 2010). Drug repurposing and haploinsufficiency profiling assay have excellent potentials in the discovery of new treatment options needed for the treatment of infections. In this chapter, haploinsufficiency profiling assay and drug repurposing were applied to prospect for new inhibitors against *C*. *albicans* biofilms.

5.2. Materials and Methods

5.2.1. Selection of target genes

Genes were selected from ontology terms in the investigation reported in Chapter 3, section 3.2.5. The genes were selected according to these expression criteria. (A) genes induced by fluconazole, but the induction was reversed in the presence of arachidonic acid (AA); (B) genes induced by fluconazole as well as by AA treatment; (C) genes induced by treatment with the combination of fluconazole and AA as indicated in Figure 5.1. The list of genes matching these criteria is listed in Table 5.1.

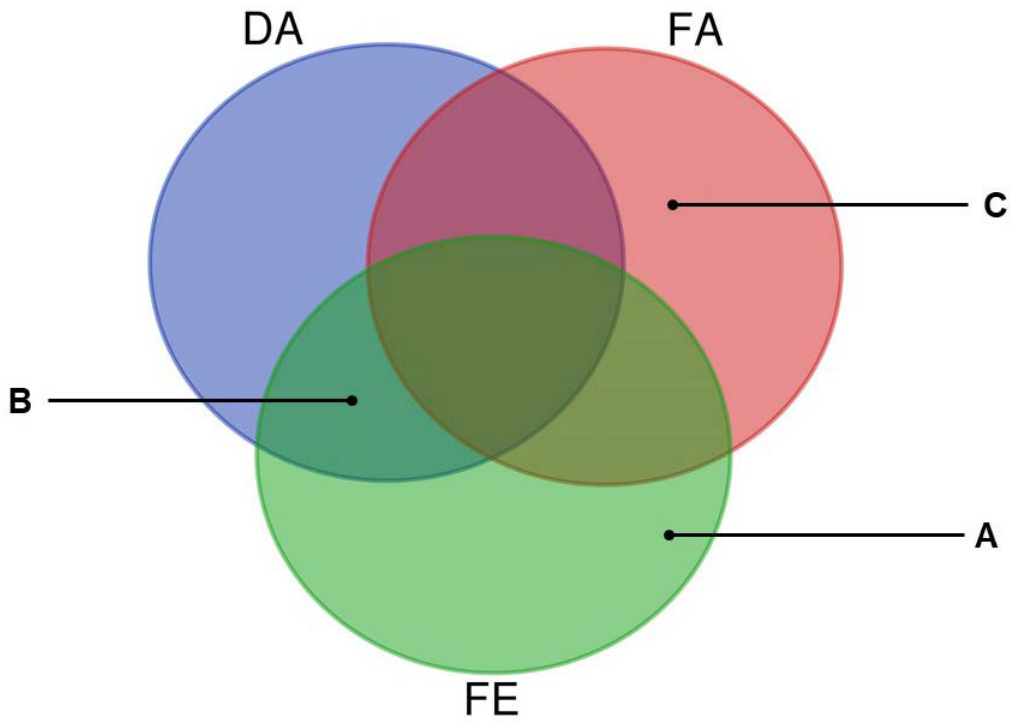

Figure 5.1. A Venn diagram representation of the association between the selected genes. DA (**D**MSO and **A**rachidonic Acid), FA (**F**luconazole and **A**rachidonic acid), FE (**F**luconazole and **E**tOH).

Table 5.1. List of genes identified from the stipulated conditions.

A		B	C				
ADH5	*MET10*	*BMT4*	*AAP1*	*EBP7*	*HOS3*	*PDB1*	*YHB1*
ARG1	*MET3*	*CSH1*	*ABC1*	*ENA21*	*HSL1*	*PEX6*	*YME1*
BGL2	*MET6*	*HSP21*	*ADE12*	*ERG2*	*IDH2*	*PHO84*	*YST1*
BIO2	*MNN15*	*KRE6*	*ADH3*	*ERG24*	*IDP1*	*RAD57*	*ZCF5*
CAT8	*PGA62*	*RNR22*	*ADK1*	*ERG251*	*ILV5*	*RCN1*	*ZCF6*
CYB5	*PRM1*	*RPN4*	*ADO1*	*FGR28*	*KRE9*	*RPS21*	*ZCF8*
DOT5	*RIM15*		*AGP2*	*FGR46*	*LCB4*	*SAP1*	*ZNC1*
ERG1	*SOD4*		*ALK8*	*FLC2*	*LEU1*	*SDS24*	
ERG11	*SOD5*		*ARA1*	*FMP45*	*LIP5*	*SGA1*	
ERG13	*SSO2*		*ATP1*	*FTH1*	*LKH1*	*SMC3*	
ERG9	*SVF1*		*ATP2*	*FUM12*	*LPD1*	*SSU1*	
GRE2	*UTR2*		*ATP3*	*GAP1*	*LYS12*	*STE13*	
HAP42	*ZCF39*		*ATP5*	*GAP6*	*LYS144*	*STE3*	
HSP70			*CBR1*	*GLN1*	*MIA40*	*STP4*	
IFR1			*CCH1*	*GNP1*	*MNN22*	*SWE1*	
KRE1			*CCN1*	*GRX3*	*NAP1*	*TAL1*	
KTR4			*CIT1*	*HGT18*	*NUP*	*THI4*	
LEU4			*CUP9*	*HGT20*	*PCK1*	*THR4*	
LYS4			*DAL8*	*HIP1*	*PCL1*	*TNA1*	
MCA1			*DAL9*	*HOM6*	*PCL5*	*YCF1*	

