

**Investigating the influence of arachidonic acid on *Candida albicans* and its interaction with *Pseudomonas aeruginosa***

**by**

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Philosophiae Doctor**

**In the**

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## **DISSERTATION SUMMARY**

Infection causes the release of arachidonic acid (AA) by the host that not only alters pathogen clearance by the immune system, but can also affect virulence and the antimicrobial susceptibility of pathogens. Furthermore, it may alter the interaction between co-infecting agents. *Candida albicans* exhibits multiple phenotypes and morphological plasticity, that are crucial to its virulence. Furthermore, it is known to form associations with commensal and co-infective bacteria in polymicrobial biofilms that can affect patient outcomes. The interkingdom interaction between *C. albicans* and the ubiquitous bacterium, *Pseudomonas aeruginosa*, can be regarded as a model for polymicrobial infection with detrimental cause to the host. The role that AA plays during this interaction is unknown. Arachidonic acid is not endogenously produced by *C. albicans* and *P. aeruginosa*, however, exogenously added AA has been reported to increase the susceptibility of *C. albicans* to antifungal agents, with reduction in virulence associated characteristics. The mechanism of this is unknown.

To address this lack of knowledge, transcriptomic profiles of single species and polymicrobial biofilms, in the absence or presence of a sub-inhibitory concentration of AA, were compared. Focusing on *C. albicans*, genes of interest were identified, and homozygous deletion mutants were constructed. The roles of these genes were then evaluated during *in vitro* characterisation of biofilms as well as virulence in a *Caenorhabditis elegans* infection model.

Regulatory mechanisms of *C. albicans* phenotypes and morphologies were evaluated in the presence of *P. aeruginosa*, along with their contribution to virulence during polymicrobial infection. Deletion of a component of the Set3/Hos2-histone deacetylase complex, involved in morphogenesis via chromatin remodelling, showed limited effect on the interaction between fungus and bacterium *in vitro*, but abrogated virulence, even in the presence of *P. aeruginosa*. Furthermore, deletion of *WOR1*, the principle regulator of phenotypic switching, did not modify population dynamics or virulence of *C. albicans* with *P. aeruginosa*.

Genes associated with membrane organisation and antifungal resistance were shown to be induced by AA in *C. albicans* single species biofilms. Three genes, *CDR1*, *IPT1* and *RTA3*, were chosen due to their importance in antifungal susceptibility. In addition

to AA, co-incubation with *P. aeruginosa* induced their expression. However, only deletion of *IPT1* was able to affect survival of *C. albicans* in the presence of *P. aeruginosa*. Furthermore, deletion of *IPT1* and *CDR1*, but not *RTA3*, altered the virulence of *C. albicans*. Deletion of *CDR1*, a multi-drug transporter, increased oxidative stress, possibly due to lipid peroxidation, in the presence of AA. This may indicate that Cdr1p may be responsible for the efflux of AA. Although AA increased *CDR1* mRNA and protein levels in a dose dependent manner, its function was diminished. This may be due to a combination of factors, including mislocalisation and subsequent loss of function of Cdr1p, reduced mitochondrial function, as well as competitive inhibition of AA with xenobiotic compounds. As Cdr1p provides antifungal resistance to *C. albicans* by efflux of antifungal agents, this provides a possible mechanism whereby AA increases antifungal susceptibility in *C. albicans*.

This study addressed the influence of AA, a polyunsaturated fatty acid (PUFA), present in ample amounts during infection, on *C. albicans*. The influence of this fatty acid is frequently ignored during *in vitro* studies. Furthermore, the results obtained in this study prompts investigation into other PUFAs and their effects on pathogenic yeast, and their associations with pathobionts.

**Keywords:** Arachidonic acid; Biofilms; *Candida albicans*; CRISPR-Cas9; Polymicrobial; *Pseudomonas aeruginosa*; Transcriptomics

## **DECLARATIONS**

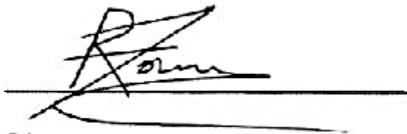
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- My sister, **Camille Fourie**
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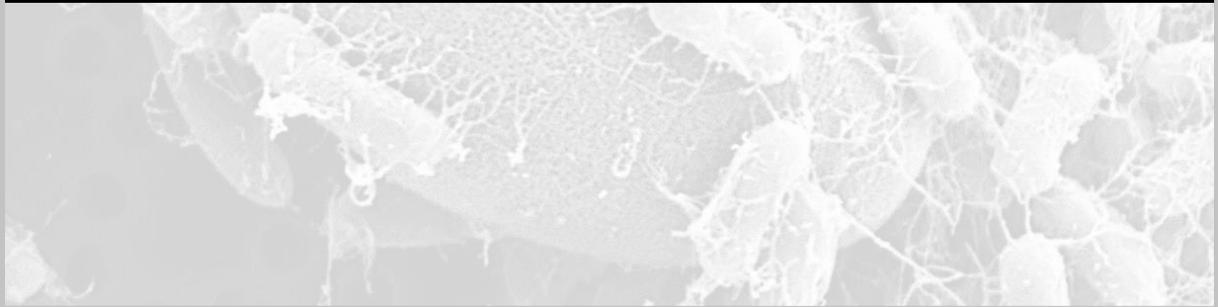
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**CHAPTER 1**

**Literature review**



## SECTION A

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### MOTIVATION

Polymicrobial interactions between microorganisms are not only ubiquitous in nature, but significantly affect human health, with the ability to prevent or promote disease through commensalism, synergism as well as antagonism (Krüger *et al.*, 2019; Murray *et al.*, 2014; Peters *et al.*, 2012; Stacy *et al.*, 2016). These interactions are driven by physical interaction, secreted molecules and nutrient availability that can not only alter population dynamics of microorganisms in the human host, but also affect the ability of the human host to clear infection.

*Candida albicans* is a polymorphic opportunistic pathogen that possesses unparalleled versatility in colonising the human host and frequently forms associations with co-inhabitants and co-infective agents (Ranjan and Dongari-Bagtzoglou, 2018; Shirtliff *et al.*, 2009). This phenomenon may occur through the ability of this yeast to form antifungal resistant biofilms through exhibition of morphological transition between yeast, pseudohyphal and hyphal morphologies that intertwine to form three-dimensional structures on biotic and abiotic surfaces (Chandra *et al.*, 2001; Lohse *et al.*, 2018). During biofilm formation, bacterial cells may get trapped, enabling interaction through proximity and contact (Fox *et al.*, 2014; Harriot and Noverr, 2009). One of the bacteria that *C. albicans* forms associations with is *Pseudomonas aeruginosa* (Méar *et al.*, 2013). The co-isolation of *C. albicans* and *P. aeruginosa* from infection sites, such as the lungs of cystic fibrosis patients, sparked extensive research into their interaction (Hogan and Kolter, 2002; Kerr, 1994; Lindsay and Hogan, 2014; McAlester *et al.*, 2008; Méar *et al.*, 2013; Trejo-Hernández *et al.*, 2014). A striking feature of polymicrobial interactions is the propensity of these associations to elicit increased resistance towards antimicrobial agents and treatment of infections (Harriot and Noverr, 2009; Murray *et al.*, 2014; Peters *et al.*, 2012). This necessitates the identification and research of alternative treatment strategies or novel antimicrobial compounds.

Research into fatty acids has gained momentum due to their antibacterial, antifungal and antiviral properties (Kohn *et al.*, 1980; Pohl *et al.*, 2011; Yoon *et al.*, 2018).

Arachidonic acid (AA) is a C-20  $\omega$ -6 PUFA, forming a crucial part of mammalian cell membranes (Hanna and Hafez, 2018; Martin *et al.*, 2016). It is released during infection by both bacterial and fungal pathogens. This is mediated through host-derived phospholipase A<sub>2</sub> and pathogen derived enzymes with phospholipase activity, and is the precursor for various immunomodulatory lipid mediators (Dennis and Norris, 2015). In addition, AA has been proposed to be an endogenous antimicrobial molecule with antibacterial and antiviral properties and has also been associated with inhibition of morphological transition in *C. albicans* (Das, 2018; Shareck and Belhumeur, 2011). However, strikingly little information is available regarding the effect of AA on *C. albicans*, the mechanism of action and its effect on *C. albicans*' association with pathogens such as *P. aeruginosa*.

Therefore, research into the effect of AA on *C. albicans* and its interaction with *P. aeruginosa* provides a unique opportunity to determine the effect of a PUFA on *C. albicans* that may be extended to other pathogenic yeast and their associations with co-infecting species. Furthermore, it enables the characterisation of a component of pathogen-host interaction frequently overlooked during *in vitro* studies. Considering this, the aim of this research is to evaluate the effect of AA on *C. albicans* single species biofilms as well as in combination with *P. aeruginosa* in polymicrobial biofilms.

**SECTION B**

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The candidate, Ruan Fourie, wrote the manuscript. Both authors collected literature and placed it into context for the review. Ruan Fourie designed the figures and Carolina H. Pohl edited the final manuscript and supervised the first author.

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Review

# Beyond Antagonism: The Interaction Between *Candida* Species and *Pseudomonas aeruginosa*

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**Abstract:** There are many examples of the interaction between prokaryotes and eukaryotes. One such example is the polymicrobial colonization/infection by the various opportunistic pathogenic yeasts belonging to the genus *Candida* and the ubiquitous bacterium, *Pseudomonas aeruginosa*. Although this interaction has simplistically been characterized as antagonistic to the yeast, this review highlights the complexity of the interaction with various factors influencing both microbes. The first section deals with the interactions *in vitro*, looking specifically at the role of cell wall components, quorum sensing molecules, phenazines, fatty acid metabolites and competition for iron in the interaction. The second part of this review places all these interactions in the context of various infection or colonization sites, i.e., lungs, wounds, and the gastrointestinal tract. Here we see that the role of the host, as well as the methodology used to establish co-infection, are important factors, influencing the outcome of the disease. Suggested future perspectives for the study of this interaction include determining the influence of newly identified participants of the QS network of *P. aeruginosa*, oxylipin production by both species, as well as the genetic and phenotypic plasticity of these microbes, on the interaction and outcome of co-infection.

**Keywords:** *Candida albicans*; interaction; *Pseudomonas aeruginosa*

## 1. Introduction

Nature provides many examples of the interaction between prokaryotes and eukaryotes [1,2]. These interactions can have various outcomes and may provide an advantage to one party (e.g., through antagonism of the other) or both parties (mutualism). Interactions between fungi and bacteria are abundant in many habitats, including the human body [3–5]. The identification of these interactions, especially in the human host, have been performed through culture-dependent techniques or culture-independent techniques, the latter being able to identify the presence of microbial intruders at low cell counts that could play a large role in infection. One of the most well-documented bodily sites where polymicrobial interaction takes place is the gastrointestinal tract, colonized by a plethora of microbial species [6]. Furthermore, polymicrobial infections occur frequently in the urinary tract, wounds, where it can negatively affect wound healing, as well as in the lungs, especially in patients predisposed to infection by altered immunity, as in the case with cystic fibrosis (CF). The competition posed by close proximity and nutrient limitation can result in altered metabolism and virulence factor expression as well as altered host immunity that may lead to a competitive advantage to species within a polymicrobial infection [6,7]. This interaction between fungi and bacteria can cause a considerable increase in morbidity and mortality compared to single species infection [8,9].

One of the principle models for studying the interaction between eukaryotic and prokaryotic interaction in the context of human health is the association between *Candida albicans*, a polymorphic fungus, and the ubiquitous bacterium *Pseudomonas aeruginosa*. The study of this interaction dates

back to the 1970s, when several authors observed that *P. aeruginosa* had an inhibitory effect on the growth of *C. albicans* [10,11]. This phenomenon is not confined to these two species. Kerr (1994) found that *Pseudomonas cepacia* also has this inhibitory effect, while other potentially pathogenic *Candida* spp. (*C. dubliniensis*, *C. glabrata*, *C. guilliermondii*, *C. kefyr*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, *C. pseudotropicalis* and *C. tropicalis*) can also be susceptible to inhibition by *Pseudomonas* [12–14]. Today we know that many factors can mediate the interaction between these organisms, with the best-studied example being the interaction between *P. aeruginosa* and *C. albicans*.

## 2. In Vitro Interaction between *Pseudomonas aeruginosa* and *Candida* Species

### 2.1. Role of Cell Wall Components in the Interaction

Hogan and Kolter (2002) showed that *P. aeruginosa* preferentially attaches to *C. albicans* hyphae, effectively killing them, probably to gain nutrients for growth [1]. This was not the case for *C. albicans* yeast cells. In addition, they found that *P. aeruginosa* mutants lacking type IV pili, could attach to *C. albicans* hyphae, but were unable to kill them. They speculated that the role of pili might involve pilus retraction to bring the bacterium into close contact with the hyphae or that the pili act as sensors, signaling attachment to the fungal surface. Other cell wall-associated compounds of both *P. aeruginosa* and *C. albicans* are also important in this interaction. Bacterial lipopolysaccharide (LPS), including *P. aeruginosa* LPS, has adverse effects on *C. albicans* biofilms [14,15]. These include a decrease in filamentation, biofilm metabolic activity (including glycolysis) and growth. In contrast, peptidoglycan triggers filamentation in *C. albicans* [16]. Brand and co-workers (2008) investigated the role of *C. albicans* hyphal-specific cell wall proteins and mannoproteins in the selective attachment and killing of hyphae, and found that neither the major hyphal-specific proteins (Hyr1p, Hwp1p and Als3p), nor enzymes involved in *N*-glycosylation of cell wall proteins played a role, but that *O*-mannan probably protects against *P. aeruginosa* killing activity [17]. Whereas the bulk of research has been done on the interaction between *C. albicans* and *P. aeruginosa*, less is known regarding the interaction of *P. aeruginosa* with other *Candida* species. However, Bandara and co-workers (2010) did indicate a mutually suppressive interaction between *P. aeruginosa* and five non-*albicans* *Candida* species, i.e., *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. dubliniensis* and *C. krusei* in an in vitro dual species biofilm model [14]. They observed species-specific variations of inhibition of *Candida* biofilms, with almost complete inhibition of *C. albicans*, *C. glabrata* and *C. tropicalis* biofilms after 48 hours. Interestingly, although research from Brand and co-workers (2008) indicated selective attachment and killing of hyphal cells, Bandara and co-workers (2010) observed attachment of bacterial cells to blastospores, indicating that the hyphal phenotype may not be an absolute requirement for the physical association of *P. aeruginosa* to fungal cells [14,17].

### 2.2. Role of Quorum Sensing Molecules in the Interaction

*P. aeruginosa* secretes several types of molecules that may influence the growth of *Candida* spp. [18]. An important category of secreted molecules is quorum sensing molecules (QSM), produced by both organisms [19]. Gram-negative bacteria use *N*-acyl homoserine lactones (AHL) as QSM. At high population densities, when the concentration of AHL is above the threshold level, it can bind to and activate transcriptional activators to induce expression of target genes [20]. *P. aeruginosa* has two AHL-dependent QS systems, *las* and *rhl*. The *LasI* autoinducer synthase controls the production of the autoinducer, 3-oxododecanoyl-L-homoserine lactone (3-oxo-HSL), while production of butanoyl homoserine lactone is regulated by *RhII* autoinducer synthase. These autoinducers act on their respective transcriptional activators, *LasR* and *RhIR* [21,22] and, depending on the culture conditions, may regulate up to 10% of the *P. aeruginosa* genome [23,24]. 3-oxododecanoyl-L-homoserine lactone (3-oxo-HSL), which is required for the production of surface adherence proteins on *P. aeruginosa* cells, is also important for adherence of *P. aeruginosa* to *C. albicans* hyphae [25]. In addition, 3-oxo-HSL could inhibit the yeast to hyphal switch of *C. albicans* in a dose-dependent manner [26,27]. Interestingly,

Trejo-Hernández and co-workers (2014) found that hypoxia influences the ability of *P. aeruginosa* to inhibit *C. albicans* filamentation, due to a decrease in AHL production [28]. Another QS signal, 2-heptyl-3-hydroxyl-4-quinolone signal or *Pseudomonas* quinolone signal (PQS), which is released by *P. aeruginosa* during late exponential phase [29,30], is induced by the LasI/R system and inhibited by the RhlI/R system [31]. This signal not only modulates swarming motility of *P. aeruginosa* [32,33], but also induces the production of several virulence factors, including phenazines [34]. *Pseudomonas* quinolone signal and its precursor, 2-heptyl-4-quinolone, represses *C. albicans* biofilm formation [35]. These combined effects may lead to the dispersal of *C. albicans* cells in the presence of *P. aeruginosa* [25,36].

Lee and co-workers (2013) provided evidence of an additional QS network, integrated QS system or IQS, that is capable of integrating the stress cues from the environment with the QS network [37,38]. Although dependent on environmental factors, this QS system was shown to function below the *las* QS circuit in a hierarchical manner and was capable of regulating virulence factors through induction of the PQS and *rhl* QS systems. The impact of this additional player in the QS regulatory circuit in the interaction with *C. albicans* is unknown.

Recently, Martínez and co-workers (2019) discovered another cell-density dependent system that is reliant on the production of oxidized fatty acids (oxylipins), termed oxylipin-dependent quorum sensing (ODS) [39]. This system affects the motility of *P. aeruginosa*, but functions independently of the hierarchical circuit of *P. aeruginosa*. The activity of this system was induced with the addition of the polyunsaturated fatty acid, oleic acid, that may be available during infection by this pathogen. This raises the question if oxylipins may play a role in the interaction between *P. aeruginosa* and *C. albicans*, as *C. albicans* is also known to produce oxylipins from polyunsaturated fatty acids that can affect morphology in a QS manner [40]. The role of fatty acid metabolites in the interaction is discussed later.

*Candida albicans* also produces QSMs [41]. The QSM, farnesol, inhibits germ tube formation, similar to 3-oxo-HSL. This effect may be due to inhibition of the Ras1p-controlled pathway involved in hyphal growth [36]. Farnesol inhibits *P. aeruginosa* PQS and pyocyanin (PYO) production in a dose dependent manner [19]. It also influences expression of *P. aeruginosa* virulence-related proteins [42], including haemolysin production [43] and inhibits swarming motility [27]. Another *C. albicans* QSM, tyrosol, stimulates hyphal formation [44], especially during early and intermediate stages of biofilm formation [45]. Physiologically relevant concentrations of tyrosol could also inhibit haemolysin production and secretion of protease by clinical isolates of *P. aeruginosa* [43].

### 2.3. Role of Phenazines in the Interaction

Pyocyanin or methyl-1-hydroxyphenazine, is produced by *P. aeruginosa* during the early stationary phase [46,47] and is the best studied bacterial phenazine. It plays a major role in maintaining NADH/NAD<sup>+</sup> ratio stability in *P. aeruginosa* cells under oxygen limitation, since *P. aeruginosa* has a limited fermentation capability [48]. It acts as an alternative terminal electron acceptor and decreases the NADH/NAD<sup>+</sup> ratio. When oxygen becomes available again, PYO is re-oxidized, possibly leading to the production of reactive oxygen species (ROS). This production of ROS may contribute to the toxic effect of phenazines on eukaryotes, including fungi [49,50]. Pyocyanin also decreases cyclic adenosine monophosphate (cAMP) [51], which explains the observed inhibition of the *C. albicans* yeast to hyphae transition, which requires cAMP. Gibson and co-workers (2009) co-incubated *P. aeruginosa* and *C. albicans* and observed a red pigment inside *C. albicans* cells [52]. The presence of the red pigment was linked to repression of *C. albicans* viability. The precursor of this red pigment was identified as 5-methyl phenazine-1-carboxylic acid (5-MPCA). The related phenazine, phenazine-1-carboximide (PCN), also showed broad-spectrum antifungal activity, including against *C. albicans* and *C. glabrata* and inhibited the yeast to hyphal switch in *C. albicans* [53]. The mechanism of action of PCN was increased ROS production, mitochondrial membrane hyperpolarization, and eventually, apoptosis. Interestingly, Morales and co-workers (2013) found that low concentrations of PYO, phenazine methosulfate, and PCN specifically inhibited respiration, leading to increased fermentation and lower extracellular pH,

which inhibited *C. albicans* yeast-to-hyphal switch [54]. Furthermore, Briard and co-workers (2015) indicated that sub-inhibitory concentrations of phenazines were capable of reducing ferric iron, making it more bio-available for co-inhabitants, such as *Aspergillus fumigatus* [55]. This is a phenomenon that may apply to *C. albicans* as well.

Chen and co-workers (2014) studied the effect of the *C. albicans* fermentation product, ethanol, on *P. aeruginosa* [56]. They found that ethanol inhibited swarming motility, but stimulated adhesion, subsequent biofilm formation and production of 5-MPCA and PCN. This implies the existence of a positive feedback loop where *C. albicans*-produced ethanol enhances *P. aeruginosa* biofilm formation and production of antifungal phenazines, which in turn stimulates ethanol production in *C. albicans*. Several other phenazines (phenazine-1-ol, phenazine-1-carboxylic acid, and PCN) are also inhibitory towards *C. albicans* and *C. tropicalis*, with phenazine-1-carboxylic acid having the lowest minimum inhibitory concentration [57]. These phenazines also have a synergistic effect with azoles against *Candida* species. All of this suggests that the presence of phenazine-producing organisms, such as *P. aeruginosa*, may influence co-infection by *Candida* spp. as well as the treatment of such fungal co-infection.

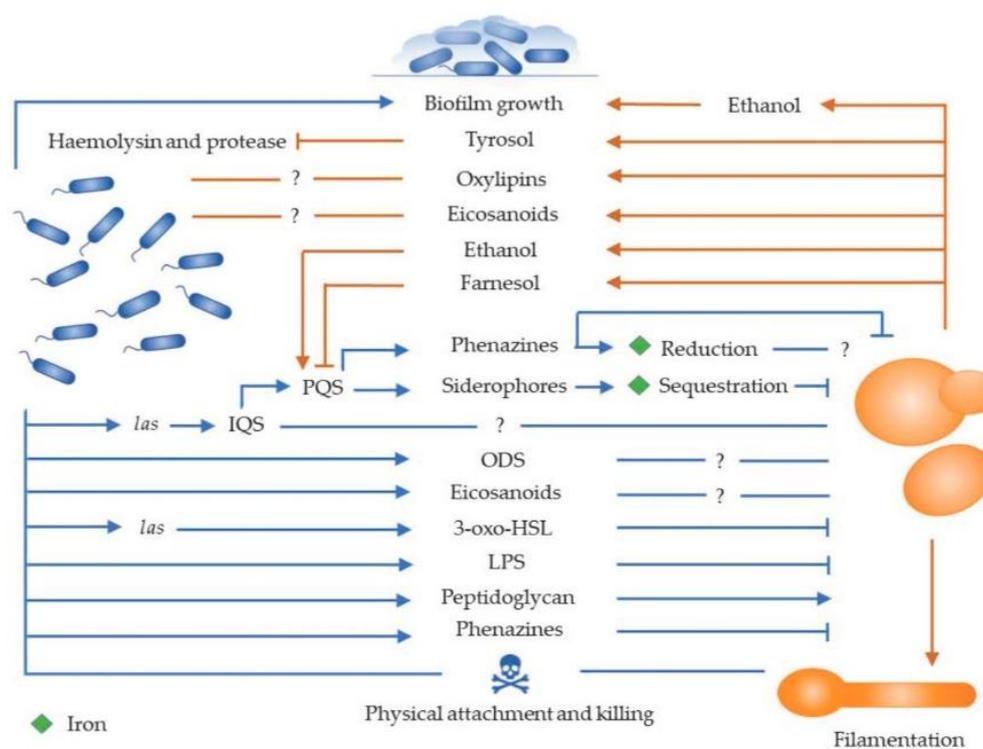
#### 2.4. Role of Fatty Acid Metabolites in the Interaction

As stated above (Section 2.2), *Candida* spp. can convert exogenous (or host-derived) fatty acids into various lipid metabolites, including hydroxy fatty acids and eicosanoids, such as prostaglandins [40,58–67], that may influence co-infecting microbes [68] or the host [69,70]. One of the best-studied *Candida* lipid metabolites is the eicosanoid, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), which has been reported in *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis*. *P. aeruginosa* also secretes an array of fatty acid metabolites, including hydroxy fatty acids [71–73] as well as PGE<sub>2</sub> and prostaglandin F<sub>2α</sub> [74]. Martínez and coworkers (2019) studied the effect of hydroxy fatty acids produced by the action of diol synthase in *P. aeruginosa* and found that the products, 10-hydroxy-octadecenoic acid (10-HOME) and 7,10-dihydroxy-octadecenoic acid (7,10-DiHOME), caused transcriptomic and proteomic alterations in *P. aeruginosa* [39,71]. This reflected in reduced swarming as well as swimming motility in a dose-dependent manner, as well as strongly promoting type IV pilus-driven twitching movement. These metabolites also promoted micro-colony and subsequent biofilm formation in vitro. Interestingly, 7,10-DiHOME has an inhibitory effect on the growth of *C. albicans* [75], while another hydroxy fatty acid, 3-hydroxy tetradecadienoic acid (3-OH 14:2), which is an endogenous linoleic acid metabolite of *C. albicans*, stimulates germ tube formation, even in the presence of farnesol [40]. Fourie and co-workers (2017) investigated the influence of co-incubation of *C. albicans* and *P. aeruginosa* on eicosanoid production from exogenous arachidonic acid by mixed species biofilms [74]. They found that, although co-incubation decreased the ability of individual colony forming units (CFUs) in the biofilm to produce eicosanoids, the final concentrations of all the studied eicosanoids (i.e., PGE<sub>2</sub>, PGF<sub>2α</sub>, and 15-hydroxy eicosatetraenoic acid) increased compared to single species biofilms. This was due to the increase in *P. aeruginosa* CFUs in the mixed species biofilms. Although it is known that PGE<sub>2</sub> enhances serum-induced germ tube formation in both *C. albicans* and *C. dubliniensis* [60,66,76], the influence of these eicosanoids on *P. aeruginosa* is unknown.