5.2.2. Drug sensitivity haploinsufficiency screen

Strains carrying a heterozygous deletion of the selected gene were selected from the double barcoded library of heterozygous *C. albicans* mutants (version 2013A) supplied by Merck Sharp & Dohme Corp. *Candida albicans* wildtype strains CA14, used in the construction of the heterozygous mutants, and SC5314 were included as controls. The mutants were revived and grown in biofilm-forming conditions as described in Chapter 2, sections 2.2.3 with fluconazole supplementation to a final concentration of 1 $mg.L^{-1}$. After 48 h incubation at 37^{o}C, metabolic activity was measured with XTT assay.

5.2.3. Identification of possible inhibitors

Protein sequences of the selected genes were obtained from the *Candida* Genome Database (CGD) (Skrzypek et al., 2017). Three-dimension structures of the proteins were

obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (Berman et al., 2000). The structure corresponding to the gene of interest with the highest identify and score was selected for each gene. The enzyme family of the structures was identified, and their relationship with that of the gene of interest was compared. The inhibitors for the different structure were identified from the BRaunschweig ENzyme Database (BRENDA) database (https://www.brenda-enzymes.org/) (Placzek et al., 2017; Jeske et al., 2019) using the respective Enzyme Commission (EC) number. The inhibitor list was screened, and only compounds approved for use in humans were selected for testing against *C. albicans* biofilms.

5.2.4. Susceptibility testing of inhibitors against *Candida albicans* biofilms

Candida albicans biofilms were tested for susceptibility to the selected drugs. The maximum serum concentration of the drug (C_{max}) was selected as the test concentration. The biofilms were grown as described earlier and supplemented with the respective test drug to the desired concentration while the equivalent volume of the drug solution was included as a control. Measurement of metabolic activity using XTT assay was used to determine the effect of the drug.

5.2.5 Statistical analyses

All experiments were conducted in biological triplicates, each with at least three technical replicates, and the averages and standard deviations calculated. The t-test was performed in order to assess the significance of differences. *P* values of at least 0.05 were considered to be significant.

5.3. Results and Discussion

5.3.1. Haploinsufficiency screening of genes as possible drug targets

The selection condition used for the genes resulted in 104 genes as potential drug targets. Haploinsufficiency screening with fluconazole was performed with only 99 of the mutants because five mutants could not be revived after several attempts. The screen produced 30 heterozygous mutants displaying a reduction in metabolic activity by at least 80% in the presence of fluconazole, and these were selected for further investigation (Figure 5.2).

The reduction in metabolic activity indicates that these genes may be involved in *C. albicans* biofilm fluconazole resistance (Giaever et al., 1999; Smith et al., 2010).

5.3.2. Inhibitors with human application

Protein structures corresponding to the selected 30 genes were searched within the RCSB protein database. Structures were obtained for 25 proteins, while there were no structures which corresponded to the amino acid sequences of *DAL8*, *DAL9*, *SVF1*, *PCL5* and *STE3*. The EC number for the enzyme family of the 25 proteins structures was used to identify the respective inhibitors in the BRENDA database. Since the target of interest is a pathogenic yeast of human importance, only inhibitors with known application in humans were selected from the obtained list. Eight such inhibitors were identified for four targets for repurposing against *C. albicans* (Table 5.2). An additional drug, the anticoagulant enoxaparin, was also included at 3.5 μg mL^{-1}.

Table 5.2. Selected approved drugs in humans, and the respective C_{max} concentration tested.