#### 2.5. Role of Competition for Iron in the Interaction

Purscke and co-workers (2012) compared the secretome of single and mixed species biofilms of *C. albicans* and *P. aeruginosa* [77]. They found an overall increase in secreted proteins of mixed species biofilms relative to single species biofilms. This increase was due to increased *P. aeruginosa* secreted proteins, many of them related to production of the *P. aeruginosa* siderophore, pyoverdine. The authors speculated that this was caused by competition for iron in multispecies biofilms. Chelation of iron by pyoverdine decreases its availability to *C. albicans*, leading to the observed inhibition of yeast growth. Because pyoverdine production is increased in mixed biofilms, the virulence of *P. aeruginosa* might also be upregulated in mixed biofilms [28]. An interesting observation was made by Nazik and co-workers (2017) who found that *P. aeruginosa* bacteriophages can bind to and inhibit *C. albicans* (and possibly

*C. kefyr*) [78]. This inhibition is likely due to the ability of the bacteriophages to bind iron into tight complexes, precipitating it out of the medium. This denies iron to the yeast, inhibiting both biofilm and planktonic cells. A summary of the interaction between *C. albicans* and *P. aeruginosa* is given in Figure 1.



**Figure 1.** Schematic of the interaction between *Candida albicans* and *Pseudomonas aeruginosa* *in vitro*. *P. aeruginosa* kills *C. albicans* hyphae through physical attachment. In addition, cell wall components such as lipopolysaccharide (LPS) and peptidoglycan affect filamentation. Furthermore, secreted factors such as phenazines and siderophores, as well as quorum sensing systems, such as *las* (controls the production of autoinducer 3-oxododecanoyl-L-homoserine lactone or 3-oxo-HSL), *Pseudomonas* quinolone signal (PQS), integrated quorum sensing (IQS), and oxylipin dependent sensing (ODS) may affect *C. albicans*. Lastly, *C. albicans* secreted factors such as farnesol, ethanol, oxylipins, and eicosanoids may affect *P. aeruginosa* growth characteristics and biofilm formation. Question mark (?) indicate unknown roles in the interaction.

### 3. In Vivo Interactions between *Candida* spp. and *Pseudomonas aeruginosa*

Although studying the interaction between organisms *in vitro* has the advantage of being able to limit the number of variables, this does not always provide a true picture of the potential interactions of co-infecting organisms in a living host with an active immune system. In addition, different body sites provide unique environments in terms of nutrient availability, presence of microbiota and immunity. Therefore, pathogens may exhibit unique patterns of growth and virulence in separate niches of the human body [79]. Due to this, a brief review of the documented interaction between *C. albicans* and *P. aeruginosa* in different body sites will be given.

#### 3.1. Interaction in the Lungs

Kerr (1994) was the first to report inhibition of *C. albicans* in the lungs of postoperative patients after subsequent colonization by *P. aeruginosa* [80]. This inhibition was reversed by antibiotic treatment of the *P. aeruginosa* infection. This observation was followed by studies that indicated that prior colonization by *Candida* spp. increases the susceptibility of the host to *P. aeruginosa* infection [81–84] and that the risk can be reduced by antifungal treatment [85]. This could be explained by Roux and co-workers (2013) who found that the *Candida*-induced Th1-Th17 immune response caused an increase in interferon-gamma

(IFN-gamma) [86]. This led to the inhibition of the expression of scavenger receptors on rat alveolar macrophages, and thus to a decrease in *P. aeruginosa* phagocytosis [87]. Interestingly, Chen and co-workers (2014) found that *C. albicans* produced ethanol promotes *P. aeruginosa* colonization of lung epithelial cells, and Greenberg and co-workers (1999) demonstrated that ethanol could inhibit the clearance of *P. aeruginosa* from the lungs in a rat model of infection by inhibiting macrophage recruitment [56,88]. Ader and co-workers (2011) established a murine infection model with short term colonization by *C. albicans* prior to *P. aeruginosa* infection and found that this leads to a reduction in *P. aeruginosa* load compared to infection in the absence of *C. albicans* [89]. Mear and co-workers (2014) found that *C. albicans* induces secretion of interleukin-17 (IL-17) and IL-22, leading to the production of antimicrobial peptides, as well as the mobilization of phagocytic cells [90]. Bergeron and co-workers (2017) developed a zebra fish swimbladder infection model to study the effect of *C. albicans*-*P. aeruginosa* co-infection on virulence [91]. This model clearly showed a synergistic virulence associated with increased *C. albicans* pathogenesis and inflammation. It is thus clear that the model and the specific methods used for infection influence the outcome of infection and should be taken into account when comparing results regarding the interactions of *Candida* spp. and *P. aeruginosa* in animal models.

Several studies of CF patients have indicated pulmonary co-infection with *C. albicans* and bacteria [92–96]. In this context, the co-infection of *C. albicans* and *P. aeruginosa* has been well documented [97]. Haiko and co-workers (2019) indicated a higher incidence of co-existence of *P. aeruginosa* with *Candida* spp. compared to other respiratory disorders [98]. Although the clinical impact of fungi in lungs is not yet fully understood [99], it is known that the presence of *C. albicans* in CF sputum is a very strong predictor of co-colonization with *Pseudomonas* spp., as well as of hospital-treated exacerbations [100]. However, Haiko and co-workers (2019) provided evidence that *Candida* spp. colonization does not exclusively lead to higher existence of pathogenic bacteria, indicating that this aspect still needs further validation [98]. Co-colonization with *C. albicans* and *P. aeruginosa* also led to a significant decline in lung function compared to patients without *C. albicans*. Kim and co-workers (2015) provided evidence of adaptation of *C. albicans* in the CF lung [101]. They isolated *C. albicans* strains that independently accumulated loss-of-function mutations in the *NRG1* gene, the product of which is a transcription factor that is known to repress filamentation in *C. albicans* [102]. Strikingly, these strains were impervious to the filamentation-suppressing effects of co-culture with *P. aeruginosa* and an analog of PYO, phenazine methosulfate. This study indicated that this adaptation may be common in the CF lung environment [101].

Patients on mechanical ventilation are also particularly susceptible to colonization by *Candida* spp. and subsequent *P. aeruginosa* ventilator-associated pneumonia (VAP) [81]. In 214 patients with *Pseudomonas* spp. VAP included in this study, who also had *Candida* spp. in their respiratory tracts, the most common *Candida* spp. were *C. albicans*, *C. glabrata* and *C. tropicalis*. Hamet and co-workers [83] found that mortality of intensive care patients with VAP was significantly increased by *Candida* spp. co-colonization and that *Candida* colonization was an independent predictor of death. According to Delisle and co-workers (2011), it is unclear whether colonization of the respiratory tract by *Candida* spp. is merely an indication of disease severity or contributes to the observed worse clinical outcomes in these patients [103]. Since respiratory colonization with *Candida* spp. alone can increase levels of tumor necrosis factor alpha and IFN-gamma in the lung [82] and, as stated above, IFN-gamma can impair the function of alveolar macrophages, it may be possible that colonization by *C. albicans* could inhibit an antibacterial immune response. In a recent review by Pendleton and co-workers [104] dealing with the significance of *Candida* in the human respiratory tract, the question regarding the clinical relevance of treating such occurrences with antifungal drugs is raised. Although treatments with antifungals in experimental models have yielded positive results [86,105], this was not always the case in human patients [85,106,107]. Due to these discrepancies in the results, no firm treatment guidelines exist, and clinical practice varies.

### 3.2. Interaction in Wounds

Chronic wounds, such as diabetic foot ulcers, pressure ulcers, and venous leg ulcers are prone to infection by polymicrobial biofilms [108,109]. Many of these are co-infections by *Candida* spp. (*C. parapsilosis*, *C. tropicalis* and *C. albicans*) and bacteria, including *P. aeruginosa* [110]. Despite this, infections in chronic wounds are typically treated with antibiotics, ignoring the fungal component, often leading to unresolved infections. Townsend and co-workers (2017) used a biofilm model, consisting of a complex cellulose matrix, to mimic the wound environment and polymicrobial biofilms consisting of *C. albicans*, *P. aeruginosa* and *Staphylococcus aureus* [109]. By treating the polymicrobial biofilms with antibiotics alone or in combination with fluconazole, they could change the compositions of the biofilms without affecting the overall cell numbers. They also found that the *C. albicans* population is an important driving force within the biofilm and is correlated to the total number of cells in the biofilm. This is due to the physical support and protection provided by the hyphae and extracellular matrix of *C. albicans*.

Another type of wound prone to infection by both *Candida* spp. and *P. aeruginosa* is thermal burn wounds. Although burn wounds are initially sterile, within the first 48 hours they typically become infected by Gram-positive bacteria. During the first week the population shifts to Gram-negative bacteria [111]. Lethal *Candida* infections often follow or occur concomitantly with bacterial infections [112]. The *Candida* spp. identified from burn wounds include *C. albicans*, *C. tropicalis*, and *C. parapsilosis* [113]. Using a burned mouse model to study the effect of a recent *P. aeruginosa* infection on the development of systemic *C. albicans* infection, Neely and co-workers (1986) found that burned mice pre-infected with a sublethal dose of *P. aeruginosa*, followed by a sublethal challenge with *C. albicans*, had a significantly higher mortality rate than either unburned mice, challenged in the same way, or burned mice infected with only one of the microbes [112]. They found that burned mice challenged with both organisms died due to *Candida* infection and indicated the importance of proteolytic enzymes secreted by *P. aeruginosa* in allowing the establishment of lethal *C. albicans* infections. This is in contrast to a study by Gupta and co-workers (2005), who found significant *in vivo* inhibition of *C. albicans* growth in the presence of *P. aeruginosa* in burn wounds [114].

### 3.3. Interaction in the Gastrointestinal Tract

*C. albicans* is a commensal member of the gastrointestinal microbiota of many mammals, including humans [115,116]. Certain immunocompromised patients, such as cancer patients, are at risk of developing invasive *Candida* infections that may originate from the gastrointestinal system. Similarly, these patients are also at higher risk of *P. aeruginosa* bloodstream infections originating from the gastrointestinal tract [117]. In order to study the interaction between *C. albicans* and *P. aeruginosa*, a neutropenic murine model for gastrointestinal co-colonization was developed by Lopez-Medina and co-workers (2015) [118]. The reported antagonism between *C. albicans* and *P. aeruginosa*, often seen *in vitro*, was not seen in this model, with levels of both *P. aeruginosa* and *C. albicans* unaffected by co-colonization. In addition, mice co-colonized with *P. aeruginosa* and *C. albicans* had significantly lower mortality compared to mice colonized with only *P. aeruginosa*. All dead mice exhibited evidence of *P. aeruginosa* dissemination. The authors showed that *C. albicans* suppressed expression of *P. aeruginosa* pyochelin and pyoverdine genes. Deletion of these genes did not affect the ability of *P. aeruginosa* to colonize the gastrointestinal tract, but did decrease the dissemination and virulence of *P. aeruginosa*. This virulence was restored after oral iron supplementation. This points to the importance of iron in the *in vivo* interaction between *C. albicans* and *P. aeruginosa*. In addition, using cultured colonocytes, they also found that *C. albicans* secreted proteins inhibited the production of cytotoxic exotoxin A. Lamont and co-workers (2002) indicated that pyoverdine regulates the production of exotoxin A, so the decrease in exotoxin A levels may also be an additional effect of *C. albicans* mediated decrease in pyoverdine production [119]. Regardless of the mechanism behind the reduction in exotoxin A, this may prevent the damage caused by *P. aeruginosa* in the gastrointestinal tract and possibly prevent dissemination. Table 1 summarizes the interaction between *C. albicans* and *P. aeruginosa* *in vivo*.

**Table 1.** Summary of the interaction between *Candida* spp. and *Pseudomonas aeruginosa* in vivo in various infection sites.

Infection Site	Type of Study/Model	Observations	Effect of <i>P. aeruginosa</i> on <i>Candida</i>	Effect of <i>Candida</i> on <i>P. aeruginosa</i>	Number(s) in Reference List
Lungs	Postoperative monitoring of surgery patients	Inhibition of <i>C. albicans</i> after subsequent colonization with <i>P. aeruginosa</i> . Reversed with antibiotic treatment	Inhibited	-	[80]
Lungs	Monitoring of patients with mechanical ventilation	Colonization with <i>Candida</i> spp. associated with increased risk of <i>P. aeruginosa</i> VAP <sup>1</sup>	-	Promoted	[81]
Lungs	Monitoring of patients with VAP <sup>1</sup>	Increase in isolation of multidrug resistant bacteria such as <i>P. aeruginosa</i> when <i>Candida</i> spp. are present. Reduced risk for <i>P. aeruginosa</i> VAP <sup>1</sup> with antifungal treatment	-	Promoted	[83,85]
Lungs	Analysis of sputum samples from CF <sup>2</sup> patients	Higher incidence of co-existence between <i>P. aeruginosa</i> and <i>Candida</i> spp. in CF <sup>2</sup> patients compared to other respiratory disorders	-	-	[98,100]
Lungs	Analysis of sputum samples from CF <sup>2</sup> patients	Presence of hyperfilamentous <i>C. albicans</i> with loss of function mutation in <i>NGR1</i> in presence of <i>P. aeruginosa</i>	No inhibition of filamentation	-	[101]
Lungs	Wistar rat model	Fungal colonization promoted pneumonia by <i>P. aeruginosa</i> Reversed with antifungal treatment	-	Promoted	[82,86]
Lungs	Mouse model	Prior <i>C. albicans</i> colonization promoted <i>P. aeruginosa</i> clearance	-	Clearance enhanced	[89,90]
Mucosa	Zebrafish swimbladder model	Synergistic increase in virulence in co-infection compared to single species infection	Increased virulence	Increased virulence	[91]
Wounds	Microbial populations of deep tissue wounds of patients with type 2 diabetes cultured	<i>C. albicans</i> not found in combination with <i>P. aeruginosa</i>	Inhibited	-	[106]

Table 1. Cont.

Infection Site	Type of Study/Model	Observations	Effect of <i>P. aeruginosa</i> on <i>Candida</i>	Effect of <i>Candida</i> on <i>P. aeruginosa</i>	Number(s) in Reference List
Wounds	Mouse model	Pre-infection with <i>P. aeruginosa</i> predisposed mice to lethal <i>C. albicans</i> infection	<i>P. aeruginosa</i> proteolytic enzymes promote lethal <i>C. albicans</i> infection	-	[108]
Wounds	Microbial analysis of wounds of burn patients	Inhibition of <i>Candida</i> spp. when <i>Pseudomonas</i> spp. were present	Inhibited	-	[110]
Wound model	<i>In vitro</i> biofilm model to mimic wounds	Biofilms of <i>C. albicans</i> , <i>Staphylococcus aureus</i> and <i>P. aeruginosa</i> created. Monotreatment with antifungal/antibiotic only shifted population dynamics without affecting overall biofilm bioburden		Provides physical support and protection to bacteria	[105]
Gastro-intestinal tract	Neutropenic mouse model	Levels of both <i>P. aeruginosa</i> and <i>C. albicans</i> unaffected by co-incubation		Decrease in siderophore production and virulence	[114]

<sup>1</sup> VAP – Ventilator-associated pneumonia; <sup>2</sup> CF – Cystic fibrosis.

#### 4. Phenotypic Plasticity in Polymicrobial Interactions

An impressive characteristic of *C. albicans* is its phenotypic plasticity, with this fungus able to exhibit up to nine distinct cell types that are dependent on environmental cues [120]. These include the classical cell types, yeast, hyphae, pseudohyphae, and chlamydospores. Furthermore, in addition to the white phenotype with heterozygosity at the mating type locus ( $a/\alpha$ ; mostly observed in laboratory conditions and infection), a white phenotype exists with either the  $a$  or  $\alpha$  genotype, as well as an opaque phenotype with heterozygosity at the mating type locus,  $a/\alpha$ , with opaque (homozygous,  $a$  or  $\alpha$ ), grey, and gastrointestinally-induced transition (GUT) phenotypes [120–123]. These non-classical phenotypes are highly dependent on the expression of the white-opaque regulator, Wor1p, that causes heritable changes, eliciting transcriptional changes, which flow over into virulence. The expression of this regulator is dependent on environmental cues, such as *N*-acetylglucosamine, a component of the bacterial cell wall, CO<sub>2</sub>, and hypoxia—an environmental circumstance frequently found in the gastrointestinal tract [122,124,125]. These phenotypes exhibit altered metabolic specialization and mating potential. For example, expression of GUT cell genes involved in iron uptake and glucose utilization is down-regulated, with an upregulation in genes involved in utilization of *N*-acetylglucosamine and short-chain fatty acids, making these cells well suited for commensal growth with resident bacteria in the gastrointestinal tract [122]. Furthermore, whereas white cells have an increased expression of genes involved in pathways associated with fermentative growth, opaque cells are more primed for oxidative respiration [121,123]. Furthermore, Malavia and co-workers (2017) described a hyper-adherent goliath phenotype induced by zinc limitation [126].

The influence of some of the classical phenotypes, such as yeast and hyphae, on the interaction with *P. aeruginosa* has been evaluated. Less information regarding the influence of non-classical phenotypic alterations on the interaction between *C. albicans* and pathobionts such as *P. aeruginosa* is available. This is especially interesting as certain niches, where co-occurrence of *C. albicans* and *P. aeruginosa* has been observed, may have the presence of environmental cues that could potentially elicit a partial or full switch to the mentioned opaque or GUT phenotypes in *C. albicans*, such as in the CF lung where bacterial cell wall components such as *N*-acetylglucosamine, are abundant due to the presence of bacteria [102], and hypoxic conditions are present [127]. However, further research is required to determine if *P. aeruginosa* could encounter other phenotypes of *C. albicans* besides the classical cell types, and whether this would cause any alterations in the interaction with this bacterium.

Similar to the functional specialization of *C. albicans*, is the remarkable phenotypic variation exhibited by *P. aeruginosa*, driven by hypermutable strains and richness in oxidative and nitrosative stresses, when in the environment of the CF lung [128–130]. Genetic and functional phenotypic variation lead to *P. aeruginosa* isolates exhibiting differences in colony morphology, extracellular polysaccharide production, motility, quorum sensing, protease activity, auxotrophy, siderophore production, and growth profiles [131]. These different phenotypes are found together, even in a single patient, and comprise a highly dynamic population of *P. aeruginosa* in chronic CF lung infections due to spatial heterogeneity and niche partitioning [131,132]. In addition, large variation in susceptibility to antimicrobials is seen with little association to morphotype. How these different phenotypes may influence the interaction of *P. aeruginosa* with pathobionts in the CF lung such as *C. albicans* has received little attention. Of special interest is the ethanol production by *C. albicans* that may be able to promote a mucoid phenotype of *P. aeruginosa* in the CF lung [56,133]. This phenotype, associated with an increase in alginate production, a component of the extracellular matrix of *P. aeruginosa*, is associated with long term chronic infection of the CF lung by *P. aeruginosa*. In addition to overproduction of alginate, the adaptation of *P. aeruginosa* to long term chronic CF infection also includes decreased expression and loss of flagellin, the type III secretion system, as well as changes in LPS structure that aid in immune evasion [128,134–137]. The possible influence of this adaptation of *P. aeruginosa* on *C. albicans* deserves further attention.

## 5. Conclusions and Future Perspectives

The interaction between *C. albicans* and *P. aeruginosa* is frequently used as a model to study the complex interplay between cross-kingdom co-infectious agents. This interaction is often characterized as antagonistic in vitro, with suppression of fungal growth by both physical association as well as secreted factors. Notably, QSMs produced by both species alter the phenotype of the other during association. The discovery of an additional *P. aeruginosa* QS participant in the *las*, *rhl*, and PQS systems, namely IQS, still warrants investigation into the influence during polymicrobial growth with *C. albicans*. Additionally, the identification of the QS activity of oxylipins and eicosanoid production in *P. aeruginosa*, as well as the known production of oxylipins and eicosanoids by *C. albicans*, further motivates research into their effects on fungal-bacterial interactions.

The in vitro antagonism of the interaction between *C. albicans* and *P. aeruginosa* may be a simplistic view of an interaction in which both participants influence the outcome of infection. In addition, this interaction is much more complex in vivo, where either species can alter colonization by the other, promoting or inhibiting disease. This seems to be highly dependent on the site of infection, immune functionality, and prior antimicrobial treatment. Both pathogens exhibit complex regulatory systems that facilitate adaptability to multiple niches within the human body. Due to this, they are rarely isolated in only one niche, as described above in the various infection sites, and exhibit alterations in their phenotypes. The occurrence of the functional specialization of both *C. albicans* and *P. aeruginosa* leading to altered phenotypes also prompts the investigation into the occurrence of these phenotypes in the presence of other microbial species, i.e., *C. albicans* in the presence of *P. aeruginosa*, and vice versa. Additionally, the influence of these phenotypes on the interaction of these two species still requires investigation.

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**SECTION C**

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**The section below forms an additional part of the complete literature review for this dissertation. This section is followed by general conclusions of *CHAPTER 1* and aims and objectives for this dissertation.**

**1.1. ANTIFUNGAL AND ANTIBIOTIC TREATMENT OF INFECTION**

The basis of treatment of infections is the inhibition or interference in various essential processes, such as cell wall biogenesis, ergosterol and DNA synthesis in fungi (Odds *et al.*, 2003; Shareck, 2011) as well as cell wall and protein synthesis and DNA replication in bacteria (reviewed by Kapoor *et al.*, 2017 and Kohanski *et al.*, 2010). Increased exposure to antibiotics and antifungals in the clinical and agricultural settings, through therapeutic and prophylactic strategies, have provided selective pressure for emergence of microbial resistance to antimicrobial agents (Hughes, 2014; Srinivasan *et al.*, 2014).