Target in *C. albicans*	Drug	C_{max} Concentration	Reference
ABC1	Gefitinib	0.35 μg mL^{-1}	(Swaisland et al., 2005)
ABC1	Roscovitine	1.8 μg mL^{-1}	(de la Motte and Gianella-Borradori, 2004)
GRE2	Warfarin	3.5 μg mL^{-1}	(Kumar et al., 2013)
GRE2	Furosemide	8.5 μg mL^{-1}	(Sica et al., 2018)
STE13	Bacitracin	2 μg mL^{-1}	(Pfizer, 2017)
STE13	Vildagliptin	0.525 μg mL^{-1}	(He, 2012)
STE13	Nateglinide	5.6 μg mL^{-1}	(Weaver et al., 2001)
ZCF5	Cimetidine	1.1 μg mL^{-1}	(Gugler et al., 1981)

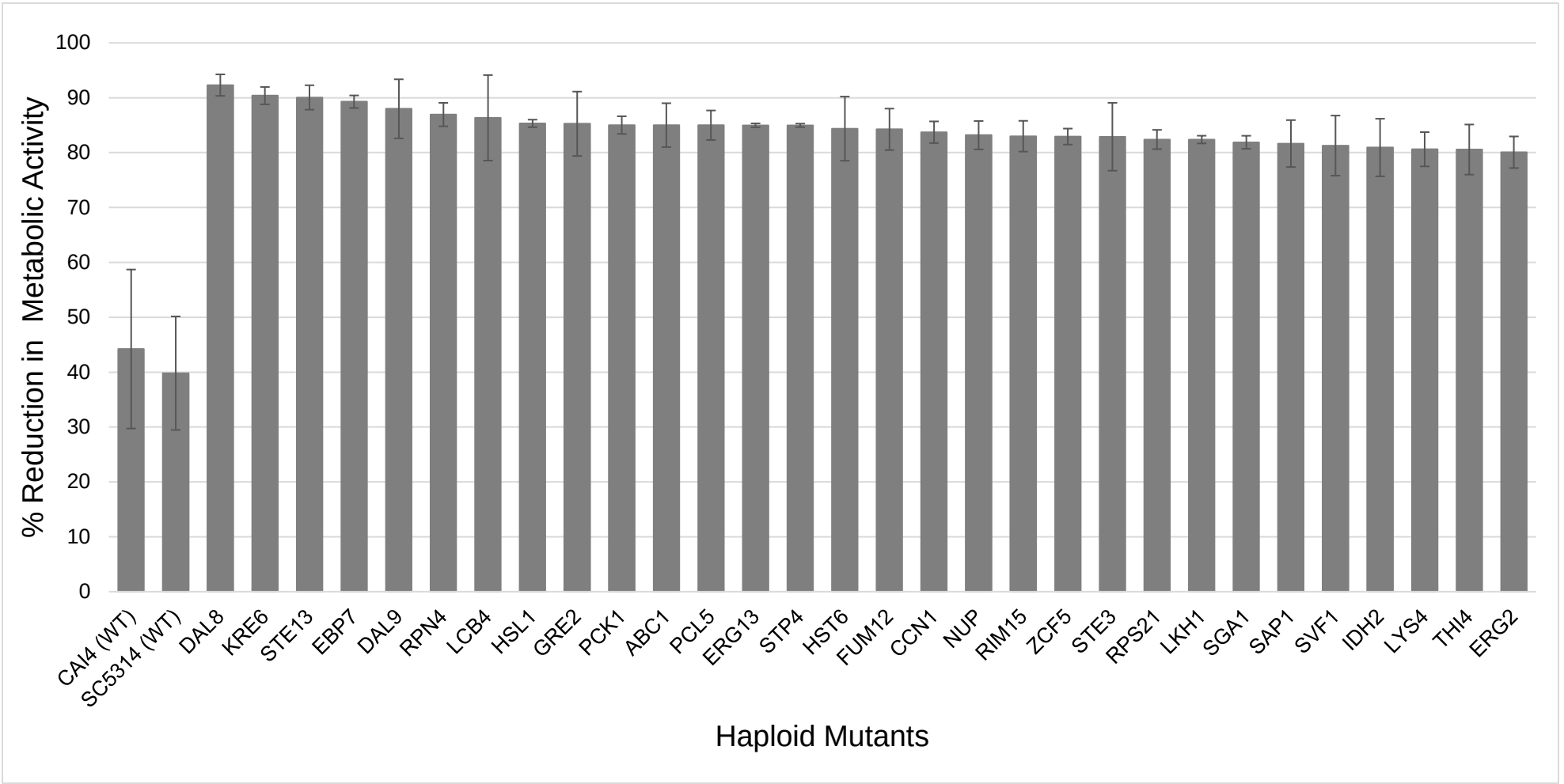

Figure 5.2. Haploinsufficiency screen showing the 30 heterozygous mutants with a greater than 80% reduction in metabolic activity. Values are a mean of three independent repetitions, and the standard deviations are indicated by the error bars.

5.3.3. Influence of inhibitors on *Candida albicans* biofilms

5.3.3.1. *ABC1* as a potential target

Gefitinib and roscovitine were identified as potential inhibitors for Abc1p, which is a putative ubiquinol-cytochrome-c reductase. Ubiquinol-cytochrome-c reductase, also known as cytochrome bc1 protein complex, transfers electron across the membrane for mitochondrial ATP production in the respiratory chain (Hunte et al., 2003). The closest structural homology to the *ABC1* protein sequence in the protein database was *ADCK3* which encodes a Human AarF Domain Containing Kinase 3 (Stefely et al., 2015). *ADCK3* is one of the five UbiB kinase genes which are atypical protein-like genes present in humans (Leonard et al., 1998; Stefely et al., 2015) and it is also a homologue of yeast *ABC1* gene (Bousquet et al., 1991; Mollet et al., 2008). Gefitinib and roscovitine both target kinases. Roscovitine, used as a purine analogue in the treatment of a broad range of cancers is a direct competitor with ATP for the active site on cyclin-dependent kinases (CDKs), including Cdk1p, Cdk2p, Cdk5p and Cdk7p which controls cell cycle progression to stimulate apoptosis in cancer cells (De Azevedo et al., 1997; Meijer et al., 1997; Cicenas et al., 2015). In addition, the CDK homologue in *C*. *albicans,* Cdc28p (Cdk1p) is also a regulator of cell cycle progression (Wang, 2016). Gefitinib is a selective inhibitor of the epidermal growth factor receptor (EGFR) tyrosine kinase, which is administered to patients with non-small cell lung cancer (Maemondo et al., 2010; Yamaguchi et al., 2015). Gefitinib has been shown to reduce fungal burden by reducing the phosphorylation of its target EGFR (Swidergall et al., 2018). Based on these, it was expected that roscovitine and gefitinib should inhibit the growth of *C*. *albicans* biofilm. However, the investigation of Butts and co-workers (2018) on the effect of oncology drugs on *Candida* species indicated that kinase inhibitors enhance the growth of *C*. *albicans* in a concentration-dependent manner, even in the presence of azoles, although the mechanism is not known. In the present study, growth of *C*. *albicans* biofilm increased in the presence of roscovitine and gefitinib (Figure 5.3). Therefore, roscovitine and gefitinib, which are kinase inhibitors may have a growth-stimulating effect on *C*. *albicans* biofilm via a similar as yet unknown mechanism.

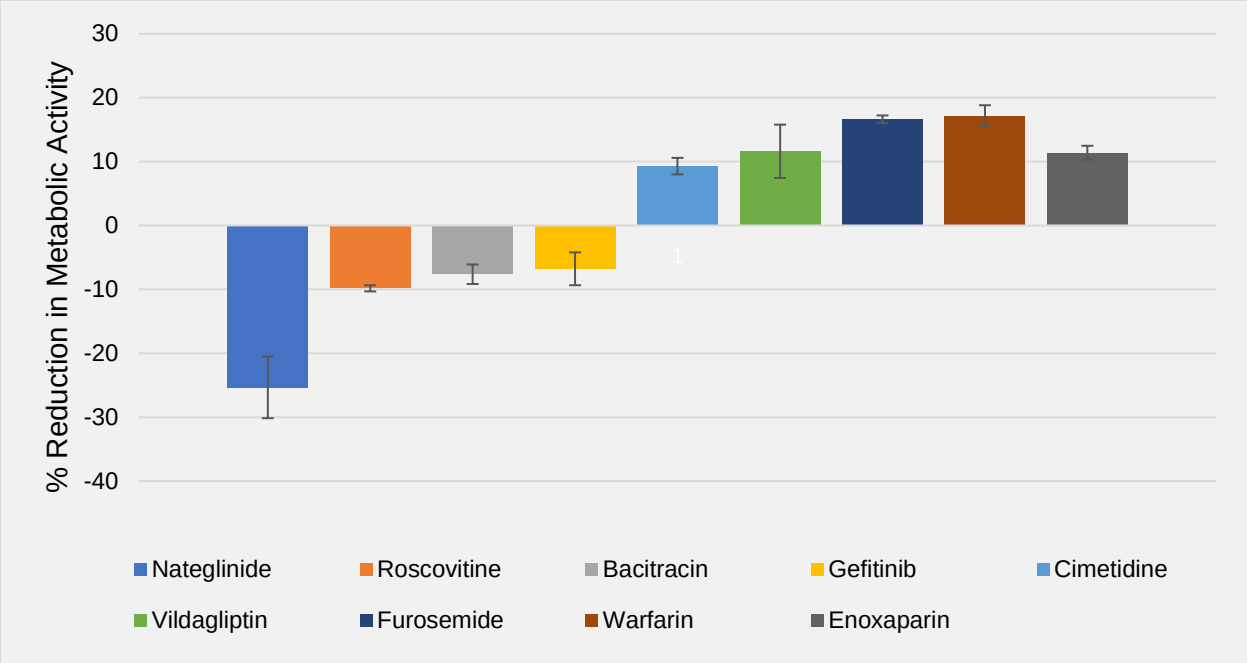

Figure 5.3. Percentage reduction in metabolic activity of *C. albicans* biofilm grown in the presence of the physiological concentration of the selected drugs. Values are the mean of three independent experiments, and the standard deviation is indicated by the error bars.