Development of resistance to antifungal agents can be due to various processes, including increased drug efflux by multidrug transporters, mutations of target proteins decreasing their susceptibility, overexpression of drug targets, or altered pathways to circumvent inhibitory effects (Marichal *et al.*, 1999; Nolte *et al.*, 1997; Perea *et al.*, 2001; Sanglard *et al.*, 1995; 1997; Wirsching *et al.*, 2002). Similarly, bacterial antibiotic resistance mechanisms include changes in outer membrane permeability, decreased uptake or increased efflux of drugs, modification of the target molecules and inactivation of antibiotics (Kapoor *et al.*, 2017). Furthermore, severe side effects in patients and the formation of hyper-resistant biofilm structures can hamper treatment (Anderson, 2005; Cowen *et al.*, 2002; Nett *et al.*, 2014; Sanglard and Odds, 2002; Shareck, 2011; Silva *et al.*, 2017). The formation of these biofilms and the interaction with co-infecting bacterial and fungal pathogens can also increase the antimicrobial resistance of infecting agents (Costa-Orlandi *et al.*, 2017; Kim *et al.*, 2018; Stacy *et al.*, 2016; Wolcott *et al.*, 2013). This then necessitates the search for new strategies in antimicrobial treatment (Shareck, 2011). This may be accomplished by development

of new antimicrobial compounds or combination therapies with compounds that may increase susceptibility to known antibiotics and antifungals.

## 1.2. FATTY ACIDS AS POTENTIAL ANTIMICROBIAL MOLECULES

The antimicrobial action of fatty acids (FAs) has been reported as early as the late 1880s, with inhibition of growth of *Bacillus anthracis* (Thormar, 2010; Joon *et al.*, 2018). In addition, fatty acids are proposed to be a component of the skin's innate immune system (Chen and Tsao, 2013; Yoon *et al.*, 2018). Fatty acids are components of membranes and involved in energy storage and signalling pathways (Pohl *et al.*, 2011). They are characterised as a hydrocarbon chain (hydrophobic) and a carboxylic acid functional group (hydrophilic) and thus exhibit amphipathic properties. Fatty acids are naturally even numbered with 4 to 28 carbon atoms, with less than 8 carbon atoms regarded as short chain, more than 12 carbons regarded as long chain and between 8 and 12 carbon atoms regarded as medium chain FAs (Pohl *et al.*, 2011; Yoon *et al.*, 2018). Furthermore, they can be saturated, with carbon atoms linked by single covalent bonds, or unsaturated. The latter fatty acids contain one or more double bonds between carbon atoms that introduce bends into the carbon chain, with FAs with more than one double bond between carbon atoms termed polyunsaturated fatty acids (PUFAs). Interestingly, medium to long chain FAs exhibit the greatest efficacy as antimicrobial agents against bacteria (Galbraith *et al.*, 1971; Kodicek and Worden, 1945; Yoon *et al.*, 2018). Lauric acid (C12:0) was shown to be the most potent inhibitor of growth in Gram positive bacteria when evaluating FAs with 6-18 carbons (Kabara *et al.*, 1972). Furthermore, unsaturated FAs such as oleic acid (C18:1), linoleic (C18:2) and linolenic acid (C18:3) also exhibit strong antibacterial activities (Galbraith and Miller, 1973).

The antibacterial activity of FAs is mainly due to alterations in cell membranes, interrupting cellular processes due to membrane destabilisation and subsequent increased membrane fluidity and permeability (Greenway and Dyke, 1979; Yoon *et al.*, 2018). This is, in part, due to incorporation of FAs into membrane phospholipids, causing alterations in phospholipid structure (Baker *et al.*, 2018; Giles *et al.*, 2011; Wier *et al.*, 2010). The main processes that FAs can affect in bacteria, include disruption of electron transport chain and oxidative phosphorylation, where FAs bind to electron carriers as well as decrease membrane potential and proton gradient.

Furthermore, FAs can directly inhibit membrane-bound enzymes such as glucosyltransferase, inhibit nutrient uptake and induce loss of ribonucleic acid (Speert *et al.*, 1979; Won *et al.*, 2007; Yoon *et al.*, 2018). Fatty acids can also influence virulence associated characteristics (Baker *et al.*, 2018; Kenny *et al.*, 2009; Liaw *et al.*, 2004; Moravec *et al.*, 2017; Sun *et al.*, 2012).

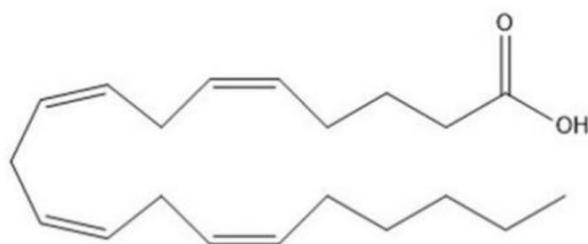
In addition to bacteria, these antimicrobial effects are reported against enveloped viruses, malaria parasites and fungi (Das, 2018; Kohn *et al.*, 1980; Krugliak *et al.*, 1995). In fungi, FAs induce cytotoxicity (Bergsson *et al.*, 2001; Carballeira, 2008; Deva *et al.*, 2000). In addition, they can reduce the virulence of *C. albicans* through inhibition of morphogenesis (Clement *et al.*, 2007; McLain *et al.*, 2000; Murzyn *et al.*, 2010; Noverr and Huffnagle, 2004; Shareck and Belhumeur, 2011). The mechanism in fungi is similar to that observed in bacteria, with FAs interacting and inserting themselves in the lipid bilayer of fungal membranes, increasing fluidity, eliciting disorganisation of the membrane and affecting the function of membrane proteins (Avis and Bélanger, 2001; Pohl *et al.*, 2011). Furthermore, FAs can inhibit lipid synthesis and protein synthesis (Carballeira *et al.*, 2004; McLain *et al.*, 2000; Pohl *et al.*, 2011). In addition, FAs have potential in antifungal therapies, where they can increase antifungal susceptibility (Ells *et al.*, 2009; Pohl *et al.*, 2011). Interestingly, FAs, especially PUFAs, can act as pro- or anti-inflammatory mediators, with  $\omega$ -6 PUFAs associated with pro-inflammatory effects, whereas  $\omega$ -3 PUFAs are associated with anti-inflammatory and immunosuppressive effects (Calder, 2009; 2013; Rutting *et al.*, 2019).

### 1.2.1. Arachidonic acid

Arachidonic acid (AA) (**Figure 1.1**), or all-*cis*-5,8,11,14-eicosatetraenoic acid is an  $\omega$ -6 C20 fatty acid present in mammalian cell types mostly esterified to membrane phospholipids in the sn-2 position (Brash, 2001; Hanna and Hafez, 2018; Martin *et al.*, 2016; Rodríguez *et al.*, 2014). It can be obtained by mammalian cells through diet or elongation of C18 fatty acids such as linoleic acid (Chilton *et al.*, 1996). Due to the presence of four double bonds, it is important for normal cellular membrane fluidity, and readily reacts with molecular oxygen (Yin *et al.*, 2011).

Arachidonic acid is released in large amounts from the sn-2 position of phospholipids through the action of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) during inflammation (Rodríguez *et al.*, 2014). Among the 22 genes encoding PLA<sub>2</sub> categorized into three types (secreted

PLA<sub>2</sub>, intracellular group VI calcium-independent PLA<sub>2</sub> and group IV cytosolic PLA<sub>2</sub>, group IV cytosolic PLA<sub>2α</sub> possesses specificity to AA and mediates its release (Park *et al.*, 2006; Schaloske and Dennis, 2006). The release of AA is tightly controlled through the cytosolic PLA<sub>2α</sub> post-translational modification, including phosphorylation and Ca<sup>2+</sup>-induced translocation from the cytosol to membranes with esterified AA (Rodríguez *et al.*, 2014). The release of AA and downstream oxygenated products can be stimulated by bacterial lipopolysaccharide and fungal cell wall components such as β(1,3)-glucans and play substantial roles as lipid signals (Chilton *et al.*, 1996; Rodríguez *et al.*, 2014). Infection by *P. aeruginosa* can induce the release of AA through ExoU and intracellular phospholipase (Agard *et al.*, 2013; König *et al.*, 1996; Sadikot *et al.*, 2007; Saliba *et al.*, 2005). Similarly, *C. albicans* can release AA by the action of its own enzymes that show PLA<sub>2</sub> activity (Deva *et al.*, 2000).



**Figure 1.1.** Structure of arachidonic acid (C20:4)

#### 1.2.1.1. Oxygenated products of AA

Oxygenated products of AA can form via non-enzymatic processes, forming isoprostanes or through the action of various enzymatic processes yielding eicosanoids (Dennis and Norris, 2015). These products display functions in various physiological processes such as inflammation, allergy and pain. Eicosanoids are formed through the oxidation of arachidonic acid by lipoxygenases, cyclooxygenases and cytochrome P450s. The route of AA can give rise to various products, e.g. leukotrienes, epoxyeicosatrienoic acids via modification through lipoxygenases and cytochrome P450s, and thromboxanes and prostaglandins through the action of cyclooxygenases (Dennis and Norris, 2015; Liu *et al.*, 2016; Peters-Golden and Henderson, 2007; Spector, 2009). The most noteworthy prostaglandin being prostaglandin E<sub>2</sub>, the predominant eicosanoid generated in response to pro-inflammatory cytokines, fungal pathogen-associated molecular patterns and toll-like receptor ligands (Agard *et al.*, 2013; Dennis and Norris, 2015). Prostaglandin E<sub>2</sub> is

involved in homeostasis, inflammation, pain and tumorigenesis (Davies *et al.*, 1984; Sugimoto *et al.*, 2000; Wang and Dubois, 2010). Interestingly, inhibition of prostaglandin synthesis primes the innate immune system and increases leukocyte killing of bacteria (Stables *et al.*, 2010). Furthermore, PGE<sub>2</sub> promotes *C. albicans* biofilm formation and inhibition of PGE<sub>2</sub> production reduces *C. albicans* biofilm formation and increases susceptibility to fluconazole (Mishra *et al.*, 2014).

#### 1.2.1.2. Free fatty acid form of AA

Although the oxygenated products of AA are tightly involved in the inflammatory response towards infection (Dennis and Norris, 2015), the non-oxygenated fatty acid may also play important roles during infection and is proposed to be an endogenous antimicrobial molecule that is released by immune cells as a defence mechanism to invading pathogens (Das, 2018). Arachidonic acid exhibits toxic effects in various bacterial species, including *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Baker *et al.*, 2018; Beavers *et al.*, 2019; Eijkelkamp *et al.*, 2018). Similar to other PUFAs, this is due to insertion in the membrane and subsequent increase in membrane fluidity. Furthermore, AA causes toxicity in *S. aureus* due to lipid peroxidation (Beavers *et al.*, 2019). Interestingly, exposure to AA did not alter the susceptibility of *S. pneumoniae* to antibiotic or metal ion stress (Eijkelkamp *et al.*, 2018). Furthermore, *S. pneumoniae* treated with AA was more resistant to killing by human antimicrobial peptide LL-37. Exposure of *P. aeruginosa* induced an increase in antibiotic resistance as well as a decrease in swimming motility and increase in biofilm formation (Baker *et al.*, 2018). This suggests that PUFAs, such as AA, can both assist in pathogen killing and inhibition of their growth and virulence, as well as negatively affect patient outcomes and exacerbate resistance of bacterial pathogens to antibiotic treatment.

Contrary to this, AA has been shown to decrease antifungal resistance in pathogenic yeast such as *C. albicans* and *C. dubliniensis* (Ells *et al.*, 2008). Furthermore, it is able to inhibit morphogenesis in *C. albicans* (Shareck and Belhumeur, 2011). The reason for these effects on *C. albicans* is still unknown, however, AA incorporates into phospholipids and has also been shown to modify ergosterol levels in *C. albicans* (Ells *et al.*, 2009). Therefore, the effects observed may be due to alterations in membrane fluidity and disorganisation.

### 1.3. GENERAL CONCLUSIONS FOR CHAPTER 1

In **Section B**, a detailed overview of the interaction between *Candida* species (especially *C. albicans*) with *P. aeruginosa* is given, highlighting several aspects of interaction that still requires further investigation, for example, the influence of phenotypic plasticity of both pathogens on their interaction. Widespread use of antibiotics and antifungals in the clinical sector has provided selective pressure of microorganisms to develop resistance towards these substances. Therefore, a need to identify novel therapeutic strategies has arisen. Multiple reports have provided evidence of the antimicrobial properties of fatty acids towards both bacterial and fungal species. Arachidonic acid is an especially interesting fatty acid, as it is available in ample amounts during infection and *in vitro* studies frequently overlook its contribution to the host environment. Furthermore, it plays a crucial role during infection through acting as a precursor for immune modulating eicosanoids. When *P. aeruginosa* is exposed to AA it not only enhances biofilm formation, but also decreases susceptibility to antibiotic compounds. In contrast, AA can increase the susceptibility of *C. albicans* towards antifungals. However, investigation into the mechanisms of this is still required. Furthermore, the effect of AA on mixed infections of *C. albicans* and *P. aeruginosa* is still unknown.

#### 1.4. RESEARCH AIMS AND OBJECTIVES

With the above information as background, the aims of this dissertation are, firstly, to identify novel facets of interaction between *C. albicans* and *P. aeruginosa*, focused on *C. albicans*. Secondly, the effect of a sub-inhibitory concentration of arachidonic acid on *C. albicans* will be evaluated. Lastly, the effect of arachidonic acid on the interaction between *C. albicans* and *P. aeruginosa* will be assessed.

The following objectives will be used to accomplish the above-mentioned aims:

**Objective 1:** Determine the transcriptional response of *C. albicans* towards arachidonic acid and co-incubation with *P. aeruginosa* in polymicrobial biofilms (RNAseq).

**Objective 2:** Taking the results obtained in Objective 1 in mind, genes of interest in *C. albicans* will be identified and homozygous deletion mutants constructed with a CRISPR-Cas9 system published by Nguyen *et al.* (2017).

**Objective 3:** The homozygous deletion mutants will be used to determine the role of selected genes in the response of *C. albicans* to arachidonic acid and *P. aeruginosa*. This will entail assessment of biofilm formation and survival of *C. albicans* in the presence of *P. aeruginosa*. Furthermore, the effect on virulence and innate immune induction will be evaluated using the A549 Dual™ lung epithelial cell line and *Caenorhabditis elegans* infection model.

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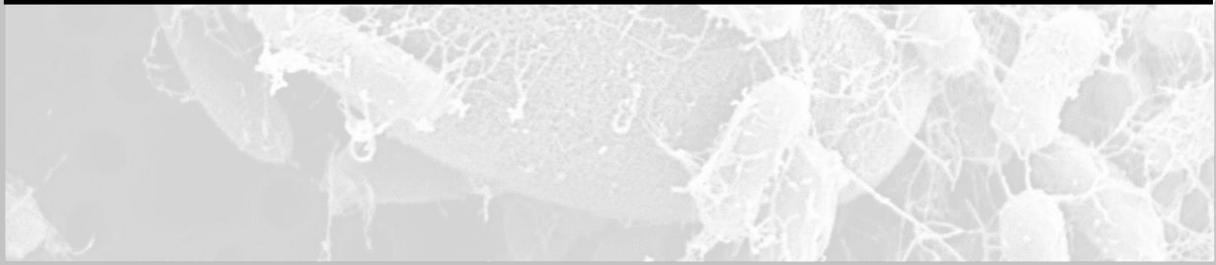
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## **CHAPTER 2**

**Transcriptomic analysis of the influence of arachidonic acid on polymicrobial biofilms of *Candida albicans* and *Pseudomonas aeruginosa***



**PLEASE NOTE**

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The following chapter provides a comprehensive overview of the response of *Candida albicans* towards arachidonic acid in the presence and absence of *Pseudomonas aeruginosa*. *Chapter 3* and *Chapter 4* are based on the *Results and Discussion* presented in this Chapter. However, *Chapter 3* and *Chapter 4* are written as standalone research articles. Therefore, duplication of some information in *Chapter 3* and *Chapter 4* is unavoidable.

## 2.1. ABSTRACT

Interkingdom microbial interactions, in the clinical setting, can alter infection dynamics and patient outcomes. This is especially true for the polymorphic yeast *Candida albicans*. Although the interaction between *C. albicans* and *Pseudomonas aeruginosa* is characterised as antagonistic *in vitro*, the association between the two species can exacerbate patient morbidity and mortality. *Candida albicans* and *P. aeruginosa* both exhibit resistance towards antifungal and antibiotic treatments respectively. This necessitates the identification of novel treatments. Polyunsaturated fatty acids (PUFAs) possess antimicrobial properties. The PUFA, arachidonic acid (AA), is released during infection with *C. albicans* and *P. aeruginosa* and has been shown to increase susceptibility of *C. albicans* to azole antifungal agents. However, the mechanism of this is unknown. To gain insight into the mechanism of action of AA on *C. albicans*, both alone, or in combination with *P. aeruginosa*, we used RNAseq to determine the transcriptomic response of the yeast. Overrepresented Gene Ontology (GO) terms (with PANTHER) and transcription factors (with PathoYeasttract) were used to identify processes in *C. albicans* that were affected by co-incubation with *P. aeruginosa* and treatment with AA. In addition, Nanostring (nCounter<sup>®</sup>) was used to confirm differential expression of selected genes. The response of *C. albicans* towards *P. aeruginosa* was dominated by a response to hypoxia. In addition, genes associated with regulation of the yeast-to-hyphal switch and white-opaque switching were differentially expressed. Genes associated with membrane organisation were differentially expressed when *C. albicans* biofilms were treated with AA. Interestingly, several of these genes involved in membrane organisation were not differentially expressed when polymicrobial biofilms were exposed to AA, however, exacerbated stress responses (including chemical, xenobiotic, drug, hydroxy compound and oxidative) were seen. The present study not only identified novel facets of interaction between *C. albicans* and *P. aeruginosa*, but also provides information regarding the response of *C. albicans* to AA.

## 2.2. INTRODUCTION

Interactions between microorganisms can alter their growth through shifting population dynamics and fostering synergism between co-inhabitants (Polke *et al.*, 2015; Stacey *et al.*, 2016). This is accomplished through secreted molecules, nutrient availability as well as physical interaction (De Sordi and Mühlischlegel, 2009; Elias and Banin, 2011; Stacey *et al.*, 2016). In the context of human health, these interactions can prevent or promote disease (Braga *et al.*, 2016; Morales and Hogan, 2010). The opportunistic fungal pathogen, *Candida albicans*, is known to form associations with commensal as well as co-infecting bacteria (Diaz *et al.*, 2012; Neville *et al.*, 2015). At the core of its virulence, is the ability to interchangeably switch between yeast, pseudohyphal and hyphal morphologies (Polke *et al.*, 2015). In addition, it can form robust, antifungal resistant biofilms on biotic and abiotic surfaces, such as medically implanted devices. These biofilms can either be monomicrobial, or can be formed together with other microbial species, such as the Gram-negative bacterium *Pseudomonas aeruginosa* (Morales and Hogan, 2010). Co-infection by these pathogens increases morbidity and mortality (Bergeron *et al.*, 2017). Interestingly, an antagonistic interaction between them is observed *in vitro* (Fourie and Pohl, 2019). The close-proximity and contact provided by the biofilm structure enables both physical and indirect interaction between the fungus and bacterium. *Pseudomonas aeruginosa* was indicated to physically kill *C. albicans* hyphal cells as well as inhibit morphogenesis through the action of secreted compounds such as phenazines and *N*-acylhomoserine lactones (Brand *et al.*, 2008; McAlester *et al.*, 2008). In addition, overall suppression of fungal metabolic activity and respiration is seen (Trejo-Hernández *et al.*, 2014).

During infection by *C. albicans* and *P. aeruginosa*, a large amount of arachidonic acid (AA) is released from host cells through the action of phospholipase A<sub>2</sub> and microbial phospholipase activity (Castro *et al.*, 1994; Deva *et al.*, 2000; Kirschnek and Gulbins, 2006; McDermott *et al.*, 2013; Parti *et al.*, 2010; Saliba *et al.*, 2005; Smeekens *et al.*, 2010; Smith *et al.*, 2002). This fatty acid acts as a precursor for immune modulating compounds (eicosanoids) within the host (Dennis and Norris, 2015). Interestingly, both *C. albicans* and *P. aeruginosa* are reported to produce significant quantities of eicosanoids *in vitro* with supplementation of AA (Erb-Downward and Noverr, 2007; Fourie *et al.*, 2017; Lamacka and Sajbidor, 1995; Noverr *et al.*, 2001). In addition to acting as a precursor for eicosanoids, AA has been proposed to act as an antimicrobial

molecule with antibacterial and antiviral properties (Das, 2018). Furthermore, it has been observed to inhibit the yeast-to-hyphal switch, as well as increasing susceptibility of *C. albicans* to antifungal agents (Ells *et al.*, 2009; Shareck and Belhumeur, 2011). However, the mechanism of this is still unknown.

The advent of high throughput sequencing technologies, such as RNAseq, enables the monitoring and analysis of the transcriptomic response of a cellular population towards a stimulus. Through using this technique, the aim of this research chapter is to elucidate the effect of AA on *C. albicans* in both monomicrobial biofilms as well as in polymicrobial biofilms with *P. aeruginosa*. In addition, we aim to identify novel facets of interaction between *C. albicans* and *P. aeruginosa*.

## 2.3. MATERIALS AND METHODS

### 2.3.1. Formation of mono- and polymicrobial biofilms

#### 2.3.1.1. Monomicrobial biofilm formation by *C. albicans*

*Candida albicans* SC5314 (wild type) was grown on YM agar for 24 h at 30°C and was inoculated into 10 mL yeast nitrogen base (YNB) broth (10 g/L glucose, 16 g/L YNB) and incubated at 30°C for 24 h. Cells were harvested at 1878g for 5 minutes and the supernatant removed. This was followed by washing the cells twice with phosphate buffered saline (PBS) (Oxoid, England). The cells were then counted with a hemocytometer and diluted to  $1 \times 10^6$  cells/mL in 20 mL filter sterilized (0.22  $\mu$ m nitrocellulose filter, Merck Millipore, Ireland) RPMI-1640 medium (Sigma-Aldrich, USA) and dispensed into 90 mm polystyrene petri dishes (Merck, Germany). In addition to the cells, 0.1 mM of AA (Sigma-Aldrich, USA) (Stock of 1 g in 25 mL of absolute ethanol reaching a concentration of 131.4 mM) or the equivalent ethanol vector (0.076% total ethanol) was added to each petri dish containing medium plus cells (adapted from Ells *et al.*, 2011; Fourie *et al.*, 2017). Petri dishes were covered with parafilm and incubated for 6 h at 37°C to allow biofilm formation. Biofilms were harvested at 6 hours to combat transcriptional changes due to glucose starvation, as glucose was depleted after 6 hours incubation in RPMI-1640 medium (data not shown).

### 2.3.1.2. Polymicrobial biofilm formation by *C. albicans* and *P. aeruginosa*

*Pseudomonas aeruginosa* PAO1 (wild type) was grown on Luria-Bertani (5 g/L yeast extract, 10 g/L tryptone, 10 g/L sodium chloride and 15 g/L agar) plates for 24 h at 37°C. Cells were inoculated into 5 mL nutrient broth (1 g/L malt extract, 2 g/L yeast extract, 5 g/L peptone and 8 g/L sodium chloride) and incubated at 37°C for 24 h with shaking (150 rpm). These cells were washed (X3) and diluted to an optical density (OD<sub>600</sub>) of approximately 0.05 in 20 mL RPMI-1640 medium containing 1 x 10<sup>6</sup> cells/mL *C. albicans* (prepared as described in Section 2.3.1.1) and dispensed into 90 mm polystyrene petri dishes. In addition to the cells in the medium, 0.1 mM of AA (or 0.076% total ethanol) was added to each petri dish (adapted from Fourie *et al.*, 2017). Petri dishes were covered with parafilm and incubated for 6 h at 37°C to allow biofilm formation.

### 2.3.2. Biofilm collection and storage

After incubation to allow biofilm formation, supernatant was aspirated from mono- and polymicrobial biofilms and 2 mL RNA*later* (Invitrogen) was added to each biofilm to combat degradation of RNA. Biofilms were scraped off and a total of 5 biofilms were collected and pooled per sample. This was done in triplicate for both mono- and polymicrobial biofilms. Samples were frozen at -80°C until RNA extraction.

### 2.3.3. Total RNA extraction

Samples were thawed on ice, centrifuged at 4000g to collect cells and RNA*later* was aspirated. Total RNA was extracted from samples using RNeasy protect mini kit (Qiagen) with DNA removal (Rnase-Free Dnase Set, Qiagen) according to manufacturer's instructions. The RNA samples were evaluated by the Centre for Proteomic and Genomic Research (CPGR) prior to sequencing. This included checking for contaminants with the use of the NanoDrop ND1000, determining absolute concentration using the Qubit<sup>®</sup> RNA HS Assay Kit, as well as evaluating the integrity using the Agilent Bioanalyzer Nano Assay. As genomic DNA may interfere with subsequent processes, contamination with genomic DNA was included. Samples with contaminants were cleaned up with an Agencourt RNAClean XP Kit (Beckman Coulter) and the quality was evaluated again. A total of 1 µg of RNA per sample was treated with the Illumina Ribo-Zero rRNA Removal Kit to remove ribosomal RNA. After ribosomal RNA removal, samples were purified (Agencourt RNAClean XP Kit,

Beckman Coulter) and indexed libraries were prepared using the ScriptSeq™ v2 RNA-Seq Library Preparation Kit and ScriptSeq™ Index PCR Primers – Set 1 (Illumina). The sizes of the libraries were profiled with the Bioanalyzer High Sensitivity Assay Kit (Agilent) and quantified (Qubit® HS DNA Assay Kit). Samples were diluted and a 1% Phix control library (Illumina) was spiked into samples. Sequencing was completed on the Nextseq 500 (Illumina) using Nextseq 500 High Output (150 cycle) Kits which yielded paired-end reads with between 96% and 97% of total data having bases above Q30.

#### 2.3.4. Analysis of differentially expressed genes

The resultant fastq files were analysed for quality by FastQC (v0.11.5; Andrews, 2010) and low-quality reads and bases were discarded using PRINSEQ-lite (v0.20.4; Schmieder and Edwards, 2011). *Candida albicans* samples (monomicrobial and polymicrobial) were aligned to the *C. albicans* SC5314 genome (assembly 21; The *Candida* Genome Database; Skrzypek *et al.*, 2017) via TopHat2 (Kim *et al.*, 2013; Trapnell *et al.*, 2012) using the fr-secondstrand option which gave the best overall alignment rate (Dutton *et al.*, 2016). In addition, unaligned reads of the polymicrobial biofilms that did not map to the *C. albicans* genome, were mapped to the *P. aeruginosa* PAO1 genome (NCBI Resource Coordinators, 2016). Aligned files (*C. albicans* and *P. aeruginosa*) of polymicrobial biofilms were merged with SAMtools (Li *et al.*, 2009). The resultant BAM files constructed with TopHat2, were used to construct gene expression count tables with the use of the BEDTools multicov command (Quinlan and Hall, 2010). Differences between expression of genes can be determined by ranking them according to a fold change of expression between control and experimental samples. However, this can lead to gross inaccuracies due to the low sample size of RNAseq data, such as  $n=3$  in this case (Love *et al.*, 2014). Therefore, gene counts were analysed with DESeq2 (Love *et al.*, 2014). This resource considers the high variance of lowly expressed genes with small sample sizes (which could generate many false positives) by comparing single gene variance with the variance of the total dataset and concurrent shrinkage of the dispersion (within sample variance) to more accurately determine significant differential expression across expression sizes. This was done for the conditions listed in **Table 2.1**. After differential expression analysis, heatmaps and principle component analysis plots were constructed with DESeq2 (Love *et al.*,

2014), with modified commands described at “<https://gist.github.com/stephenturner/f60c1934405c127f09a6>”.

**Table 2.1:** Conditions used to determine effect of arachidonic acid (AA) or equivalent ethanol control on *Candida albicans* (CA) monomicrobial biofilms or polymicrobial biofilms with *Pseudomonas aeruginosa* (PA).

No.	Experimental condition	Control condition	Abbreviations
1	CA polymicrobial biofilms with PA treated with 0.076 % ethanol	CA monomicrobial biofilms treated with 0.076 % ethanol	CPET/CET
2	CA monomicrobial biofilms treated with 0.1 mM AA	CA monomicrobial biofilms treated with 0.076 % ethanol	CAA/CET
3	CA polymicrobial biofilms with PA treated with 0.1 mM AA	CA polymicrobial biofilms with PA treated with 0.076 % ethanol	CPAA/CPET

To determine the effect of the mentioned conditions on the transcriptome of *C. albicans*, an approach was utilised that includes the functional analysis of gene lists to determine overrepresented functional classes of genes. The functional classes, or Gene Ontology (GO) terms, are organised into three branches, namely biological process, molecular function and cellular component (The Reference Genome Group of the Ontology Consortium, 2009). For this research, GO cellular component will be used to evaluate localisation of genes of interest, as well as the function of genes via GO biological process. The functional classification of genes is reliant on direct experimental evidence supporting the function of genes, or homology (e.g. sequence homology) to related gene products of organisms with biomedical impact (The Reference Genome Group of the Ontology Consortium, 2009). It is necessary to mention that, although the functional annotation of genes is ever expanding, only a portion of *C. albicans* genes are annotated, with many genes with unknown function (as of January 27, 2020, 70.20 % or 4365 of open reading frames are still uncharacterised; Skrzypek *et al.*, 2017). Thus, the data represented here in terms of gene enrichment analysis do not incorporate many genes currently without GO annotations. To determine which functional classes of genes are overrepresented in our datasets, PANTHER (Protein Alignment Through Evolutionary Relationship) was utilised (Mi *et al.*, 2013; 2016). Overrepresented, in this case, means that the number of genes contributed to a term is higher than expected by chance for that term, if a

random sample of genes were taken. This is evaluated statistically with a Fisher's exact test with False Discovery Rate (FDR) multiple test correction (q-value), dependent on the number of genes associated to a term and the number of genes in your dataset. All results shown have an overall FDR (calculated by the Benjamini-Hochberg procedure) of below 0.05, even if individual values are above this value. Functional classification of datasets was performed by dividing the induced and repressed genes to find functional classes of genes that were either induced or repressed.

*Candida albicans* possesses a complex transcriptional circuit that enables rapid and genome-wide adaptation to the changing environment of the human host. Analysis of transcription factors (TFs) may give insight into the change of cellular processes as well as virulence associated characteristics (Pérez *et al.*, 2013). Transcription factor gene expression level, rather than its regulatory response, is a more accurate predictor of function (Xu *et al.*, 2015). Therefore, gene lists of differentially expressed genes were evaluated for overrepresented TFs. Two tools are currently available online to assist researchers in this for *C. albicans*. Firstly, the Transcription Factor binding site Search Tool (TfbsST) by Nina Konstantinidou (2016) searches for 47 binding motifs upstream or downstream of genes, however, this is not corroborated with experimental data and gives an indication of potential regulation. In contrast, Pathogenic Yeast Search for Transcriptional Regulators And Consensus Tracking (PathoYeasttract) is a curated repository of all known regulatory associations between TFs and target genes of *C. albicans* that is based on bibliographic references (Monteiro *et al.*, 2016). In addition, the overrepresentation of TFs can be evaluated statistically, where a *P*-value is given, indicative of the probability of a TF regulating an equal or higher number of genes than expected by chance in a dataset of equal size (Monteiro *et al.*, 2016). This probability is modelled by a hypergeometric distribution and undergoes Bonferroni correction for multiple testing. For this research, PathoYeasttract was used to determine overrepresented TFs.

### 2.3.5. Confirmation of differential expression with nCounter®

Confirmation of differential expression was performed using nCounter® with Elements™ XT Reagents according to manufacturer's specifications. A multiplexed probe library (nCounter® elements CodeSet) was designed with two sequence-specific

probes for genes of interest (**Supplementary Table S2.1**). Probes were mixed with approximately 100 ng of purified total RNA (see *Section 2.3.3*) and allowed to hybridize (20 hours, 67°C). Samples were loaded on an nCounter® SPRINT™ Cartridge and processed with an nCounter® SPRINT Profiler (NanoString Technologies, USA). Results were processed in nSolver 4.0 software. This was done for one sample of each condition (see **Table 2.1** for conditions).

### 2.3.6. Data availability

The data in this chapter have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO Series accession number **GSE136726** (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE136726>). Datasets are private until publication; however, access can be granted from the author, or from Prof. C. H. Pohl-Albertyn for the purpose of the revision process (See **Supplementary Text S2.1**).

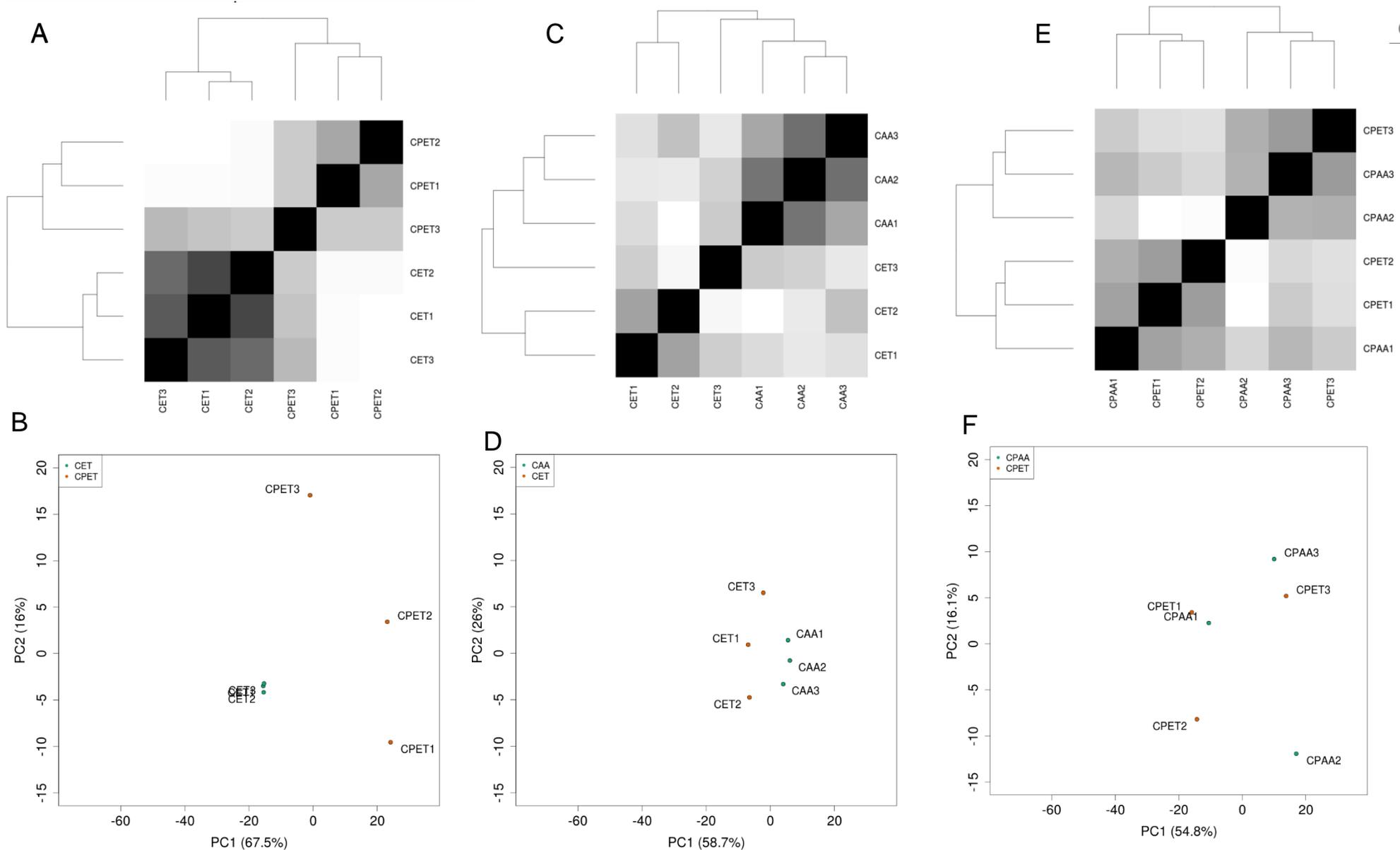
## 2.4. RESULTS AND DISCUSSION

### 2.4.1. Exploratory analysis of RNAseq data

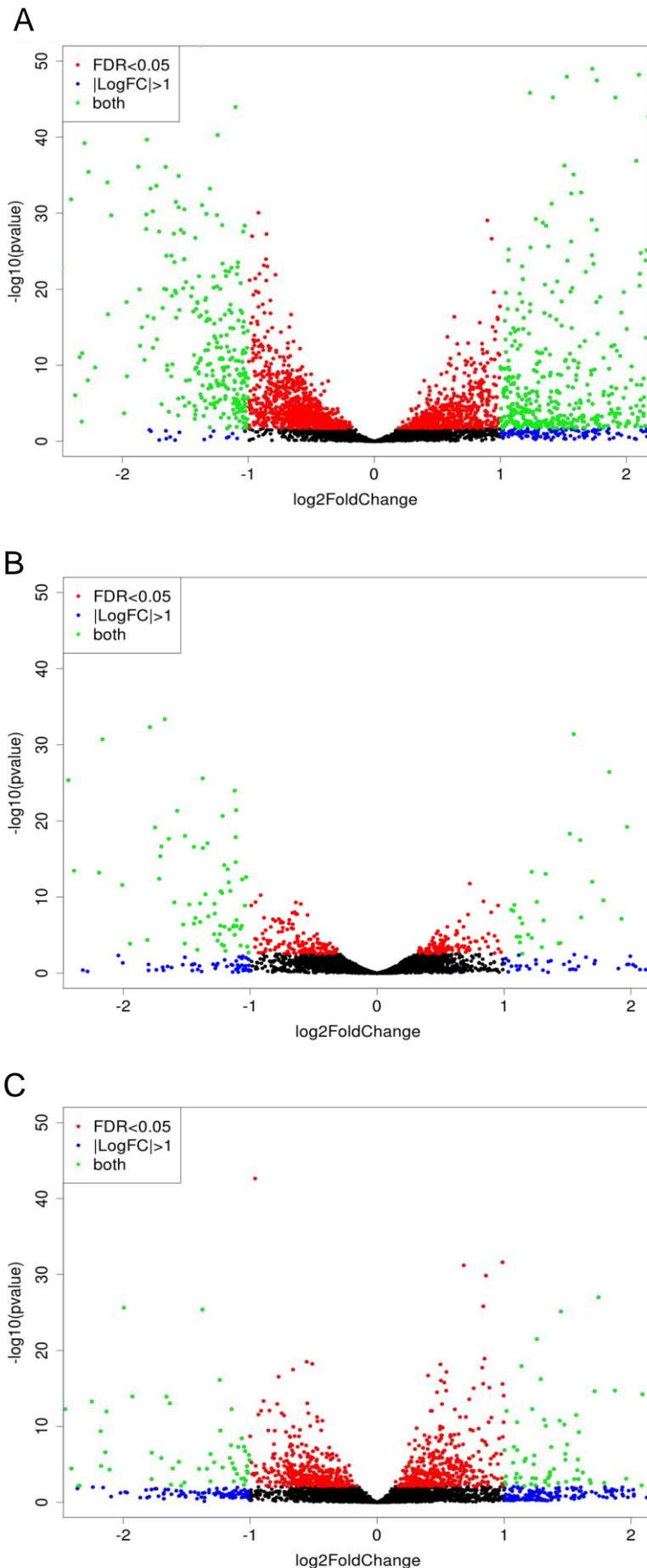
Analysis of raw data revealed excellent quality data, with approximately 92.6% of sequences having a Phred-score of above Q30. An average of 4.3% of low-quality sequences were removed (PRINSEQ-lite; Schmieder and Edwards, 2011), yielding an average of  $126.2 \times 10^6$  sequences (150 bp paired-end) per sample. As explained in *Section 2.3.4*, these raw reads were aligned to the genome of *C. albicans* (SC5314) and further evaluated for differential expression. Complete expression profiles were compared to determine overall differences. **Figure 2.1** indicates the distance between control and experimental biofilms using sample distance heat maps as well as principle component analysis plots (Love *et al.*, 2014). As seen in **Figure 2.1**, monomicrobial biofilms of *C. albicans* exposed to the ethanol vector are very similar in terms of their expression profiles with low distance between replicates, compared to polymicrobial biofilms. *Candida albicans* co-cultured with *P. aeruginosa* (exposed to ethanol vector) also cluster together (**Figure 2.1A**), however, large variation is seen between replicates (**Figure 2.1B**) compared to monomicrobial counterparts. These differences in gene expression profiles are expected due to large variation in population dynamics and microcolony formation in polymicrobial biofilms (Stacy *et al.*, 2016). The large distance between mono- and polymicrobial biofilms is apparent through the amount of

significantly differentially expressed genes (**Figure 2.2A**) with a total of 2537 open reading frames (ORFs) (with a  $\text{padj} < 0.05$ ) corresponding to approximately 40% of the *C. albicans* transcriptome being altered due to the presence of *P. aeruginosa* (**Supplementary Table S2.2**). Furthermore, 917 ORFs are significantly differentially expressed at a  $\log_2$  fold change (L2FC) threshold of above 1 or below -1 in the presence of *P. aeruginosa* (**Figure 2.2A**). In comparison to the influence of co-incubation, a modest response of *C. albicans* monomicrobial biofilms towards 0.1 mM AA is seen (**Supplementary Table S2.3**), with only 354 ORFs significantly differentially expressed ( $\text{padj} < 0.05$ ) and only 63 above the L2FC threshold (**Figure 2.2B**). Furthermore, less variation between samples when comparing monomicrobial biofilms in the presence and absence of AA is seen (**Figure 2.1C and D**).

Although the response of monomicrobial biofilms towards AA does not reveal a large number of significantly differentially expressed genes, a larger number of significantly differentially expressed genes (969 ORFs) is seen for *C. albicans* in polymicrobial biofilms exposed to AA (**Supplementary Table S2.4; Figure 2C**). However, only 89 of ORFs are significantly differentially expressed above the L2FC threshold, indicating that most of the significantly differentially expressed may not cause significant phenotypic differences due to small changes in expression. Interestingly, when evaluating the distance between samples of polymicrobial biofilms exposed to AA or equivalent ethanol control, large distances between replicates in the same condition is seen. In addition, samples from different conditions seem to cluster together (**Figure 2.1E and F**), reflecting a very heterogenous change in expression of genes of polymicrobial biofilms exposed to AA. These large differences between samples may be due to the biogeography of the polymicrobial biofilms. As physical interaction and nutrient availability may be drastically different in each biofilm due to the formation of microcolonies, large variation between samples concerning gene expression may be expected (Traven *et al.*, 2012). This heterogeneity in biofilm formation by different microbes is also seen *in vivo* and may cause large differences during dynamics of microbial growth and infection (Stacy *et al.*, 2016).

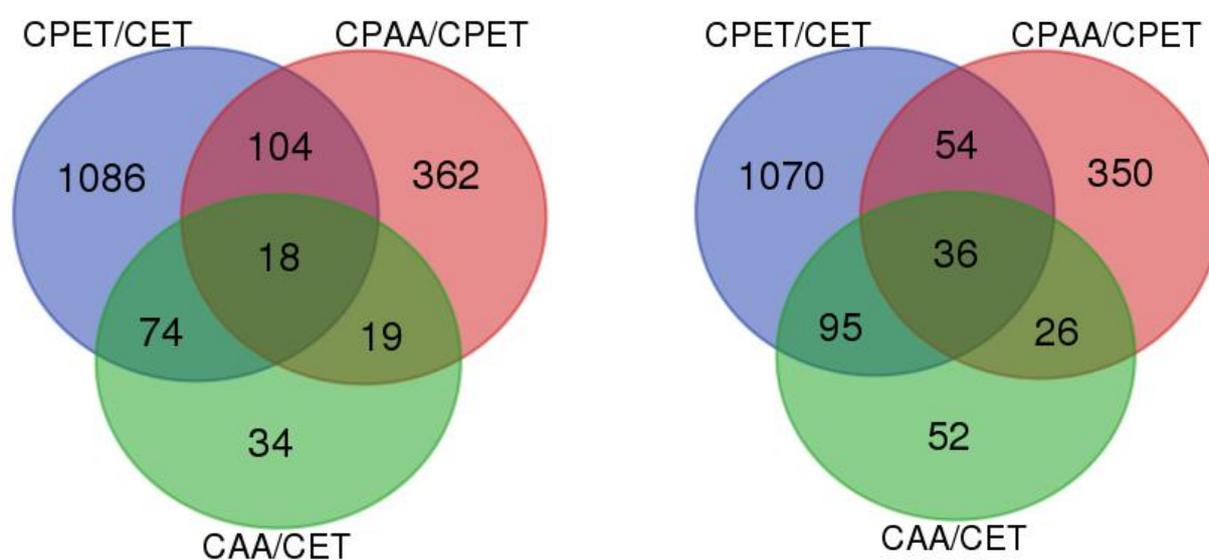


**Figure 2.1. Distance between control and experimental biofilms using sample distance heat maps and principle component analysis plots constructed with DESeq2 (Love *et al.*, 2014).** A, C and E represent sample distance heat maps. Principle component analysis plots are indicated by B, D and F. CPET – polymicrobial biofilms of *Candida albicans* and *Pseudomonas aeruginosa* exposed to ethanol vector; CET – *C. albicans* monomicrobial biofilms exposed to ethanol vector; CAA – *C. albicans* monomicrobial biofilm exposed to 0.1 mM arachidonic acid; CPAA – polymicrobial biofilms exposed to 0.1 mM arachidonic acid.



**Figure 2.2. Volcano plots of significant differentially expressed genes of *Candida albicans*.** Log<sub>10</sub>(p-value) of differentially expressed genes on y-axis with the x-axis representing the log<sub>2</sub> fold change of these genes. Red dots indicate differential expression with a False Discovery Rate (FDR) of less than 0.05. Blue dots indicate genes that are differentially expressed with a log<sub>2</sub> fold change of above 1. Green represents genes adhering to both criteria. **A** – Differentially expressed genes of *C. albicans* exposed to *Pseudomonas aeruginosa* versus monomicrobial biofilms; **B** – *Candida albicans* monomicrobial biofilms exposed to 0.1 mM arachidonic acid versus control biofilm with ethanol vector; **C** – polymicrobial biofilms exposed to 0.1 mM arachidonic acid versus polymicrobial biofilms with ethanol vector.

The data obtained by the current transcriptomic analysis of the various conditions enable us to obtain a set of genes that are differentially expressed in response to *P. aeruginosa*, as well as in response to AA in mono- and polymicrobial biofilms. This is of importance as *C. albicans* is exposed to both conditions *in vivo* in the cystic fibrosis lung where it may encounter AA as well as co-infecting pathogens such as *P. aeruginosa* (Fourie *et al.*, 2016). Genes that are significantly differentially expressed irrespective of L2FC were compared across conditions (CPET/CET; CAA/CET; CPAA/CPET). **Figure 2.3** represents the overlap of genes across conditions.