5.3.3.2. *STE13* as a potential target

Bacitracin, nateglinide and vildagliptin were identified as potential inhibitors for Ste13p, which is a dipeptidyl aminopeptidase, involved in the maturation of α-factor mating pheromone (Bautista-Muñoz et al., 2005). Heterozygous mutants of *STE13* exhibited reduced filamentation (Uhl et al., 2003). The closest structural homology to *STE13* amino acid sequence was a dipeptidyl peptidase IV from a pig's kidney (Engel et al., 2003) which is identical to *Saccharomyces cerevisiae* dipeptidyl aminopeptidase (Anna-arriola and Herskowitz, 1994). Bacitracin is a *Bacillus subtilis* derived cyclic polypeptide antibiotic, which targets C_{55}-isoprenyl pyrophosphate and is used for the treatment of superficial bacterial skin infections (Stone and Strominger, 1971; Williamson et al., 2017). It has also been reported to inhibit disulphide isomerase, an endoplasmic reticulum enzyme which helps in the folding of disulphide bonds (Hatahet and Ruddock, 2009) as well as mammalian aminopeptidases (Arrebola et al., 2019). Administration of antibiotics, such as bacitracin, has been reported to enhance the growth of *Candida* sp. during oral and vaginal candidiasis (Xu et al., 2008; Azevedo et al., 2015). According to Seelig (1966),

enhancement of *C*. *albicans* could be either via direct stimulation, removal of organisms competing for nutrient or removal of organisms secreting antifungal substances. However, given that bacitracin was tested *in vitro*, it may have a direct stimulatory effect on *C*. *albicans* biofilm, resulting in the increased growth. This is interesting, given that Pierce and co-workers (2014) reported on the inhibitory effect of bacitracin on *C. albicans* biofilms. The difference between the current data and this previous study may be explained by the lower concentration used in the current study (2 $\mu g\ l^{-1}$ vs 3.03 $\mu g\ l^{-1}$).

Nateglinide is a hypoglycemic, type 2 diabetes mellitus treatment drug derived from D-phenylalanine. It competitively binds to sulphonylurea receptors (SURs) of the pancreatic β-cells to inhibit K_{ATP} channels. These results in the depolarisation of the β-cells membrane, an influx of calcium and the stimulation of a quick and short insulin secretion response from β-cells (Hu et al., 2003; Tentolouris et al., 2007; Maideen and Balasubramaniam, 2018). Sulphonylurea receptors are atypical ABC transport proteins which mediate other processes rather than membrane transport (Burke et al., 2008). Although, considerably less potent than specific dipeptidyl peptidase 4 (DPP-4) inhibitors (such as vildagliptin), McKillop and co-workers (2009) showed that nateglinide could also inhibit this protein. Nateglinide also increased biofilm formation of *C. albicans* (Figure 5.3). The mechanism behind the stimulatory effect of this compound is still unknown, but it may be an exciting area of future research, especially since diabetes is a known risk factor for *C. albicans* infection (Gonçalves et al., 2016).

In contrast to the other two drugs, vildagliptin [an oral antidiabetic, which improves glucose acceptance by increasing glucagon-like peptide-1, resulting in high insulin levels (Balas et al., 2007)], was able to inhibit *C. albicans* biofilms by approximately 11% (Figure 5.3). Vildagliptin is a selective inhibitor of human dipeptidyl peptidase 4 (DPP-4) present in plasma, kidney and intestinal membrane (He et al., 2009; He, 2012). Although it has been reported that gliptins, including vildagliptin, can inhibit biofilm formation of *Streptococcus mutans* at high concentration (De et al., 2018) and may influence the gut microbiome (Olivares et al., 2018), this is the first report of the inhibitory effect of *C. albicans* biofilms.

5.3.3.3. *GRE2* as a potential target

Furosemide and warfarin were identified as the inhibitors of Gre2p, an NADPH dependent methylglyoxal reductase (Chen et al., 2003; Nguyen et al., 2019). Methylglyoxal reductase is involved in methylglyoxal detoxification, a toxic dicarbonyl compound consisting of an aldehyde and a ketone produced in alkaline conditions during glycolysis from glyceraldehyde or dihydroxyacetone (Maeta et al., 2005; Dafre et al., 2015). In *C. albicans*, *GRE2* is induced in response to oxidative stress (Arana et al., 2010). The protein shares high sequence homology with similar proteins in other yeasts and has similarity to human UDP-galactose 4-epimerase (GALE) (Nguyen et al., 2019). *Candida glabrata* ketoreductase 1, which catalyses the reduction of keto esters to chiral alcohol (Qin et al., 2016) had the closest structural homology to *GRE2* amino acid sequences in the database.