**Figure 2.3. Venn diagram of overlap of differentially expressed genes across conditions.** Left – number of repressed genes; right – number of induced genes. CPET/CET – Polymicrobial biofilms of *Candida albicans* and *Pseudomonas aeruginosa* compared to *C. albicans* monomicrobial biofilms (without 0.1 mM arachidonic acid, AA); CAA/CET – monomicrobial *C. albicans* biofilms with 0.1 mM AA compared to monomicrobial biofilms with ethanol vector; CPAA/CPET – polymicrobial biofilms with 0.1 mM AA compared to polymicrobial biofilms with ethanol vector.

As expected, across all conditions, polymicrobial versus monomicrobial biofilms (CPET/CET) have the most (induced and repressed) unique differentially expressed genes (1086 repressed; 1070 induced) with 223 genes (92 repressed; 131 induced) overlapping with monomicrobial biofilms exposed to AA (CAA/CET) (63.0% of differentially expressed genes) and 212 genes (122 repressed; 90 induced) overlapping with polymicrobial biofilms exposed to AA (CPAA/CPET) (21.88% of

differentially expressed genes). When evaluating the overlap of mono- and polymicrobial biofilms exposed to AA, only 99 genes are found to be differentially expressed (37 repressed; 62 induced) in both mono- and polymicrobial biofilms corresponding to 28.0% of genes in monomicrobial biofilms and 10.22% of genes in polymicrobial biofilms exposed to AA. Furthermore, monomicrobial biofilms exposed to AA have 86 (24.29%) unique elements (34 repressed; 52 induced) whereas polymicrobial biofilms have 712 (73.48%) unique elements (362 repressed; 350 induced). Interestingly, this indicates that a larger overlap between monomicrobial biofilms exposed to AA and polymicrobial biofilms exposed to only the ethanol vector is found.

## 2.4.2. Effect of *P. aeruginosa* on *C. albicans* during co-incubation

### 2.4.2.1. Metabolism

Analysis of functional classes of genes that are differentially expressed in response to co-incubation with *P. aeruginosa* is given in **Table 2.2** (repressed genes) and **Table 2.3** (induced genes). These tables do not include parent GO-terms due to limited space. PANTHER (GO cellular component) revealed that products of repressed genes are localised to mitochondria, small ribosomal subunit, cell periphery and extracellular environment. Genes involved in ATP synthesis, tricarboxylic acid cycle as well as electron transport chain are repressed. Interestingly, induction of genes related to carbohydrate metabolic process is induced. This response is expected during fermentative growth (Askew *et al.*, 2009). Glycolysis is central to both respiration and fermentation to acquire energy and to metabolise hexose phosphates into pyruvate with the production of ATP. In the presence of oxygen, pyruvate is oxidised to carbon dioxide via the tricarboxylic acid cycle, whereas fermentation is used in the absence of oxygen for regeneration of NAD<sup>+</sup> through the production of acetate and ethanol as by-products. The repression of the tricarboxylic acid cycle and electron transport chain, together with the induction of the carbohydrate metabolic process and alcohol biosynthetic process indicate that *C. albicans* relies more on fermentation for energy acquisition during co-incubation with *P. aeruginosa*. Several genes involved in ethanol production (*ADH5*, *ADH3*, orf19.4504 and *ADH2*) are induced in our dataset (**Supplementary Table S2.2**). The rapid aerobic growth of *P. aeruginosa* in polymicrobial biofilms with *C. albicans* may quickly deplete available oxygen in the biofilm, forcing hypoxia quicker than in *C. albicans* monomicrobial biofilms. During this

hypoxic growth, fermentation is utilised for energy acquisition. The carbohydrate metabolism of *C. albicans* in this hypoxic environment is regulated by Tye7p and Gal4p (Askew *et al.*, 2009; Bonhomme *et al.*, 2011). Confirming our observation, is the induction of *TYE7* (**Table 2.4**) as well as *GAL4* (**Supplementary Table S2.2**), the former of which is needed for growth and virulence in a hypoxic environment. Interestingly, genes involved in glycolysis, fermentation, stress response, cell wall, fatty acid, iron metabolism and hyphae specific genes are induced during hypoxia, whilst the tricarboxylic acid cycle, respiration and ATP synthesis are repressed (Askew *et al.*, 2009). This response is similar to what is observed in polymicrobial biofilms of *C. albicans* and *P. aeruginosa* in this dataset, suggesting that the response of *C. albicans* towards *P. aeruginosa* may be dominated by the response towards hypoxia. In addition to induced hypoxia, inhibition of *C. albicans* metabolic activity and aerobic respiration by *P. aeruginosa*, due to the production of the redox-active phenazine compounds by *P. aeruginosa*, has been reported previously (Morales *et al.*, 2013). Morales *et al.* (2013) reported an increase in fermentation products by *C. albicans*, such as ethanol due to the action of these phenazine compounds. Considering this, the observed effect on *C. albicans* carbohydrate metabolism may be due to not only hypoxia, but also phenazine production by *P. aeruginosa*. Interestingly, ethanol stimulates *P. aeruginosa* biofilm formation and phenazine production (Chen *et al.*, 2014).

**Table 2.2. Overrepresented GO Terms of *Candida albicans* repressed genes in response to co-incubation with *Pseudomonas aeruginosa* in a polymicrobial biofilm.** Overrepresented Gene Ontology (GO) terms with PANTHER (Overrepresentation Test released 2017-12-05; GO Ontology database released 2017-12-27) (Mi *et al.*, 2013) of significantly ( $\text{padj} < 0.05$ ) differentially expressed genes with a  $\log_2$  fold change threshold of 1/-1. Significance based on Fisher's Exact with False Discovery Rate (FDR) multiple test correction.

<b>GO cellular component of repressed genes</b>				
GO term	No. of genes	Fold enrichment	<i>P</i> -value	FDR
cytosolic small ribosomal subunit (GO:0022627)	3	24.04	0.000873	0.0157
mitochondrial respiratory chain complex III (GO:0005750)	3	24.04	0.000873	0.0153
proton-transporting ATP synthase complex, catalytic core F(1) (GO:0045261)	3	19.23	0.00137	0.0221
proton-transporting ATP synthase complex, coupling factor F(o) (GO:0045263)	4	12.82	0.000641	0.0118
hyphal cell wall (GO:0030446)	16	7.54	2.78E-09	1.07E-07
cell surface (GO:0009986)	26	5.02	7.59E-11	4.09E-09
yeast-form cell wall (GO:0030445)	8	4.75	0.000506	0.00952
plasma membrane (GO:0005886)	41	3.4	1.54E-11	8.91E-10
extracellular region (GO:0005576)	21	3.32	3.32E-06	9.94E-05
cellular component (GO:0005575)	174	1.26	5.94E-10	2.4E-08
Unclassified	18	0.33	5.94E-10	2.53E-08
<b>GO biological process of repressed genes</b>				
GO term	No. of genes	Fold enrichment	<i>P</i> -value	FDR
cellular zinc ion homeostasis (GO:0006882)	3	32.05	0.00051	0.02
hydrogen peroxide catabolic process (GO:0042744)	3	32.05	0.00051	0.0199
ATP synthesis coupled proton transport (GO:0015986)	8	15.08	4.23E-07	4.1E-05
tricarboxylic acid cycle (GO:0006099)	8	12.21	1.51E-06	0.000118
cellular iron ion homeostasis (GO:0006879)	6	11.31	4.66E-05	0.00234
electron transport chain (GO:0022900)	13	7.71	7.19E-08	8.48E-06

cellular oxidant detoxification (GO:0098869)	6	7.69	0.000278	0.0115
cellular response to reactive oxygen species (GO:0034614)	5	7.28	0.00113	0.0404
translation (GO:0006412)	46	6.55	5.97E-23	2.6E-19
cell adhesion (GO:0007155)	9	3.61	0.00139	0.0488
symbiosis, encompassing mutualism through parasitism (GO:0044403)	13	3.39	0.000219	0.0092
pathogenesis (GO:0009405)	27	2.57	1.13E-05	0.000695
response to external stimulus (GO:0009605)	20	2.26	0.000881	0.0318
filamentous growth of a population of unicellular organisms (GO:0044182)	28	2.06	0.000345	0.0139
Biological process (GO:0008150)	182	1.16	3.66E-07	3.63E-05
Unclassified	10	0.29	3.66E-07	3.71E-05

**Table 2.3. Overrepresented GO Terms of *Candida albicans* induced genes in response to co-incubation with *Pseudomonas aeruginosa* in a polymicrobial biofilm.** Overrepresented Gene Ontology (GO) terms with PANTHER (Overrepresentation Test released 2017-12-05; GO Ontology database released 2017-12-27) (Mi *et al.*, 2013) of significantly ( $\text{padj} < 0.05$ ) differentially expressed genes with a  $\log_2$  fold change threshold of 1/-1. Significance based on Fisher's Exact with False Discovery Rate (FDR) multiple test correction.

GO cellular component of induced genes				
GO term	No. of genes	Fold enrichment	<i>P</i> -value	FDR
membrane raft (GO:0045121)	6	10.76	8.6E-05	0.0058
anchored component of membrane (GO:0031225)	23	5.05	2.12E-09	4.28E-07
cell surface (GO:0009986)	31	4.02	4.41E-10	1.19E-07
extracellular region (GO:0005576)	35	3.71	2.09E-10	8.44E-08
fungus-type cell wall (GO:0009277)	21	3.2	8.29E-06	0.000745
plasma membrane (GO:0005886)	40	2.22	4.6E-06	0.000465
macromolecular complex (GO:0032991)	20	0.47	7.33E-05	0.00539

GO biological process of induced genes				
GO term	No. of genes	Fold enrichment	<i>P</i> -value	FDR
xenobiotic transport (GO:0042908)	4	21.51	0.000232	0.0361
organic hydroxy compound transport (GO:0015850)	6	10.76	8.6E-05	0.0156
drug export (GO:0046618)	5	10.76	0.000347	0.0421
lipid catabolic process (GO:0016042)	9	6.45	3.88E-05	0.00995
fatty acid biosynthetic process (GO:0006633)	7	6.27	0.000332	0.0414
alcohol biosynthetic process (GO:0046165)	8	5.55	0.000254	0.0357
adhesion of symbiont to host (GO:0044406)	10	4.58	0.000172	0.0279
cellular response to oxidative stress (GO:0034599)	14	3.76	5.97E-05	0.0124
pathogenesis (GO:0009405)	45	2.87	8.65E-10	1.26E-06
biofilm formation (GO:0042710)	17	2.79	0.000276	0.0365
carbohydrate metabolic process (GO:0005975)	19	2.59	0.000296	0.0379
cellular response to drug (GO:0035690)	28	2.08	0.000415	0.0476
oxidation-reduction process (GO:0055114)	40	2.04	3.23E-05	0.0101
biological process (GO:0008150)	258	1.1	0.000238	0.0358
Unclassified	28	0.54	0.000238	0.0346

#### 2.4.2.2. Stress responses

GO biological process reveals a response towards stress (e.g. chemical) with response to xenobiotic as well as drug and organic hydroxy compound transport being overrepresented. This response may be due to toxic phenazines produced by *P. aeruginosa*, that *C. albicans* may be attempting to remove to circumvent deleterious effects. Additionally, the cellular response to oxidative stress is overrepresented in induced genes (**Table 2.3**). This may indicate that toxic oxidative and nitrosative radicals may be formed by the redox-active phenazines produced by *P. aeruginosa*. The glutathione-dependent S-nitrosoglutathione reductase *FDH3*, the nitric oxide dioxygenase *YHB1*, as well as the transcriptional regulator *CTA4* (**Table 2.4**), playing a role in nitrosative radical detoxification, is induced in our dataset (**Supplementary Table S2.2**). This indicates a response to nitrosative stress of *C. albicans* in the presence of *P. aeruginosa* (Chiranand *et al.*, 2008; Tillmann *et al.*, 2015; Ullmann *et al.*, 2004). Nitrosative radicals may be from both endogenous and exogenous origin, such as the use of nitrite as an alternative electron acceptor as well as from the redox-active phenazines produced by *P. aeruginosa* (Chiranand *et al.*, 2008; O'Malley *et al.*, 2003).

Interestingly, the gene encoding for catalase, *CAT1*, playing a role in oxidative stress response, is repressed in our dataset, however, the genes encoding for superoxide dismutase (SOD) *SOD1* and *SOD6* are significantly induced (**Supplementary Table S2.2**). SODs protect cells against reactive oxygen species generated by the mitochondrial respiratory chain and external sources such as the oxidative burst during phagocytosis. Only *SOD1* has been linked to virulence (Martchenko *et al.*, 2004). The function of *SOD6* still needs to be elucidated (Frohner *et al.*, 2009). Interestingly, the mitochondrial SOD, *SOD2*, is not differentially regulated, indicating that the possible increase in oxidative stress is localised in the cytosol, and not due to mitochondrial activity. This may indicate that the source of oxidative stress is not due to cellular activity, but due to external factors, such as phenazines, entering the cell.

**Table 2.4. Overrepresented transcription factors of *Candida albicans* in response to co-incubation with *Pseudomonas aeruginosa* in a polymicrobial biofilm for 6 hours.** Overrepresented transcription factors with a log<sub>2</sub> fold change (L2FC) above 1 and below -1 generated by PathoYeasttract (Monteiro *et al.*, 2016). Descriptions according to *Candida* Genome Database (Skrzypek *et al.*, 2017).

Transcription factor	ORF	No. of genes	P-value	L2FC	Process/Description
Wor2p	orf19.5992	40	2.52E-07	-1.55	White-opaque switching; required or maintenance of opaque state
Ume6p	orf19.1822	72	2.02E-06	-1.50	Filamentation
Zfu2p	orf19.6781	9	7.68E-06	-1.38	Negative regulation of filamentation; Adhesion
Csr1p	orf19.3794	155	<1.00E-15	-1.37	Zinc homeostasis; Filamentation
Ahr1p	orf19.7381	95	1.82E-09	-1.32	Adhesion
Mcm1p	orf19.6017	306	3.17E-13	-1.19	Filamentation
Tcc1p	orf19.6734	4	<1.00E-15	-1.06	Filamentation
Cta4p	orf19.7374	1	<1.00E-15	1.06	Response to nitric oxide
Tye7p	orf19.4941	289	1.58E-08	1.16	Glycolysis; Hypoxia
Ifh1p	orf19.1639	55	3.66E-12	1.46	Ribosomal transcription
Rpn4p	orf19.1069	29	1.27E-06	1.62	Proteasome
Upc2p	orf19.391	142	8.45E-11	1.66	Ergosterol biosynthesis; Sterol uptake
Stp4p	orf19.909	1	<1.00E-15	2.41	Core caspofungin response
Fcr1p	orf19.6817	1	<1.00E-15	2.60	Antifungal resistance
Sut1p	orf19.4342	23	1.69E-08	2.72	Sterol uptake
Wor1p	orf19.4884	98	4.21E-10	2.84	White-opaque switching
Try4p	orf19.5975	29	3.16E-08	3.47	Negative regulation of filamentation; Adhesion

#### 2.4.2.3. Genes involved in membrane formation and organisation

Lipid catabolic processes, including lipase genes *LIP1* to *LIP3* and *LIP6*, *LIP8*, *LIP9* and *LIP10*, catalysing hydrolysis or synthesis of triacylglycerols (Gácsér *et al.*, 2007; Schofield *et al.*, 2005) is overrepresented in induced genes (**Table 2.3**). In addition, fatty acid biosynthetic process is induced, possibly to counteract physical damage to *C. albicans* plasma membrane by *P. aeruginosa* (Brand *et al.*, 2008). Importantly, several TFs, with roles in membrane and cell wall metabolism, such as *UPC2*, *STP4* and *SUT1* are overrepresented in our dataset and are significantly differentially expressed (**Supplementary Table S2.2**). Upc2 is associated with the regulation of a hypoxic response, inducing genes involved in ergosterol biosynthesis (MacPherson *et al.*, 2005; Synnott *et al.*, 2010). Sut1p is involved in sterol uptake during hypoxia in *S. cerevisiae* (Foster *et al.*, 2013). Interestingly, Sut1p is also involved in zinc acquisition in *C. albicans* (Xu *et al.*, 2015).

#### 2.4.2.4. Proteinases

In addition to lipases, secreted aspartyl proteinases (SAPs) are also differentially regulated. These SAPs have proteinase activity and play a role in nitrogen acquisition and tissue damage during infection (Hube *et al.*, 1997; Kumar *et al.*, 2015). *SAP3*, *SAP4* and *SAP6* are upregulated, possibly indicating a response to nitrogen starvation (**Supplementary Table S2.2**). Interestingly, the biofilm specific *SAP5* is downregulated in our dataset, possibly due to inhibition of biofilm formation by co-incubation with *P. aeruginosa* (Bandara *et al.*, 2010; Joo *et al.*, 2013). The increased production of several SAPs may also play a role in competition with *P. aeruginosa*, attacking the bacterial cells.

#### 2.4.2.5. Iron acquisition

Genes associated with cellular iron ion homeostasis is repressed (**Table 2.2**). Previous research suggests that *C. albicans* in combination with *P. aeruginosa* elicits an iron-deprivation response in *C. albicans* due to rapid iron-sequestration by *P. aeruginosa* siderophores (Purschke *et al.*, 2012; Singh *et al.*, 2011; Trejo-Hernández *et al.*, 2014). This iron-deficient response is mediated by a Cap2-HAP complex, affecting various genes including three iron uptake pathways and repression of iron utilisation and storage (Singh *et al.*, 2011). However, closer examination of the data revealed that only a modest response towards iron deprivation is seen in terms of repression of iron

utilisation genes, including *ACO1* and *IDH2* (involved in aerobic respiration), *QCR2* (respiratory electron transport chain) and haem containing proteins like *CAT1* (hydrogen peroxide detoxification), indicating a possible reduced dependency on iron. This repression of iron usage genes is similar to the results obtained by Trejo-Hernández *et al.* (2014) in a proteomic study of monomicrobial and polymicrobial biofilms. None of the transcription factors (*SFU1*, *SEF1* or *HAP43*) involved in iron regulation was differentially regulated at 6 hours of co-incubation (Chen *et al.*, 2011; Singh *et al.*, 2011). Strikingly, several genes involved in iron uptake and usage are repressed in our dataset, resembling an abundance of iron compared to monomicrobial biofilms. This may indicate that less iron is required for cellular growth when *C. albicans* is co-incubated with *P. aeruginosa* due to growth repression. These genes include components of the reductive iron pathway, namely the high affinity iron permease *FTR1* along with the multicopper ferroxidase *FET34*, the ferric/cupric reductase *CFL2*, the ferritin receptor *ALS3* and the heme oxygenase *HMX1*. This contrasts with what has been reported before (Trejo-Hernández *et al.*, 2014), although this may be due to differences in incubation conditions and duration of biofilm formation, as biofilms were only allowed to form for 6 hours in this study.

#### 2.4.2.6. Zinc acquisition

Interestingly, a repression of cellular zinc ion homeostasis is seen (**Table 2.2**). Zinc is essential for normal cellular function, as it is part of zinc-finger motifs (approximately 116 zinc finger transcription factors in *C. albicans*), as well as forming the catalytic core of various cellular enzymes (Kim *et al.*, 2008; Staats *et al.*, 2013). Genes involved in zinc acquisition are repressed in our dataset (**Supplementary Table S2.2**), including components of a “zincophore” system, namely, the zinc transporters *ZRT1*, *ZRT2* and *ZRT3*, as well as the extracellular zinc binding protein *PRA1*, responsible for zinc sequestration (Böttcher *et al.*, 2015; Łoboda and Rowińska-Żyrek, 2017). This “zincophore” system is under positive regulation of Csr1p (also known as Zap1p). *CSR1* (**Table 2.4**) is repressed in our dataset, indicating that, compared to monomicrobial biofilms consisting of only *C. albicans*, more zinc is available, and the fungus is under less zinc-limitation during co-incubation with *P. aeruginosa*, similar to the effect on iron discussed above. As this phenomenon has not been reported during co-incubation of *C. albicans* with *P. aeruginosa* previously (Holcombe *et al.*, 2010; Konstantinidou, 2016), the effect may be due to incubation conditions. *Pseudomonas*

*aeruginosa* has been reported to inhibit hyphae-formation in *C. albicans*, and additionally, *CSR1* may be repressed during inhibition of filamentous growth (Ballou and Wilson, 2016; Fourie *et al.*, 2016). Therefore, the phenomenon on zinc-acquisition may be morphology dependent. Another possibility is that an alternative mechanism may be in use for zinc acquisition, decreasing the requirement for a zinc-deficiency response. The secreted aspartyl protease Sap6p contributes to autoaggregation in *C. albicans* and possesses amyloid domains that bind zinc (Kumar *et al.*, 2017). Double deletion mutants of *SAP6* have been shown to have lower intracellular zinc. Interestingly, an overexpression of *SAP6* was observed in our dataset in response to co-incubation with *P. aeruginosa*. This overexpression of *SAP6* may contribute to the increased uptake of zinc by *C. albicans* and additionally, this may implicate zinc as a contributing factor to polymicrobial aggregation observed (El-Azizi *et al.*, 2004; Klotz *et al.*, 2007).

#### 2.4.2.7. Morphogenesis

Previous research suggests that *C. albicans* morphogenesis is inhibited in the presence of *P. aeruginosa* due to the *N*-acyl-homoserine lactone (AHL) 3-oxo-homoserine lactone produced by the bacterium (Hogan *et al.*, 2004). *Candida albicans* yeast and pseudohyphal morphologies are characterised by a morphology dependent set of expressed genes. Similarly, true hyphae formation is transcriptionally unique (Bensen *et al.*, 2002). In true hyphae, several genes are expressed, including *HWP1*, *ECE1*, *HYR1*, *RBT1*, *RBT4*, *RBT5* and *WAP1* which encode GPI-modified cell wall proteins. A number of these proteins are repressed in the presence of *P. aeruginosa* in our dataset, including *HWP1* and *ECE1*, associated with a predominant yeast and pseudohyphal transcriptional profile (**Supplementary Table S2.2**). Interestingly, *HYR1* and *CHT2*, associated with a true hyphae-morphology, is induced in our dataset.

#### 2.4.2.8. Regulation of biofilm formation and morphogenesis

Adhesion is a crucial component of the process of biofilm formation of *C. albicans*. In our dataset, overrepresentation of genes associated with adhesion is observed as being repressed in the presence of *P. aeruginosa* (**Table 2.2**). In support of this, several TFs are overrepresented with roles in adhesion, including *ZFU2*, *AHR1* and *TRY4* (**Table 2.4**). *ZFU1* and *TRY4* are TFs that negatively regulate filamentation and

promote persistence in a germ-free mouse gut through promotion of a yeast phenotype (Böhm *et al.*, 2017). However, *ZFU2* is repressed (L2FC of -1.38), whereas *TRY4* is induced (L2FC of 3.47) with contrasting effects. In addition, Ahr1p recruits Mcm1p and induce initial surface adhesion during biofilm formation (Askew *et al.*, 2011). Interestingly, both *AHR1* and *MCM1* are repressed in our dataset. The yeast wall protein *YWP1*, with expression negatively correlating with adhesion (Granger *et al.*, 2005), is induced in response to *P. aeruginosa*, further providing evidence for an effect on adhesion.

Biofilm formation in *C. albicans* is under control of six master regulators namely *BCR1*, *BRG1*, *EFG1*, *NDT80*, *ROB1* and *TEC1* (Nobile *et al.*, 2014). One of the common targets of all six regulators is the DNA-binding protein Nrg1p, a key regulator of biofilm dispersal through interaction with the Tup1p repressor (Nobile *et al.*, 2014; Uppuluri *et al.*, 2010). The transition from yeast to hyphal morphogenesis is a crucial part in biofilm development. Four of these six master regulators (*NRG1*, *EFG1*, *BRG1* and *TEC1*) also form a core regulatory circuit that control the yeast-to-hyphal transition in *C. albicans* (Hnisz *et al.*, 2012). During growth as in the yeast phase, *NRG1* is overexpressed. This, in turn represses hyphal specific genes. *NRG1* overexpression is in turn repressed by the cAMP/PKA pathway. The decrease in *NRG1* transcripts lead to expression of *BRG1* and *TEC1* and results in a hyphae-specific program. This program also includes various TFs. A number of external stimuli is found in the presence of *P. aeruginosa* (Fourie *et al.*, 2016). This includes hypoxia (induces filamentation), presence of LPS (represses filamentation), cell wall and membrane stress (induces filamentation) and quorum sensing molecules. These large amounts of stimuli may present as an exaggerated response that could be detrimental to adaptation to a host environment. Therefore, in addition to TFs, chromatin-remodelling has been shown to affect biofilm formation and filamentation through integrating signals from external stimuli and acting as a transcriptional buffer affecting transcription kinetics (Garnaud *et al.*, 2016; Hnisz *et al.*, 2010; 2012; Kim *et al.*, 2012). This chromatin-remodelling includes histone modifications such as acetylation, methylation and phosphorylation. The Set3/Hos2 complex (Set3C) is a NAD-dependent histone deacetylation complex that binds directly to 5 out of 6 master regulators of biofilm formation (*BRG1*, *TEC1*, *EFG1*, *NDT80* and *ROB1* as well as *NRG1*). The complex is involved in the dispersal of cells from mature biofilms, via the

modulation of *NRG1* expression levels (Nobile *et al.*, 2014). On solid medium, mutants of the Set3C show a hyperfilamentous phenotype in the presence of hyphae-inducing signals (Hnisz *et al.*, 2010). The *S. cerevisiae* Set3C consists of 4 core subunits, including Set3p, Hos2p, Snt1p and Sif2p as well as 3 peripheral subunits Hos4p, Hst1p and Cpr1p (Hnisz *et al.*, 2010). This complex is conserved in *C. albicans*. *SET3* is significantly induced (L2FC of 2.11) in our dataset. In addition, the peripheral subunit *HST1* is also induced. The induction of *SET3* in our dataset raises the question if Set3C may play a role in modulating filamentation of *C. albicans* in the presence of *P. aeruginosa* and inducing the dispersion of *C. albicans*, possibly to escape the hostile activity of *P. aeruginosa* during co-incubation. Cells dispersed from biofilms have increased virulence compared to planktonic cells in a murine disseminated infection model (Uppuluri *et al.*, 2010), therefore, the virulence of *C. albicans* may be affected by co-incubation with *P. aeruginosa*.

#### 2.4.2.9. Phenotypic switching

In addition to affecting biofilm formation in *C. albicans*, the Set3C is also a regulator of white-opaque switching (Garnaud *et al.*, 2016; Hnisz *et al.*, 2010). Strikingly, the white-opaque regulator, *WOR1*, is induced in our dataset (**Supplementary Table S2.2**). *WOR1* regulates the epigenetic transition between white cells, which form round, white colonies and is associated with systemic infection, and opaque cells, which are elongated with pimples, forming flat, opaque colonies (Huang *et al.*, 2006; Xie *et al.*, 2013). The latter phenotype is associated with cutaneous infection and is mating competent when cells are homozygous at the mating type locus (MTL). The regulation by *WOR1* is in an all or nothing manner (Huang *et al.*, 2006). This is because little to no expression of *WOR1* is seen in white cells. Spontaneous switching to the opaque state can occur, albeit with a low frequency ( $10^{-4}$  generations). However, this frequency can be increased through environmental stimuli such as the presence of *N*-acetylglucosamine and 5% carbon dioxide. These stimuli can cause the overexpression of *WOR1* in some cells and this concentration can be high enough to initiate a feedback loop (Huang *et al.*, 2006; Zordan *et al.*, 2007). In addition, anaerobic conditions have been shown to induce switching to the opaque state, even in the presence of high temperature (37°C), normally shown to induce mass reversion to the white state in oxic conditions (Dumitru *et al.*, 2007; Hnisz *et al.*, 2009). However, the opaque phenotype is unstable and overexpression of another regulator, *WOR2*, is

needed for stabilising it, but this phenomenon is dependent on the carbon source (Tong *et al.*, 2013).

Since we observed an overexpression of *WOR1* in *C. albicans* exposed to *P. aeruginosa*, it may indicate that white-opaque switching may take place. However, *WOR2* expression is significantly repressed (**Table 2.4**), indicating that switching may be unstable and transient. In addition, whereas *WH11* and *EFG1* expression is associated with white-phase and *OP4* and *SAP1* expression is associated with the opaque phase (Tao *et al.*, 2014), contradicting results are obtained in this dataset, with both *WH11* and *OP4* being induced. In addition to white and opaque phenotypes, a gray phenotype can also manifest, however, comparison with expression results obtained by Tao and co-workers (2014) revealed little overlap between our dataset and opaque or gray specific transcriptional responses with only 16.8% of genes associated with the gray phenotype being significantly differentially expressed in our dataset and 24.5% with the opaque phenotype.

The overexpression of *WOR1* in the presence of bacteria has been reported before and is required for commensal fitness (Fox *et al.*, 2014; Pande *et al.*, 2013). The research showed that the opaque specific gene *WOR1* was induced when *C. albicans* was co-cultured with bacteria such as *Klebsiella pneumoniae*, *Escherichia coli* and *Enterococcus faecalis*, however, the full opaque program was not induced (Fox *et al.*, 2014). The authors speculate that additional signals may be required for the display of the heritable opaque phenotype. Interestingly, Ramirez-Zavala *et al.* (2008) showed that switching could be induced in *C. albicans* by an anaerobic environment in the presence of high temperature (37°C), such as those found in the mammalian gastrointestinal tract. However, this only occurred in the WO-1 and CAI-4 strains, whereas this could not be replicated in the SC5314 strain that is heterozygous at the MTL ( $a/\alpha$ ). This is since WO-1 and CAI-4 experienced a duplication of chromosome 1, therefore containing 3 copies of the *WOR1* gene (Ramirez-Zavala *et al.*, 2008). In addition to this, other chromosomal events, such as homozygosity of the MTL, enabled the induction of these strains to undergo phenotypic switching in these circumstances and display the full opaque program. The authors further speculated that these chromosomal alterations are required for switching to take place during hypoxia at high temperature. Due to this, the strain of *C. albicans* (SC5314) may experience an induction of *WOR1* in the presence of *P. aeruginosa* in a polymicrobial biofilm due to

hypoxia, as well as other environmental factors such as the presence of *N*-acetylglucosamine, however, the full opaque program may not be induced as a trisomy of chromosome 1 and homozygosity of the mating type locus is needed or further stimuli to induce *WOR1* may be needed. It is however interesting to note that an increase in expression of hydrolytic enzymes, such as *SAP3* and lipases (*LIP1* to *LIP3*, *LIP6*, *LIP8* to *LIP10*) associated with the opaque phenotype (Lan *et al.*, 2002), is seen in our dataset. This provides evidence that the opaque expression profile, at least in part, is induced by co-incubation of *C. albicans* with *P. aeruginosa*.

Since our dataset does not strictly adhere to a specific phenotype, it may indicate that biofilms consist of heterogenous populations of phenotypes. Alterations of conditions encountered by other phenotypes, such as carbon source may alter expression profiles of these biofilms. Nevertheless, the overexpression of *WOR1* in polymicrobial biofilms compared to monomicrobial biofilms of *C. albicans* raises the question if this may alter the interaction of *C. albicans* and *Pseudomonas aeruginosa*.

#### 2.4.2.10. Comparison with microarray data (Holcombe *et al.*, 2010)

In addition to GO term enrichment and TF analysis, differentially expressed genes were compared to known molecular interactions. Holcombe *et al.* (2010) evaluated the influence of *P. aeruginosa* on *C. albicans* cells. However, differences in incubation circumstances lead to little overlap in the data. Holcombe *et al.* (2010) evaluated the transcriptome of cells grown in a planktonic culture, with the addition of *P. aeruginosa* supernatant. In addition, a difference in medium possibly caused further deviation from their data. It should be noted that the medium used in this study (RPMI-1640) and the biofilm growth of cells more accurately represent *in vivo* conditions. Our data was compared to the Holcombe and co-workers' dataset with updated GO annotation provided by Konstantinidou and Morrissey (2016). This indicated a 31.7% overlap of differentially upregulated genes, and 19.97% differentially down regulated genes between the two datasets. In addition, Bandara and co-workers (2013) evaluated the effect of *P. aeruginosa* derived LPS on *C. albicans* and found an upregulation in hypha-specific genes and *EFG1*. *EFG1* is not significantly differentially regulated in our dataset, together with only one hypha-specific gene (*HYR1*) being differentially upregulated compared to the genes evaluated by Bandara and co-workers. This may indicate that the response of *C. albicans* to *P. aeruginosa* in early biofilm development

(6 hours), is not dominated by the response to LPS, as is expected, as little cell death and concomitant release of cell membrane components of *P. aeruginosa* is expected during early biofilm development.

#### **2.4.3. Effect of 0.1 mM arachidonic acid on *C. albicans* monomicrobial biofilms**

To mimic the availability of AA *in vivo*, the free fatty acid form of AA is usually supplemented to media *in vitro* to assess influence on microorganisms such as *C. albicans*. The concentrations used frequently in *in vitro* research represent the minimum inhibitory concentration (MIC), 1 mM in the case of *C. albicans*, or 0.5 mM AA ( $\frac{1}{2}$ MIC) (Fourie *et al.*, 2017; Mishra *et al.*, 2014). However, the free fatty acid precipitates out of solution in aqueous media at high concentrations such as these and is rendered unavailable to the studied microorganism (Brash, 2001). *In vivo*, AA is rarely found in its free form, but is bound by proteins so that the available concentration of free AA is only approximately 1  $\mu$ M during an uninduced state. However, disruption in homeostasis caused by genetic factors, e.g. cystic fibrosis, or environmental factors, such as infection, can cause a dramatic increase in AA concentration (Seegmiller, 2014), and can reach up to 0.1 mM in local skin inflammation (Brash, 2001; Pompeia *et al.*, 2003). Therefore, 0.1 mM of AA may be a more accurate representation of physiological AA levels during infection than the MIC concentrations. Due to this reason, *C. albicans* was exposed to 0.1 mM of AA or the equivalent ethanol vector (final concentration of 0.076 % in medium) to elucidate the effect of AA on this fungus.

Differential analysis revealed a total of 354 significantly differentially expressed ORFs of *C. albicans* exposed to AA. Among these, only 63 genes were significantly differentially expressed above a L2FC threshold of 1 (**Supplementary Table S2.3**). Due to this, it was decided to include significantly differentially expressed genes irrespective of the L2FC to allow identification of affected processes and TFs that can govern the effect of this fatty acid on *C. albicans*. Previous research suggests that even minor changes in TFs may cause phenotypic differences in *C. albicans* (Glazier and Krysen, 2018).

To confirm that the addition of AA does not have a significant effect on glucose metabolism, differentially expressed genes were evaluated, and it was found that no repression of glucose metabolism is seen, but an expected upregulation in  $\beta$ -oxidation for fatty acid catabolic process is observed, including *FAA21*, *FOX2*, *POX1-1*, *POX1-*

3, *POT1* and *FOX3* (Lorenz *et al.*, 2004). *Candida albicans* is known to utilise both preferred and non-preferred carbon-sources simultaneously, reflected also in this dataset (Lorenz, 2013).

Evaluation of overrepresented GO terms of repressed genes with PANTHER did not reveal any overrepresented GO terms involved in biological process (**Table 2.5**). This may be due to many repressed genes not annotated for function. However, an overrepresentation of gene products associated with the plasma membrane is observed (GO cellular component). Analysis of overrepresented GO terms of induced genes (**Table 2.6**) revealed a localisation of gene products to membrane raft, yeast form cell wall, cell surface, extracellular region, plasma membrane and integral component of membrane, indicating that AA significantly induces alterations in the periphery of *C. albicans* cells.

**Table 2.5. Overrepresented GO Terms of *Candida albicans* repressed genes in response to 0.1 mM arachidonic acid in a monomicrobial biofilm.**

Overrepresented Gene Ontology (GO) terms with PANTHER (Overrepresentation Test released 2017-12-05; GO Ontology database released 2017-12-27) (Mi *et al.*, 2013) of significantly ( $p_{adj} < 0.05$ ) differentially expressed genes. Significance based on Fisher's Exact with False Discovery Rate (FDR) multiple test correction.

<b>GO cellular component of repressed genes</b>				
GO term	No. of genes	Fold enrichment	<i>P</i> -value	FDR
plasma membrane (GO:0005886)	15	3.31	4.19E-05	0.0169
<b>GO biological process of repressed genes</b>				
GO term	No. of genes	Fold enrichment	<i>P</i> -value	FDR
No significantly overrepresented GO terms	-	-	-	-

**Table 2.6. Overrepresented GO Terms of *Candida albicans* induced genes in response to 0.1 mM arachidonic acid in a monomicrobial biofilm.**

Overrepresented Gene Ontology (GO) terms with PANTHER (Overrepresentation Test released 2017-12-05; GO Ontology database released 2017-12-27) (Mi *et al.*, 2013) of significantly ( $p_{adj} < 0.05$ ) differentially expressed genes. Significance based on Fisher's Exact with False Discovery Rate (FDR) multiple test correction.

<b>GO cellular component of induced genes</b>				
GO term	No. of genes	Fold enrichment	<i>P</i> -value	FDR
membrane raft (GO:0045121)	6	22.96	1.26E-06	0.000204
yeast-form cell wall (GO:0030445)	8	6.8	4.43E-05	0.00299
cell surface (GO:0009986)	18	4.98	5.12E-08	2.07E-05
extracellular region (GO:0005576)	15	3.39	5.46E-05	0.0034
plasma membrane (GO:0005886)	25	2.97	1.42E-06	0.000164
integral component of membrane (GO:0016021)	44	1.63	0.000698	0.0376

<b>GO biological process of induced genes</b>					
GO term	No. of genes	Fold enrichment	<i>P</i> -value	FDR	
drug export (GO:0046618)	4	18.37	0.000167	0.0332	
drug transmembrane transport (GO:0006855)	5	16.4	3.73E-05	0.0148	
lipid transport (GO:0006869)	6	11.02	3.98E-05	0.0134	
polysaccharide metabolic process (GO:0005976)	8	6.93	3.93E-05	0.0143	
DNA replication (GO:0006260)	9	6.26	2.65E-05	0.0144	
organic anion transport (GO:0015711)	9	5.23	9.53E-05	0.0231	
cell wall organisation (GO:0071555)	12	4.34	3.56E-05	0.0173	
single-species biofilm formation (GO:0044010)	11	4.01	0.000147	0.0306	
interspecies interaction between organisms (GO:0044419)	21	2.54	9.63E-05	0.0221	
cellular response to stress (GO:0033554)	24	2.27	0.000168	0.0319	

### 2.4.3.1. Membrane organisation

Evaluation of biological processes indicate an overrepresentation of genes involved in, among others, transport, including drug export and transmembrane transport as well as lipid transport in induced genes. These genes include the ABC superfamily multidrug transporters *CDR1*, *CDR2*, *CDR4* and *CDR11*. *CDR1* and *CDR2* are involved in phospholipid translocation. In addition, they have previously been shown to be overexpressed in azole resistant isolates of *C. albicans* and overexpression is the predominant cause of development of antifungal resistance (Coste *et al.*, 2004; Prasad *et al.*, 2015; Siikala *et al.*, 2010; Smriti *et al.*, 2002). *PDR16*, linked to overexpression of *CDR1* and *CDR2* (Whaley *et al.*, 2016), is also induced (**Supplementary Table S2.3**). Interestingly, AA increases susceptibility to antifungal agents including amphotericin B and clotrimazole (Ells *et al.*, 2009). However, the observed overexpression of the multidrug efflux pumps would arguably decrease susceptibility to antifungal compounds. Additionally, the fluconazole resistance 1 (*Fcr1p*) regulator is known to induce susceptibility of *C. albicans* to fluconazole through repression of *CDR1* expression, however, *FCR1* is induced in our dataset (**Table 2.7**), indicating that *FCR1* may not be involved in the effect of AA on *C. albicans* *CDR1* expression (Shen *et al.*, 2007). Pasrija and co-workers (2005) indicated that changes in ergosterol or sphingolipid composition affects membrane microdomains/membrane raft structure and causes improper surface localisation of membrane bound proteins such as *Cdr1p*, therefore this phenomenon requires further investigation.

Along with the ABC superfamily multidrug transporters (*CDR1* and *CDR2*), other *Tac1p* regulated genes that play roles in lipid homeostasis (Whaley *et al.*, 2016) are differentially expressed. These are *PDR16*, *LCB4*, *ROA1* and plasma membrane associated *RTA3* involved in translocation of lipids. *RTA3* (Resistance to 7-aminocholesterol) showed the most significant induction in this dataset (L2FC of 4.38). This membrane bound protein maintains asymmetric distribution of the phospholipid phosphatidylcholine across the plasma membrane (Srivastava *et al.*, 2017). In addition, it functions upstream of *Bcr1p* regulating biofilm formation, as well as maintaining mitochondrial membrane energetics. The asymmetric arrangement of phospholipids on the plasma membrane is dynamic and is dependent on inward and outward translocation of phospholipids across the membrane. The loss of *RTA3*

**Table 2.7. Overrepresented transcription factors of *Candida albicans* in response to 0.1 mM arachidonic acid in a monomicrobial biofilm.** Overrepresented transcription factors generated by PathoYeasttract (Monteiro *et al.*, 2016). Descriptions according to Candida Genome Database (Skrzypek *et al.*, 2017).

Transcription factor	ORF	No. of genes	P-value	log <sub>2</sub> FC	Process/Description
Wor2p	orf19.5992	27	8.81E-11	-0.63	White-opaque switching; required or maintenance of opaque state
Rfg1p	orf19.2823	23	6.50E-06	-0.60	Filamentation
Ahr1p	orf19.7381	48	2.14E-09	-0.46	Adhesion
Sfu1p	orf19.4869	10	5.86E-07	-0.46	Iron homeostasis
Ume6p	orf19.1822	34	3.24E-05	-0.42	Filamentation
Tye7p	orf19.4941	137	5.83E-12	0.64	Glycolysis; Hypoxia
Nrg1p	orf19.7150	164	6.5E-14	0.68	Filamentation
Sut1p	orf19.4342	11	1.0E-06	1.41	Sterol uptake
Fcr1p	orf19.6817	1	<1.00E-15	1.60	Antifungal resistance

increases the inwardly directed movement of phospholipids across the plasma membrane which leads to enrichment of phospholipids in the inner leaflet of the membrane. These phospholipids are then transferred to intracellular membranes via passive diffusion. As Rta3p is a membrane bound protein, it is possible that its localisation may be influenced by AA, affecting its function and causing a change in membrane organisation. This may lead to overexpression of *RTA3* to compensate for this effect. In addition, the transcription factor *SUT1* is induced and our dataset (**Table 2.7**). Sut1p modifies the intrinsic property of membranes by increasing their permeability by positive regulation of sterol uptake and intracellular sterol trafficking (Joshua and Höfken, 2017; Ness *et al.*, 2001). This indicates that Sut1p may be induced to combat changes in membrane organisation caused by AA.

#### 2.4.3.2. Inhibition of yeast-hyphal transition by AA

Significantly, other fatty acids such as docosahexaenoic acid and conjugated linoleic acid (CLA) also cause changes in membrane organisation (Collett *et al.*, 2001; Seo *et al.*, 2006; Shareck *et al.*, 2011). Shareck *and* co-workers (2011) indicated that the delocalisation of the plasma membrane bound GTPase, Ras1p, partially contributes to the inhibition of hyphae formation by CLA in *C. albicans*. In addition, CLA affects transcription of *RAS1*, inhibiting the increase in *RAS1* transcription associated with hyphal formation, as well as lowering the Ras1p concentration in the cells. Considering that AA has also been shown to inhibit the yeast-to-hyphal switch in *C. albicans*, this inhibition may have a similar cause. Although no significant differential expression of *RAS1* was observed in the presence of AA, this may have occurred at an earlier time point, as Shareck and co-workers (2011) evaluated transcription up to 90 min in contrast to the 6 hours utilised in this study. In addition, CLA induced the transcription of the Tup1p-Nrg1p repression complex, which inhibits hyphae formation via repression of Ume6p. The Tup1p repressor blocks expression of hyphal specific genes. The repression activity of Tup1p is facilitated by the DNA-binding proteins Nrg1p and Rfg1p, with Nrg1p playing the major role. In our dataset (**Supplementary Table S3**), an induction of *NRG1* as well as a repression of *UME6* is seen, possibly indicating a contribution to repression of hyphal growth by the Nrg1p-Tup1p repression complex. This data indicates that repression of hyphae-formation by AA may follow the same route as CLA, through induction of the Nrg1p-Tup1p repression complex. Interestingly, the gene encoding for glycosylphosphatidylinositol (GPI)-anchored

glycoprotein *YWP1* is also significantly upregulated. This protein is associated with an antiadhesive property, with mutants being more adhesive and forming thicker biofilms (Granger, 2012). Due to this antiadhesive property of *YWP1*, it may promote dispersal of yeast. In addition, Nrg1p is also associated with the dispersal of yeast cells from mature biofilms. Notably, biofilms exposed to AA show less adherence to abiotic surfaces (unpublished observation) and are easily disrupted by mechanical force, possibly indicating that AA induces dispersal of *C. albicans*, facilitated by less adhesive biofilms.

#### 2.4.3.3. Cell wall

In addition to effects on membrane organisation, genes involved in cell wall organisation are induced (**Table 2.6**). This may not only alter cellular function, but immunogenicity as well. Two genes involved in protein glycosylation of mannoproteins, possibly playing roles in cell wall remodelling and immunogenesis, were differentially expressed. These were the alpha-1,3-mannosyltransferase proteins *MNN1* (induced) and *MNN2* (repressed). However, previous research indicated that the *MNN1* family contains redundancies, where mutation of these proteins does not affect *C. albicans* phenotype or virulence (Bates *et al.*, 2013). However, in the study by Bates and co-workers (2013), the effect of single deletion mutants only was evaluated, raising the question if differential expression of more than one member of the *MNN1* family may alter the phenotype or virulence of the fungus.

#### 2.4.3.4. Comparison with effect of PGE<sub>2</sub>

Previous research has also indicated the response of *C. albicans* to the AA oxidation product PGE<sub>2</sub> (Levitin and Whiteway, 2007). Our data indicates that the response towards AA is different from that of PGE<sub>2</sub> as found by Levitin and Whiteway (2007) with the response to AA having a unique transcriptional profile. This may indicate that not enough PGE<sub>2</sub> is produced during the time of incubation used. It is worth noting that the transcriptional profile obtained by the authors is that of *C. albicans* cells exposed to PGE<sub>2</sub> for only 10 minutes.

### 2. 4.4. Effect of 0.1 mM arachidonic acid on *C. albicans* polymicrobial biofilms

Polymicrobial infection by pathogens such as *C. albicans* in combination with *P. aeruginosa* lead to increased morbidity and mortality compared to single-species

infection (Fourie *et al.*, 2016). During infection, a large amount of AA is released acting as precursor for immunomodulatory eicosanoids. Information regarding the effect of the released AA on the interaction of *C. albicans* and *P. aeruginosa* is still lacking, however, our group recently assessed the eicosanoid production of *C. albicans* and *P. aeruginosa* in a polymicrobial biofilm when exposed to exogenous arachidonic acid (Fourie *et al.*, 2017). Co-incubation of these species led to a higher production of eicosanoids compared to single species counterparts.