The diuretic drug furosemide, a known inhibitor of NADPH dependent carbonyl reductases (Iwata et al., 1989), was observed to inhibit the growth of *C. albicans* biofilm by approximately 16% (Figure 5.3). Furosemide has also been reported to inhibit *Staphylococcus aureus* small colony variants (Trombetta et al., 2018) and to disrupt biofilm formation by *Pseudomonas aeruginosa* in a concentration-dependent manner at pH8 (Cross et al., 2007). The mechanism of action of furosemide biofilm inhibition was suggested to be via the inhibition of potassium and sodium chloride cotransport channels which results in a decrease in sodium and water absorption, ultimately leading to death (Passàli et al., 2003; Cross et al., 2007). Furosemide may have a similar mode of action against *C. albicans* biofilm because the interference with potassium homeostasis in *C. albicans* results in apoptotic markers such as cell shrinkage and DNA fragmentation (Li et al., 2018).

The highest percentage reduction (approximately 17%) in biofilm formation was observed for warfarin (Figure 5.3), an oral anticoagulant drug, which inhibits vitamin K-epoxide reductase complex (a cytochrome P450 enzyme essential enzyme for vitamin K production) (Harrington et al., 2004; Harter et al., 2015). Warfarin is classified as a 4-hydroxycoumarin, a derivative of the aromatic oxygen-containing heterocyclic compound, coumarin, which has various biological functions (Jain and Joshi, 2012) including

antibiofilm activity against *Escherichia coli* (Ojima et al., 2016). Coumarins and their derivatives have also been reported to have antifungal effects against *C. albicans* by several possible mechanisms. These include damaging the cell wall, resulting in the leakage of cytoplasmic content (Montagner et al., 2008; Widodo et al., 2012), causing apoptosis associated with mitochondrial Ca^{2+} influx (Jia et al., 2019), disrupting the sterol biosynthetic pathway (Kovvuri et al., 2018) and disrupting the synthesis of cytochromes, leading to inhibition of respiration (Thati et al., 2007). Therefore, the mode of action of warfarin may be similar to one or any combination of these mechanisms.

Since the administration of warfarin as an antifungal drug may not be free of severe side effects, the antibiofilm activity of the heparin, enoxaparin, an anticoagulant with a lower risk of bleeding (Meyer et al., 2002), was also tested and was found to inhibit biofilms of *C. albicans* (Figure 5.3). Micelli and co-workers (2012) found that heparin could inhibit both biofilm formation as well as mature biofilms of *C. albicans in vitro*. However, at concentrations more reflective of those routinely used in central venous catheters, heparin was shown to enhance biofilm formation in rat venous catheter models (Green et al., 2013). This highlights the importance of selecting the correct dosage when attempting to study drugs for potential repurposing as well as selecting models that are reflective of the conditions of the targeted infections.

5.3.3.4. *ZCF5* as a potential target

Cimetidine was identified as an inhibitor for Zcf5p, a zinc finger protein transcription factor. The closest structural homology to Zcf5p was CYP1, a transcriptional activator for aerobic metabolism in *S. cerevisiae* (Timmerman et al., 1996). Cimetidine is a competitive histamine H_2 receptor antagonist which is used in the treatment of peptic ulcers and gastroesophageal reflux disease (Polizzi et al., 1996; Singh et al., 2018). In addition, cimetidine enhances a variety of immunologic functions and because of this has been used for the treatment of mucocutaneous candidiasis, six common variable immunodeficiency and various viral infections (Jorizzo et al., 1980; Yilmaz et al., 1996). Acid reducing therapy, such as oral cimetidine, has been associated with increased gastric fungal growth (Goenka et al., 1996), however, in another study, short term use of cimetidine did not promote *C. albicans* gastrointestinal invasion (Minoli et al., 1987). In

the present study, cimetidine inhibited *C. albicans* biofilms by approximately 9% (Figure 5.3), indicating a direct inhibition of *C. albicans* independent of its immunomodulatory effects.

5.4. Conclusions

According to this investigation, several genes were identified to be haploinsufficient in the presence of fluconazole. The increased sensitivity indicates that the genes may be associated with a role in *C. albicans* biofilm fluconazole resistance and may be investigated further as drug targets. Although, an attempt at repurposing some of the existing drugs against *C. albicans* biofilms based on similarities in protein structure did not yield any drugs able to inhibit up to 50% of biofilms. However, at the concentration tested cimetidine, vildagliptin, furosemide, warfarin and enoxaparin did show inhibitory activity. A combination of the identified drugs with other existing drugs may improve their efficacy; however, this should be considered with care due to drug-drug interactions. These interactions, which involve the alteration of the pharmacological effect of a prescribed drug by another drug, have been reported for the identified drugs (Huang et al., 2013). Drug-drug interaction was reported between cimetidine, warfarin and fluconazole and also between furosemide and amphotericin B (Lazar and Wilner, 1990; Depont et al., 2007; de Filette and Michiels, 2018). Furthermore, vildagliptin, which was approved for use in Japan, was banned in Germany by a joint federal committee due to adverse benefit-risk (May and Schindler, 2016). Therefore, the challenge with the treatment of *C. albicans* biofilms still requires more investigations to identify more existing drugs for repurposing.