#### 2.4.4.1. Effect on metabolism and growth

When *C. albicans* is exposed to *P. aeruginosa* in a polymicrobial biofilm, genes involved in respiration and energy generation are repressed (**Table 2.2**). Similar to monomicrobial biofilms exposed to AA, polymicrobial biofilms exposed to AA do not exhibit overrepresentation of genes associated with gluconeogenesis or the tricarboxylic acid cycle (**Table 2.8**). However, a slight reduction is seen in the expression of *GAL4* (L2FC of -0.44), a regulator of glycolysis, although *TYE7* is not differentially expressed (**Supplementary Table S2.4**). In addition, an induction of response to stress (including chemical, xenobiotic, drug and organic hydroxy compound) and drug transmembrane transport is seen in response to co-incubation with *P. aeruginosa*. This effect seems to be exacerbated by the addition of AA (**Table 2.9**), with GO terms associated with stress and drug response being induced, however, these processes are also induced in monomicrobial biofilms exposed to AA, indicating that this may be an additive effect.

#### 2.4.4.2. Stress responses

Notably, oxidative stress is induced in polymicrobial biofilms exposed to AA, a phenomenon not seen when monomicrobial biofilms are exposed to AA. This may indicate that *C. albicans* is exposed to increased oxidative stress in polymicrobial biofilms with added AA. Furthermore, Baker and co-workers (2018) indicated that AA can affect phenotypes associated with virulence, with a decrease in motility and increase in biofilm formation. Further research is required to determine if other virulence factors, such as phenazine production may be affected by AA that may increase oxidative stress in *C. albicans*. Interestingly, *CAT1* is repressed by *P. aeruginosa*, but this effect is partially rescued by the addition of AA.

**Table 2.8. Overrepresented GO Terms of *Candida albicans* repressed genes in response to 0.1 mM arachidonic acid in a polymicrobial biofilm.**

Overrepresented Gene Ontology (GO) terms with PANTHER (Overrepresentation Test released 2017-12-05; GO Ontology database released 2017-12-27) (Mi *et al.*, 2013) of significantly ( $\text{padj} < 0.05$ ) differentially expressed genes. Significance based on Fisher's Exact with False Discovery Rate (FDR) multiple test correction.

<b>GO cellular component of repressed genes</b>				
GO term	No. of genes	Fold enrichment	<i>P</i> -value	FDR
cellular bud neck septin ring (GO:0000144)	4	14.24	0.000664	0.0224
cellular bud neck contractile ring (GO:0000142)	4	14.24	0.000664	0.0215
cell septum (GO:0030428)	10	10.17	5.26E-07	0.000106
ER to Golgi transport vesicle membrane (GO:0012507)	4	9.5	0.00203	0.0421
mating projection (GO:0005937)	7	5.16	0.000906	0.0262
fungus-type vacuole membrane (GO:0000329)	7	4.53	0.00174	0.0401
hyphal tip (GO:0001411)	7	4.27	0.00233	0.0448
anchored component of membrane (GO:0031225)	16	3.49	4.1E-05	0.00221
hyphal cell wall (GO:0030446)	11	3.46	0.000705	0.0211
cell surface (GO:0009986)	22	2.83	2.92E-05	0.00197
extracellular region (GO:0005576)	21	2.21	0.00133	0.0326
cytosol (GO:0005829)	24	2.13	0.00112	0.0292
<b>GO biological process of repressed genes</b>				
GO term	No. of genes	Fold enrichment	<i>P</i> -value	FDR
cell septum assembly (GO:0090529)	3	21.36	0.0016	0.0404
GDP-mannose biosynthetic process (GO:0009298)	3	21.36	0.0016	0.0401
regulation of flocculation (GO:0060256)	4	12.21	0.00101	0.0309
cellular response to lipid (GO:0071396)	4	10.68	0.00146	0.0397
aromatic amino acid family biosynthetic process (GO:0009073)	6	10.68	8.93E-05	0.00541

histidine biosynthetic process (GO:0000105)	4	10.68	0.00146	0.0392
cell wall chitin biosynthetic process (GO:0006038)	4	10.68	0.00146	0.039
septin ring organisation (GO:0031106)	5	10.68	0.000358	0.0146
endoplasmic reticulum organisation (GO:0007029)	4	10.68	0.00146	0.0381
cellular biogenic amine biosynthetic process (GO:0042401)	4	9.5	0.00203	0.0487
flocculation (GO:0000128)	4	9.5	0.00203	0.0484
entry into host (GO:0044409)	5	7.63	0.0012	0.0339
cell-cell adhesion (GO:0098609)	5	7.12	0.00154	0.0392
development of symbiont in host (GO:0044114)	6	7.12	0.000516	0.0186
invasive filamentous growth (GO:0036267)	6	5.34	0.00181	0.0442
establishment or maintenance of cell polarity (GO:0007163)	13	4.96	8.82E-06	0.00101
cell-substrate adhesion (GO:0031589)	9	4.81	0.000267	0.0119
regulation of protein kinase activity (GO:0045859)	8	4.38	0.000994	0.0312
positive regulation of filamentous growth of a population of unicellular organisms in response to biotic stimulus (GO:1900445)	8	4.07	0.00151	0.039
positive regulation of multi-organism process (GO:0043902)	9	4.01	0.000855	0.028
protein glycosylation (GO:0006486)	10	3.82	0.000623	0.0218
negative regulation of filamentous growth (GO:0060258)	10	3.68	0.000794	0.0266
cellular response to biotic stimulus (GO:0071216)	10	3.56	0.001	0.031
single-species biofilm formation on inanimate substrate (GO:0044011)	18	3.43	1.64E-05	0.0017
regulation of response to stress (GO:0080134)	13	3.43	0.000251	0.0115
fungus-type cell wall organisation (GO:0031505)	16	3.32	6.98E-05	0.00491
intracellular signal transduction (GO:0035556)	13	3.31	0.000345	0.0146
hyphal growth (GO:0030448)	14	3.29	0.000217	0.0106
pathogenesis (GO:0009405)	51	3.23	1.01E-12	1.47E-09
positive regulation of transcription, DNA-templated (GO:0045893)	15	2.97	0.000353	0.0148

anatomical structure development (GO:0048856)	12	2.95	0.00143	0.0394
homeostatic process (GO:0042592)	15	2.61	0.0012	0.0337
response to abiotic stimulus (GO:0009628)	19	2.4	0.00085	0.0281
cellular response to starvation (GO:0009267)	24	2.31	0.000372	0.0147
filamentous growth of a population of unicellular organisms in response to starvation (GO:0036170)	22	2.21	0.000977	0.0309
filamentous growth of a population of unicellular organisms in response to biotic stimulus (GO:0036180)	26	2.19	0.000318	0.0138
cellular macromolecule localisation (GO:0070727)	21	2.15	0.00164	0.0408
regulation of transcription from RNA polymerase II promoter (GO:0006357)	24	2.14	0.0011	0.0328
cellular response to drug (GO:0035690)	27	1.99	0.00113	0.033
organic substance transport (GO:0071702)	31	1.85	0.00141	0.0388
RNA processing (GO:0006396)	1	0.09	0.000234	0.0111

**Table 2.9. Overrepresented GO Terms of *Candida albicans* induced genes in response to 0.1 mM arachidonic acid in a polymicrobial biofilm.**

Overrepresented Gene Ontology (GO) terms with PANTHER (Overrepresentation Test released 2017-12-05; GO Ontology database released 2017-12-27) (Mi *et al.*, 2013) of significantly ( $p_{adj} < 0.05$ ) differentially expressed genes. Significance based on Fisher's Exact with False Discovery Rate (FDR) multiple test correction.

<b>GO cellular component of induced genes</b>				
GO term	No. of genes	Fold enrichment	P-value	FDR
small-subunit processome (GO:0032040)	9	8.44	5.45E-06	0.00147
preribosome, large subunit precursor (GO:0030687)	6	6.91	0.000527	0.0387
nucleolus (GO:0005730)	14	4.17	1.91E-05	0.00257
cell wall (GO:0005618)	17	2.66	0.000422	0.0341
plasma membrane (GO:0005886)	35	2.29	8.69E-06	0.00176
protein complex (GO:0043234)	6	0.3	0.0004	0.0404

<b>GO biological process of induced genes</b>					
GO term	No. of genes	Fold enrichment	<i>P</i> -value	FDR	
ribosomal large subunit biogenesis (GO:0042273)	9	7.6	1.11E-05	0.00484	
cellular response to oxidative stress (GO:0034599)	12	3.8	0.000168	0.0457	
oxidation-reduction process (GO:0055114)	44	2.64	9.38E-09	1.36E-05	
cellular response to drug (GO:0035690)	29	2.53	1.21E-05	0.00479	
pathogenesis (GO:0009405)	29	2.18	0.000176	0.0453	
organic substance metabolic process (GO:0071704)	112	1.39	4.02E-05	0.0125	

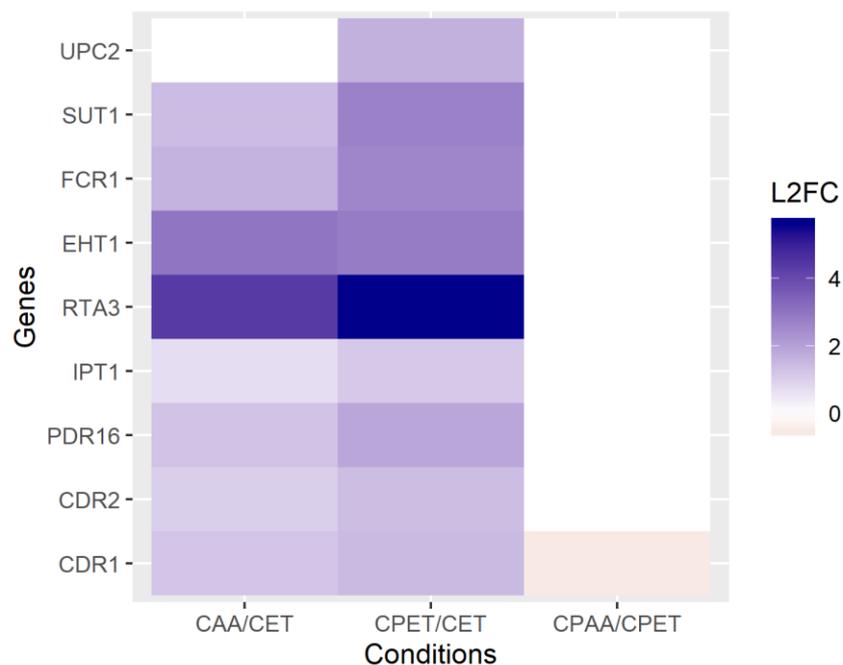
Additionally, *SOD3*, encoding for the cytoplasmic manganese-containing superoxide dismutase (Lamarre *et al.*, 2001) is repressed by *P. aeruginosa* but is induced in the presence of AA in polymicrobial biofilms.

#### 2.4.4.3. Cell wall and membrane organisation

Like monomicrobial biofilms of *C. albicans* exposed to AA, gene products localised to the cell wall and membrane are overrepresented in both repressed and induced genes (**Table 2.9**). This may also indicate an alteration of membrane and cell wall organisation, similar to monomicrobial biofilms. Strikingly, a number of genes that play a role in membrane organisation and that are induced in monomicrobial biofilms exposed to AA as well as in response to *P. aeruginosa* (**Supplementary Table S2.3, S2.4**), are weakly or not differentially expressed in polymicrobial biofilms exposed to AA (**Figure 2.3**). These genes are discussed in the section *Effect of 0.1 mM arachidonic acid on Candida albicans monomicrobial biofilms*. This may indicate that *P. aeruginosa* utilised the available AA, decreasing the concentration of the available fatty acid so that it does not induce these genes further. An alternate possibility is that an induction limit of these genes was reached in response to *P. aeruginosa*. Therefore, the stimulus of addition of AA did not cause further upregulation of the genes.

#### 2.4.4.4. Morphogenesis and biofilm formation

The previous section discussing the *Effect of P. aeruginosa on C. albicans during co-incubation* highlights the convergence of multiple stimuli to affect morphogenesis in *C. albicans*. This includes stimuli with contrasting effects on the morphogenesis, that may occur through multiple signalling pathways including the morphogenesis inducing pathways: mitogen-activated protein (MAP) kinase pathway, the cyclic-AMP (cAMP)-PKA pathway, the pH-responsive pathway (Rim101) as well as the morphogenesis suppressing pathways namely Nrg1p-Tup1p repression (Shareck, 2011). Evidence is presented that the inhibition of morphogenesis due to *P. aeruginosa* might rely, in part, on the Set3C. Indeed, *SET3* is overexpressed in response to *P. aeruginosa*. The addition of AA to polymicrobial biofilms did not elicit differential expression of components of the Set3C. This is similar to what is observed in monomicrobial biofilms exposed to AA. Interestingly, whereas *NRG1* is not differentially expressed in response to *P. aeruginosa*, it is induced in both monomicrobial and polymicrobial biofilms exposed to AA, similar to what is observed for CLA (Shareck, 2011).



**Figure 2.3. Heatmap of differential expression of selected gene involved in membrane organisation.** CAA/CET – Monomicrobial biofilms of *Candida albicans* exposed to 0.1 mM arachidonic acid (AA) compared to ethanol control; CPET/CET – polymicrobial biofilms of *C. albicans* and *Pseudomonas aeruginosa* compared to monomicrobial biofilms of *C. albicans* in the absence of AA; CPAA/CPET – polymicrobial biofilms exposed to 0.1 mM AA compared to polymicrobial biofilms exposed to ethanol control.

The downstream target of Nrg1p, a transcription factor *BRG1*, a positive regulator of filamentation, is repressed in response to AA in a polymicrobial biofilm, further confirming *NRG1s* induction in response to AA (Clearly *et al.*, 2012). In addition, *UME6* (Table 2.10) is repressed in response to AA in polymicrobial biofilms along with repression of *SFL2*, *EFG1*, *FKH2* and *RIM101*, characteristic of the inhibition of morphogenesis (Bensen *et al.*, 2002, 2004; Zeidler *et al.*, 2009; Znaidi *et al.*, 2013). This may indicate that further inhibition of morphogenesis occurs with the supplementation of AA. The response in terms of morphogenesis when AA is added to polymicrobial biofilms seems to be more pronounced, as processes linked to septin formation and hyphal growth are repressed (Table 2.8). Septins function in membrane remodelling, cytokinesis and cell polarity (Bridges and Gladfelter, 2015; Gonzales-Novo *et al.*, 2008; Warena *et al.*, 2003). In addition, septin formation is required for

**Table 2.10. Overrepresented transcription factors of *Candida albicans* in response to 0.1 mM arachidonic acid in a polymicrobial biofilm with *Pseudomonas aeruginosa*.** Overrepresented transcription factors generated by PathoYeasttract (Monteiro *et al.*, 2016). Descriptions according to Candida Genome Database (Skrzypek *et al.*, 2016).

Transcription factor	ORF	No. of genes	P-value	log <sub>2</sub> FC	Process/Description
Ume6p	orf19.1882	98	1.50E-14	-0.97	Filamentation
Brg1p	orf19.4056	72	1.17E-06	-0.95	Filamentation
Sfl2p	orf19.3969	254	1.05E-11	-0.54	Filamentation
Rlm1p	orf19.4662	36	4.43E-08	-0.46	Cell wall damage
Efg1p	orf19.610	273	2.23E-07	-0.43	Filamentation
Ahr1p	orf19.7381	82	3.37E-05	-0.42	Adhesion
Bcr1p	orf19.723	91	1.48E-08	-0.37	Adhesion
Fkh2p	orf19.5389	196	2.16E-09	-0.30	Filamentation; Cell cycle
Rim101	orf19.7247	116	1.42E-07	-0.30	pH inducible filamentation
Csr1p	orf19.3794	140	3.87E-13	0.65	Zinc homeostasis; Filamentation

morphogenesis and invasive growth. Therefore, the inhibition of morphogenesis may lead to the observed reduction in septin formation.

#### 2.4.4.5. Overlap of genes being differentially expressed in mono- and polymicrobial biofilms exposed to AA

To identify if a similar response to exposure to AA in both monomicrobial and polymicrobial biofilms could be seen, differentially expressed genes that overlap in all three datasets were identified and split into repressed and induced genes (**Supplementary Table S2.5**). This response includes repression of adhesion involved in monomicrobial biofilm formation, including the genes *ALS3* (hyphae-associated transferrin receptor), as well as the transcription factors *DEF1*, *AHR1* and *FCR3*. Notably, an induction of cell wall organisation, associated with the induction of *RBE1*, *BMT4*, *PIR1*, *XOG1*, *PHR2*, *RHD1* and *DCW1*, is seen in all three conditions. This is of special importance due to the role played by the cell wall during infection, as it is not only the first contact between the fungus and host cells, playing roles in adherence of the fungus to host tissue, but also displays antigenic determinants that can elicit immune response by the host (Ruiz-Herrera *et al.*, 2005). Changes in the organisation of this, may elicit drastic changes in virulence.

#### 2.4.5. Confirmation of differential expression with nCounter®

nCounter® (Nanostring Technologies) was used to confirm differential expression (observed with RNAseq) of selected genes. This method quantifies mRNA transcripts directly with minimal sample preparation (Geiss *et al.*, 2008). The principle of the method relies on two probes (capture and reporter probes) that hybridize to a complementary target mRNA. The reporter probes carry specific fluorophores unique to each target and provide signal detection. The capture probe facilitates immobilisation to an nCounter® cartridge that enables removal of unhybridized probes and quantification of mRNA through imaging and counting of the color-coded fluorophores in an automated system. This technology was used to quantify mRNA transcripts of genes of interest in this chapter (Summarised in **Table 2.11**). To aid in normalisation of expression levels of the selected genes, housekeeping genes (*TDH3*, *ACT1* and *LSC2*) that did not show differential expression in all conditions (**Supplementary Tables S2.2**, **S2.3** and **S2.4**) were included. Although the majority of differential expression quantified with nCounter® are similar to results obtained by

RNAseq, a number of genes do show variation from RNAseq data. This could be attributed to the fact that only one sample of each condition was used to quantify differential expression with nCounter®, whereas RNAseq was performed on three biological replicates of each condition and represent the average of these replicates.

**Table 2.11. Log<sub>2</sub> fold change (L2FC) of gene expression comparing nCounter values with results obtained by RNAseq. CAA/CET – single species biofilms of *Candida albicans* exposed to 0.1 mM arachidonic acid AA compared to ethanol control (0.076 %); CPET/CET – polymicrobial biofilms of *C. albicans* and *Pseudomonas aeruginosa* (without AA) compared to single species biofilms of *C. albicans*; CPAA/CPET – polymicrobial biofilms exposed to 0.1 mM AA compared to polymicrobial biofilms exposed to ethanol (0.076%).**

L2FC							
Gene name	ORF	CAA/CET		CPET/CET		CPAA/CPET	
		nCounter	RNAseq	nCounter	RNAseq	nCounter	RNAseq
<b>*ACT1</b>	orf19.5007	0.02	0.08	-0.01	-0.41	-0.07	-0.49
<b>*TDH3</b>	orf19.6814	-0.14	-0.02	-0.22	0.14	0.1	-0.25
<b>*LSC2</b>	orf19.1860	0.12	-0.01	0.24	0.13	-0.02	-0.47
<b>CDR1</b>	orf19.6000	1.30	1.21	1.53	1.46	-0.33	-0.52
<b>CDR2</b>	orf19.5958	1.21	1.02	0.57	1.39	-0.26	-0.31
<b>PDR16</b>	orf19.1027	1.39	1.27	1.97	1.89	0.37	-0.11
<b>FCR1</b>	orf19.6817	2.12	1.61	2.95	2.60	1.08	0.21
<b>MDR1</b>	orf19.5604	0.62	0.27	3.75	2.02	1.16	0.50
<b>RTA3</b>	orf19.23	5.13	4.38	5.78	5.62	0.89	0.47
<b>IPT1</b>	orf19.4769	0.90	0.70	1.33	1.16	-0.36	-0.33
<b>NRG1</b>	orf19.7150	1.19	0.68	0.42	-0.01	1.62	1.00
<b>TAC1</b>	orf19.3188	0.32	0.23	1.18	0.61	0.06	-0.19
<b>UPC2</b>	orf19.391	-0.18	-0.07	1.04	1.66	0	-0.09
<b>SUT1</b>	orf19.4342	1.60	1.41	2.88	2.72	0.21	-0.20
<b>TYE7</b>	orf19.4941	0.88	0.64	1.77	1.16	0.55	0.14
<b>EHT1</b>	orf19.3040	2.68	3.00	2.48	2.83	0.42	0.35
<b>YWP1</b>	orf19.3618	2.78	1.81	0.26	1.18	5.42	1.50
<b>HSP12</b>	orf19.3160	1.62	1.14	1.03	0.72	1.88	0.85
<b>CSR1</b>	orf19.3794	-0.24	-0.18	-1.05	-1.37	0.7	0.65
<b>SAP6</b>	orf19.5542	0.08	0.28	1.54	1.45	-0.7	-0.65
<b>WOR1</b>	orf19.4884	0.06	0.19	1.38	2.84	0.47	-0.16
<b>SET3</b>	orf19.7221	0.01	0.14	2.05	2.11	0.38	0.04

\*Housekeeping genes used for normalisation of mRNA transcript counts.

## 2.5. CONSIDERATIONS AND DRAWBACKS

For the formation of both mono- and polymicrobial biofilms, a stationary model adapted from Fourie *et al.* (2017), was followed. Petri-dishes are wrapped in parafilm to allow the rapid depletion of oxygen and formation of microaerophilic to hypoxic biofilms, similar to the biofilms formed in the lungs of cystic fibrosis patients. The formation of microaerophilic to hypoxic biofilms may be a more accurate representation of *in vivo* conditions, compared to planktonic growth of cells with aeration, as was previously used to determine the effect of *P. aeruginosa* on *C. albicans* (Holcombe *et al.*, 2010) as well as the effect of another fatty acid, CLA, on *C. albicans* (Shareck *et al.*, 2011). However, biofilms were allowed to form in a closed system, with AA supplemented at time zero. This may not accurately represent conditions *in vivo* during infection, which may reflect a dynamic system with a continuous supply of nutrients and AA. Furthermore, AA is prone to autoxidation (Jahn *et al.*, 2008; Milne *et al.*, 2015), which could lead to a large number of oxygenated metabolites in addition to free fatty acid. Therefore, the current results obtained may not only be representative of the influence of AA, but also oxygenated products. Furthermore, harvesting the biofilms at 6 hours may have allowed some cells to reach stationary phase. More pronounced transcriptional changes may therefore have occurred at an earlier time point. Future research may include harvesting biofilms at an earlier time point during exponential phase. Lastly, although a bioinformatics pipeline was included that normalises counts, false discovery of significantly expressed genes could still occur. To alleviate this, we filtered data according to *P*-value as well as L2FC (in some instances) and confirmed differential expression with the use of nCounter®.

## 2.6. CONCLUSIONS

RNAseq was successfully used to evaluate the effect of co-incubation of *C. albicans* with *P. aeruginosa* as well as the effect of 0.1 mM AA on both monomicrobial and polymicrobial biofilms. Sequencing yielded high quality reads that were used to evaluate both overrepresented GO terms as well as TFs influenced by these circumstances.

Co-incubation of *C. albicans* with *P. aeruginosa* indicated a possible response towards hypoxia, with a shifting towards fermentative growth. This response may be due to the experimental setup with static biofilm formation and rapid depletion of oxygen in the

biofilm. Furthermore, hypoxic environment may have driven the reduced expression of genes associated with iron and zinc acquisition, as hypoxia may decrease the need for these metals. Whether this factor may play a role during infection deserves further attention. In addition, a decrease in iron and zinc acquisition systems is seen, possibly due to the decreased need for these metals as a result of repression of growth of *C. albicans* by *P. aeruginosa*. Interestingly, *SET3* transcription is upregulated in the presence of *P. aeruginosa*, indicating that the Set3C may be contributing to the interaction of *C. albicans* with *P. aeruginosa*, possibly acting as a transcriptional buffer to control filamentation and dispersal. Furthermore, the white-opaque regulator *WOR1* is upregulated in the presence of *P. aeruginosa*, possibly indicating that switching to the opaque-phenotype may be taking place, although additional signals may be required to initiate the full opaque-transcriptional profile.