5.5. References

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General Discussion and Conclusions

Candida albicans, a harmless commensal of the human microbiota, can transition into a deadly pathogen as a result of a deterioration in the host immune system. This yeast has a considerable global impact as a commonly isolated pathogen in nosocomial bloodstream infections. Also, it can form highly resistant biofilms on biotic and abiotic surfaces, a crucial contributor to its virulence which necessitates the exploration for an alternative treatment option. Nevertheless, studies have revealed that several polyunsaturated fatty acids (PUFAs), including stearidonic acid (C18:4 n-3), arachidonic acid (AA) (C20:4 n–6), eicosapentaenoic acid (EPA) (C20:5 n-3) and docosapentaenoic acid (22:5 n-3) exhibit antifungal properties against *C*. *albicans* biofilms (Thibane et al., 2010).

Similarly, a combination of PUFAs with azole and polyene antifungal classes has been shown to increase susceptibility in *C. albicans* biofilms, even though the mechanism involved is unknown (Ells et al., 2009; Thibane et al., 2012). In this study, the possible mechanisms associated with this increased azole susceptibility in the presence of PUFAs were investigated. The preliminary investigation confirmed the significant increase in *C. albicans* biofilms susceptibility to the azoles, fluconazole and clotrimazole, in the presence of either AA or EPA.

RNA sequencing (RNA-Seq), which applies high-throughput sequencing to quantify transcripts from extracted RNA, is a useful tool to probe for differentially expressed genes (Hrdlickova et al., 2017; Li and Li, 2018). The identification of differentially expressed genes was necessary to unravel the mechanisms involved in PUFAs induced increase in *C*. *albicans* biofilms antifungal susceptibility.

Azoles are primarily known to disrupt the ergosterol synthesis process, which is essential to maintain the cell's membrane integrity (Shapiro et al., 2011). However, the yeast overexpresses *ERG11* the drug target as a resistant mechanism to circumvent the effects of fluconazole (Ramage et al., 2012). As expected, in the biofilms treated with fluconazole, enrichment of gene ontology (GO) terms related to processes which increase ergosterol synthesis was observed. Also, it is known that a significant number of aneuploid cells are formed when *C. albicans* cells are exposed to fluconazole (Zacchi et

al., 2010; Harrison et al., 2014). However, the processes which are associated with DNA replication, DNA methylation or demethylation and negative regulation of helicase activity were downregulated. The downregulation of these processes results in growth defects and aneuploidy due to an abnormal cell cycle.

However, in biofilms grown with fluconazole in combination with AA, the transcriptional regulation indicated enrichment of GO terms associated with ATP synthesis. ATP generation is crucial to power several cellular processes (Bonora et al., 2012). Based on the observed regulation, it can be suggested that the presence of AA interferes with ATP synthesis processes as a possible mechanism to induce an increase in antifungal susceptibility. Furthermore, in the presence of AA (either alone or combined with fluconazole), differential downregulation of genes involved in methionine/cystine synthesis, crucial to biofilm formation was observed. The absence of methionine or cysteine has been shown to cause diminished adhesion, decreased filamentation and diminished biofilm-forming ability (Li et al., 2013). Although the pathway is regulated by catabolite repression (Care et al., 1999), the down-regulation of genes appears to be induced by the presence of AA. A closer look at the transcriptional output of *C*. *albicans* to confirm some of the differential regulation obtained will be essential to elaborate further on these possible novel mechanisms associated with the increased susceptibility.

Candida albicans employ several resistance mechanisms when exposed to fluconazole. Therefore, the effect of AA on the well-known fluconazole resistance mechanism of *C*. *albicans* was also studied. Two of the best-studied responses to fluconazole is the increased expression of *ERG11* and overexpression of *CDR1* encoded efflux pump, to mitigate the effects of fluconazole (Lockhart and Berkow, 2017). Usually, the expression of *CDR1* in *C*. *albicans* is initiated during the early stage of biofilm formation and maintained through the biofilm formation process (Lepak et al., 2006; Nett et al., 2009). In this study, *ERG11* was slightly expressed in response to fluconazole. However, the expression diminished with the addition of AA, even in the presence of fluconazole. The downregulation of *ERG11* may be a mechanism of AA to increase the effect of fluconazole on *C*. *albicans* biofilms by overriding this mechanism of fluconazole resistance.

The differential expression of *CDR1* was higher with biofilms treated with AA alone or combined with fluconazole than with fluconazole only. Generally, an increase in expression translates to enhanced protein activity. However, a significant reduction in rhodamine 6G efflux activity was observed in AA treated cells. The influence of AA on membrane fluidity and saturation may be associated with the interference of efflux activity. Furthermore, Cdr1p is an ABC protein which requires ATP for its activity. As noted earlier, the presence of AA may interfere with ATP synthesis process, and this may be another reason for the absence of Cdr1p efflux in the presence of AA.

Candida albicans also induces the expression of oxidative stress genes to mitigate the effects of fluconazole. In this study, several antioxidant expressing genes were downregulated when *C*. *albicans* biofilm was grown in the presence of fluconazole and AA. The heterozygous mutants of these genes were sensitive to 1 μg mL^{-1} fluconazole, which indicates that they play a role in fluconazole resistance. Also, the influence of fluconazole-induced oxidative stress was tested on a heterozygous mutant of *PST1*, selected a representative. There was a reduction in the effect of fluconazole oxidative stress effects observed with an increase in metabolic activity when the *PST1* mutant was grown in the presence of butylated hydroxytoluene, a known antioxidant. The observation suggests the role of these genes in fluconazole resistance. Given the observations of AA interfering with the resistance mechanism of *C*. *albicans* biofilms against fluconazole treatment, its effects on other resistance mechanisms, like the extracellular matrix, may also be investigated.

The high antifungal resistance posed by *C*. *albicans* biofilms causes the need for alternative treatment options. The option of drug repurposing is attractive because it reduces cost and time (Miró-canturri et al., 2019). In our investigation, several genes with potential as probable targets were identified based on their expression profiles in the different treatment conditions. A haploinsufficiency profiling assay of the heterozygous mutants of these genes with fluconazole showed high sensitivity. The genes with the highest response were selected and investigated as potential drug targets. While our attempt to repurpose existing drugs against *C. albicans* biofilms did not yield the expected

result, the use of combination therapy together with the identified drugs may unlock possible treatment prospects.

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Summary

A considerable proportion of infections associated with *C. albicans* are due to its ability to form biofilms. Although there are antifungals available to treat infections caused by *C. albicans*, the occurrence of antifungal resistance hampers their effectiveness. Polyunsaturated fatty acids (PUFAs) have been reported to have antifungal properties against *C. albicans* biofilms. Interestingly, the combination of PUFAs, such as arachidonic acid (AA), with existing antifungals of the azole and polyene classes has been shown to increase susceptibility in *C. albicans* biofilms, but the mechanism involved is unknown. The objective of this study was to investigate the mechanism associated with the increased antifungal susceptibility of *C. albicans* biofilms in the presence of PUFAs. Our investigation confirmed an increase of *C. albicans* biofilms susceptibility to azoles (fluconazole and clotrimazole) in the presence of PUFAs (AA and eicosapentaenoic acid). Furthermore, the transcriptional analysis of *C. albicans* biofilms grown in the presence of AA combined with fluconazole, indicated that the presence of AA might be interfering with ATP generation processes. In addition, in the presence of AA alone or combined with fluconazole, the methionine/cysteine synthesis process, which is essential for biofilm formation, was repressed. However, quantitative analysis of the metabolic products associated with these pathways is required for a comprehensive view of the effects of these compounds on *C. albicans* biofilms. Several resistance mechanisms are employed by *C. albicans* biofilms to mitigate the effect of fluconazole treatment. One of the known mechanisms is the overexpression of the *CDR1* efflux pump. Although *C. albicans* upregulates *CDR1* in the presence of fluconazole, a significant increase in expression was observed in the presence of AA alone or combined with fluconazole. Nevertheless, this increased mRNA transcription did not necessarily translate into increased efflux when tested with rhodamine 6G. The lack of efflux by Cdr1p, which is a membrane protein, indicates that the presence of AA may have altered the membrane organisation leading to the loss of efflux activity. The ergosterol content in the cell is a factor which influences membrane fluidity. The regulation of *ERG11*, the fluconazole target, was higher in the treatment containing fluconazole alone compared to those containing AA. It was predicted that the increase in *ERG11* expression would enhance the ergosterol content levels. However, the ergosterol content of *C. albicans* biofilms treated with fluconazole alone did

not differ significantly from that treated with fluconazole combined with AA. Fluconazole and PUFAs are known to induce oxidative stress in yeast. In the presence of AA and fluconazole, several oxidative stress-associated genes were downregulated. In addition, the heterozygous mutants of these genes were sensitive to fluconazole treatment, which indicates that they play a role in *C. albicans* fluconazole resistance. Therefore, the AA may increase drug susceptibility via a number of mechanisms, including inhibition of Cdr1p activity and increased oxidative stress. There is an urgent need for alternative treatment option; hence the use of drug repurposing has progressed because of its reduced cost and time for the discovery of new antifungal drugs. By exploiting drugs against gene families identified in this study, that may play a role in fluconazole resistance, some drugs leads were obtained. However, more research is still required to clarify the associated mechanisms. An understanding of these mechanisms will indicate drug targets which can be explored for the treatment of *C. albicans* biofilms infections.

Keywords: *Candida albicans*, Arachidonic acid, *CDR1*, Fluconazole, Biofilms, *Rhodamine 6G*, *ERG11*