Concerning the effect of AA on monomicrobial biofilms of *C. albicans*, genes associated with membrane organisation are upregulated. This response may be similar to the effect of other polyunsaturated fatty acids, such as docosahexaenoic acid and CLA. These changes in membrane organisation may be associated with loss of localisation and function of membrane-associated proteins, affecting various processes such as filamentation and drug susceptibility. Of special interest is the induction of *CDR1*, whose overexpression is associated with decreased susceptibility to antifungal agents such as fluconazole, although AA has been shown to decrease antifungal resistance. This phenomenon requires further investigation.

Responses to stress (including chemical, xenobiotic, drug, hydroxy compound and oxidative) are exacerbated with addition of AA to polymicrobial biofilms. Furthermore, several genes involved in membrane organisation, that are induced when exposed to AA as well as co-incubation with *P. aeruginosa*, is not further induced with exposure of polymicrobial biofilms to AA.

Induction of *SET3* and *WOR1* by *P. aeruginosa* is not affected by addition of AA to polymicrobial biofilms, similar to monomicrobial biofilms exposed to AA. However, as with monomicrobial biofilms exposed to AA, *NRG1* is induced in polymicrobial biofilms exposed to AA. This may indicate that the inhibition of morphogenesis by *P. aeruginosa* and AA may follow different routes, with AA inhibiting morphogenesis via Tup1p-Nrg1p repression, similar to the effect of CLA.

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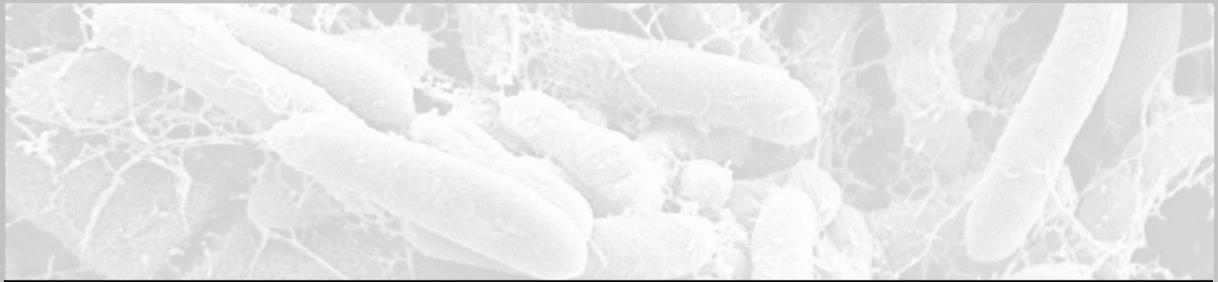
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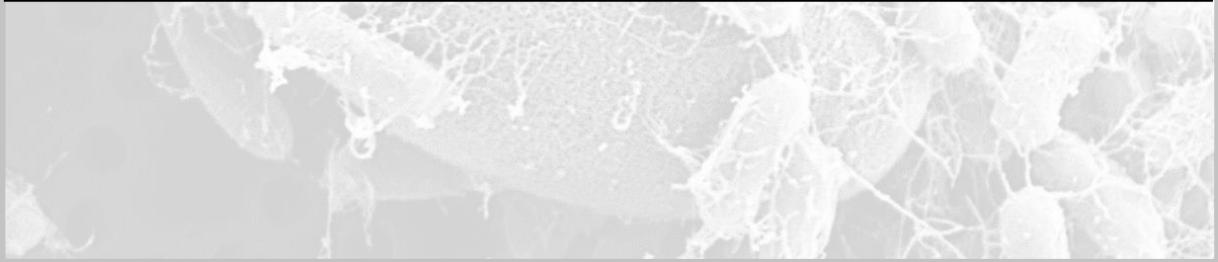
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### **CHAPTER 3**

**Role of *SET3* and *WOR1* in the interaction of *Candida albicans* with *Pseudomonas aeruginosa***



### 3.1. ABSTRACT

*Candida albicans* exhibits multiple morphologies dependent on environmental cues. Along with classical morphologies (yeast, pseudohyphae and true hyphae), *C. albicans* undergoes white-opaque switching, an epigenetic transition driven by the expression of *WOR1*. The interchangeability between the phenotypes is orchestrated by a complex transcriptional circuit, with an additional level of regulation imposed by chromatin-remodelling through the Set3/Hos2-histone deacetylase complex (Set3C). In addition to the polymorphology observed in *C. albicans*, biofilms are frequently polymicrobial in nature, enabling interspecies interaction through proximity and contact. The interaction between *C. albicans* and the bacterium, *Pseudomonas aeruginosa*, is described as antagonistic *in vitro*. *Pseudomonas aeruginosa* represses the yeast-to-hyphal switch in *C. albicans*. In addition, it preferentially kills *C. albicans* hyphae.

In *Chapter 2*, RNAseq was used to evaluate the transcriptomic response of *C. albicans* towards *P. aeruginosa* in polymicrobial biofilms and an induction of *SET3*, a component of the Set3C, was observed. Furthermore, *WOR1* was induced. The induction of *WOR1* is associated with increased commensalism with bacteria. To determine if *SET3* and *WOR1* play a role in the interaction of *C. albicans* with *P. aeruginosa*, homozygous deletion mutants were constructed and their ability to form polymicrobial biofilms with *P. aeruginosa* was evaluated. The deletion of *WOR1* did not affect polymicrobial biofilm population dynamics, biofilm morphology or virulence in *Caenorhabditis elegans* infection model. Furthermore, deletion of *SET3* did not significantly affect polymicrobial biofilm formation or morphology of *C. albicans* in the presence of *P. aeruginosa*. However, *set3Δ/Δ* did exhibit a reduction in virulence of *C. albicans* in a *C. elegans* infection model, even in the presence of *P. aeruginosa*.

### 3.2. INTRODUCTION

Interkingdom interactions are ubiquitous in nature and can affect various aspects of the growth, antimicrobial resistance and virulence of species within a consortium (Stacey *et al.*, 2016; Peters *et al.*, 2012). In the opportunistic fungal pathogen, *Candida albicans*, this is frequently encountered in polymicrobial associations formed with commensal microorganisms as well as pathobionts in humans (Diaz *et al.*, 2012; Morales and Hogan, 2010; Neville *et al.*, 2015). This is, in part, due to the ability to form antifungal resistant biofilms on both abiotic and biotic surfaces (Polke *et al.*, 2015).

*Candida albicans* exhibits polymorphism, with up to nine distinct phenotypes being formed (Noble *et al.*, 2017). This includes the classical phenotypes, yeast, hyphae, pseudohyphae and chlamydospores, as well as non-classical phenotypes dependent on the expression of the white-opaque regulator *WOR1* (Lan *et al.*, 2002; Noble *et al.*, 2017; Pande *et al.*, 2013; Tong *et al.*, 2014). In addition to the white phenotype [heterozygous at mating type locus (MTL;  $a/\alpha$ ), or homozygous at the MTL ( $a/a$  or  $\alpha/\alpha$ )], opaque phenotypes ( $a/\alpha$ ,  $a/a$  or  $\alpha/\alpha$ ), gray and gastrointestinally-induced transition (GUT) phenotypes exist. These different phenotypes show alterations in mode of growth, morphology, carbon source utilisation and virulence. Opaque and GUT are cells associated with less invasive form of infection and increased fitness in the presence of commensal bacteria (Lan *et al.*, 2002; Pande *et al.*, 2013; Tong *et al.*, 2014). The frequency of this epigenetic switch between phenotypes is dependent on environmental cues, including hypoxia and CO<sub>2</sub>, presence of bacterial-cell wall components such as *N*-acetylglucosamine as well as temperature (37°); conditions frequently found in the gastrointestinal tract (Pande *et al.*, 2013). The induction of *WOR1* expression has been observed in combination with bacteria such as *Klebsiella pneumoniae*, *Escherichia coli* and *Enterococcus faecalis* (Fox *et al.*, 2014). However, less is known about the expression of this gene with other bacteria.

*Candida albicans* is frequently co-isolated with the Gram-negative bacterium *Pseudomonas aeruginosa* from the lungs of cystic fibrosis patients (Chotirmall *et al.*, 2010; Haiko *et al.*, 2019; Leclair and Hogan, 2010). *In vitro*, the interaction is characterised as antagonistic, with both species influencing the other (reviewed by Fourie and Pohl, 2019). The bacterium was found to lyse and kill hyphal cells of *C. albicans* through physical interaction (Bandara *et al.*, 2010; Brand *et al.*, 2008; Hogan

and Kolter, 2002). In addition, it affects *C. albicans* biofilm formation and morphogenesis through various secreted factors and cell wall components (Bandara *et al.*, 2010, 2013; Hogan *et al.*, 2004; Holcombe *et al.*, 2010; Kerr *et al.*, 1999; McAlester *et al.*, 2008; Reen *et al.*, 2011; Xu *et al.*, 2008). This includes inhibition of morphogenesis from yeast to hyphal morphologies through phenazines, quorum sensing molecules, sequestration of iron and lipopolysaccharides, and promotion of morphogenesis by peptidoglycan. These stimuli elicit their effects through various signalling pathways in *C. albicans*, including stimulation of morphogenesis through the mitogen activated protein (MAP) kinase signalling pathway and the cyclic adenosine monophosphate (cAMP)/ protein kinase A (PKA), as well as repression by the transcriptional repressor (Tup1p) (Shareck and Belhumeur, 2011). Therefore, multiple stimuli from co-incubation with *P. aeruginosa* may play a role at one time to affect the morphology of *C. albicans*. In addition, the Set3/Hos2 histone deacetylase complex (Set3C) inhibits the cAMP-PKA signalling pathway as well as the white-opaque transition (Hnisz *et al.*, 2009).

This complex is conserved in *Saccharomyces cerevisiae*, consisting of 4 core subunits, namely, Set3p, Hos2p, Snt1p and Sif2p and 3 peripheral subunits Hos4p, Hst1p and Cpr1p (Hnisz *et al.*, 2010). In *S. cerevisiae* the complex has multiple functions including meiosis-specific repression of sporulation, fostering Ty1 retrotransposons integration at tRNA genes and signalling secretory stress through the PKC cell integrity pathway (Cohen *et al.*, 2008; Hnisz *et al.*, 2010; Mou *et al.*, 2006; Pijnappel *et al.*, 2001). Deletion of critical components of the complex in *C. albicans* infers a hyperfilamentous form on solid media, an increase in biofilm dry biomass and reduced dispersion in liquid medium and *in vivo* (Hnisz *et al.*, 2010; Nobile *et al.*, 2014). Interestingly, the Set3C has less than 20% identity with human or other *C. albicans* histone deacetylases, making it an attractive target for treatment of *C. albicans* infections (Garnaud *et al.*, 2016).

In *Chapter 2*, the transcriptional response of *C. albicans* towards *P. aeruginosa* during early biofilm development was evaluated and an increase in transcription of *SET3* and *WOR1* was seen (**Supplementary Table S2.2**). This raises the following questions: firstly, does the Set3C play a role in the interaction of *C. albicans* with *P. aeruginosa*, conferring a decrease in filamentation and dispersal to escape attack? Secondly, as *WOR1* is induced upon exposure to *P. aeruginosa*, does *WOR1* play a role in the

interaction of *C. albicans* with *P. aeruginosa*? This is of interest as the cystic fibrosis lung may contain triggers for induction of *WOR1* and phenotypic switching, such as hypoxia, high CO<sub>2</sub> and bacterial cell wall components including *N*-acetylglucosamine (Montgomery *et al.*, 2017). These questions will be addressed through construction of homozygous deletion mutants of *SET3* and *WOR1* and characterising polymicrobial biofilm formation, population dynamics and virulence (using a A549 reporter cell line and *Caenorhabditis elegans* infection model) of *C. albicans* in the presence of *P. aeruginosa*.

### 3.3. MATERIALS AND METHODS

#### 3.3.1. Strains used in this study

The strains used in this study are summarized in **Table 3.1**. *Candida albicans* strains were stored at -80°C with 15% glycerol. Yeast strains were revived and maintained on yeast malt (YM) agar (3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone, 10 g/L glucose, 16 g/L agar) at 30°C. *Pseudomonas aeruginosa* PAO1 was stored at -80°C with 25% glycerol and revived/maintained on Luria-Bertani (LB) agar (5 g/L yeast extract, 10 g/L tryptone, 10 g/L sodium chloride and 15 g/L agar).

#### 3.3.2. Construction of homozygous deletion mutants with CRISPR/Cas9

A CRISPR-Cas9 system published by the group of Aaron Hernday (Nguyen *et al.*, 2017) was used for the formation of homozygous mutants for *SET3* and *WOR1*. The plasmids used in this study were obtained from Addgene ([www.addgene.org](http://www.addgene.org)). All primers were purchased from Integrated DNA Technologies and are listed in **Table 3.2**. All Polymerase chain reactions (PCRs) were performed with KAPA Hifi Hotstart PCR Kit (KAPA Biosystems Inc.) and colony PCR was performed with KAPA Taq PCR Kit (KAPA Biosystems Inc.), unless otherwise stated.

##### 3.3.2.1. Plasmids and cassettes for deletion

Two microgram pADH99 (Addgene plasmid # 90979) was digested with PmeI (New England Biolabs, US) to release the CAS9 cassette (**Figure 3.1A**). For the gRNA-cassette, the 5' of the gRNA cassette was amplified from pADH110 (Addgene plasmid # 90982) with primers AHO1096-ver2 and AHO1098-ver2 (**Figure 3.1B**). To introduce target-specific CRISPR-sites into the gRNA cassette, gene sequences (for *SET3* and *WOR1*) with approximately 500 bp upstream and downstream were obtained from

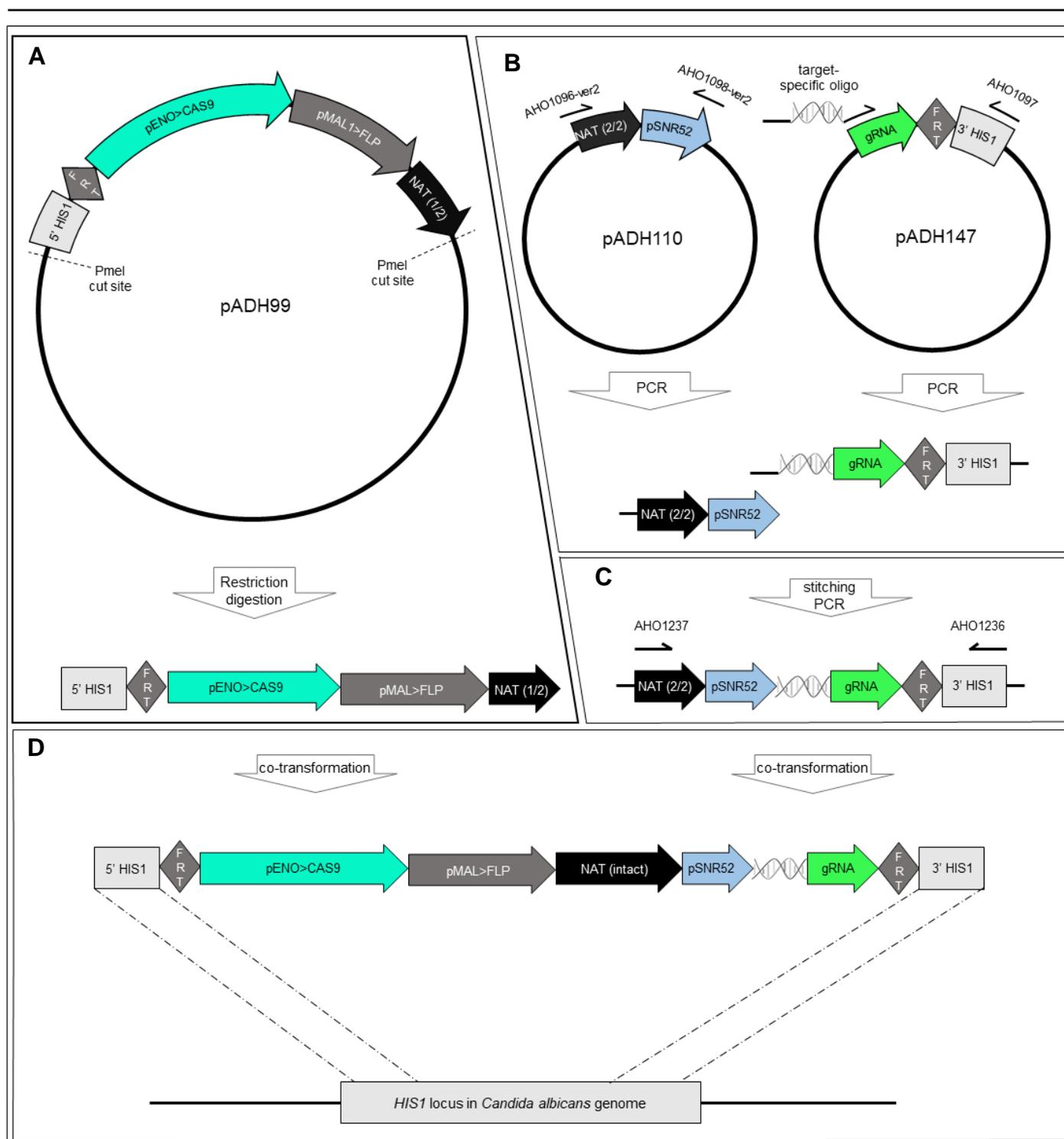
*Candida* Genome Database (CGD) and CRISPR-sites (20 bp sequence followed by a protospacer adjacent motif, PAM) for *SET3* and *WOR1* respectively were retrieved with *Geneious* (version 10.2.6; <https://www.geneious.com>). CRISPR-sites were chosen as to have the highest on-site activity score (Doench *et al.*, 2014) and off-target score (Hsu *et al.*, 2013) compared to the diploid genome of *C. albicans* SC5314 (Muzzey *et al.*, 2013; Skrzypek *et al.*, “*Candida* Genome Database” <http://candidagenome.org/> Accessed 20 July 2018) to minimize off target events in the *C. albicans* genome. In addition, CRISPR sites were chosen only if they were identical on both alleles of the diploid genome according to Assembly 22 of the *C. albicans* SC5314 genome (Muzzey *et al.*, 2013; Skrzypek *et al.*, “*Candida* Genome Database”). The flanking sequences, 5'-CGTAAACTATTTTAAATTTG-3' and 5'-GTTTTAGAGCTAGAAATAGC-3' were added to the 5' and 3' sides of the 20bp CRISPR site without the PAM sequence respectively (**Table 3**). These two regions are complementary to *SNR52* promoter on pADH110 (Addgene plasmid # 90982) and structural gRNA sequence on pADH147 (Addgene plasmid # 90991) respectively. The 3' of the gRNA cassette was constructed through amplification of pADH147 with the oligos with flanking sequences described above as forward primer (**Figure 3.1B**) and AHO1097 as reverse primer. The 3' of the gRNA cassette (product size of 704 bp) was fused with the amplification product (1066 bp) of pADH110 with a stitching polymerase chain reaction with primers AHO1237 and AHO1236 (**Figure 3.1C**). The cassettes containing the *CAS9* as well as the gRNA is co-transformed into *C. albicans* to form an intact *CAS9*-gRNA cassette for integration into the *HIS1* locus (**Figure 3.1D**).

Table 3.1. Strains used in this study

Name	Species	Genotype	Parental strain	Description	Origin
SC5314	<i>C. albicans</i>	Wild type	N/A	Reference strain	N/A
<i>set3Δ/Δ</i>	<i>C. albicans</i>	<i>set3Δ;set3Δ</i>	SC5314	Homozygous deletion of <i>SET3</i>	This study
<i>set3Δ/Δ::SET3</i>	<i>C. albicans</i>	<i>set3Δ::SET3;set3Δ::SET3</i>	<i>set3Δ/Δ</i>	Homozygous add-back of <i>SET3</i> gene into <i>set3Δ/Δ</i> to restore wild type	This study
<i>wor1Δ/Δ</i>	<i>C. albicans</i>	<i>wor1Δ;wor1Δ</i>	SC5314	Homozygous deletion of <i>WOR1</i>	This study
<i>wor1Δ/Δ::WOR1</i>	<i>C. albicans</i>	<i>wor1Δ::WOR1;wor1Δ::WOR1</i>	<i>wor1Δ/Δ</i>	Homozygous add-back of <i>WOR1</i> gene into <i>wor1Δ/Δ</i> to restore wild type	This study
PAO1	<i>P. aeruginosa</i>	Wild type	N/A	Reference strain	N/A

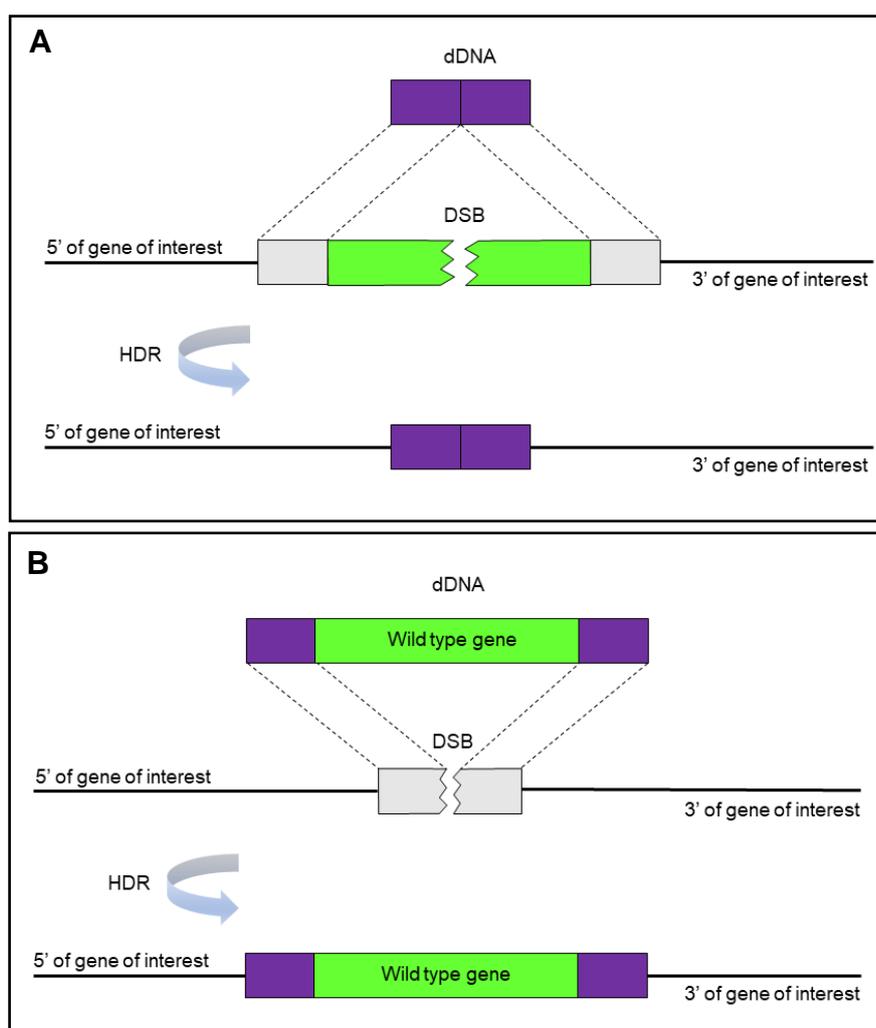
Table 3.2. List of primers used in this study

Name	Sequence (5' to 3')	Reference
SET3-2F	GCAACATGAAATAGATGGCTG	This study
SET3-2R-overlap	CTGTCGAGGCAGTATCGACAAACCAAACA	This study
SET3-3F-overlap	TGTCGATACTGCCTCGACAGTTACAGGAGAA	This study
SET3-3R	AGGCAAGAGACTGTGATTTGA	This study
SET3-CRISPR-1-deletion	CGTAAACTATTTTTAATTTGATACTTATGGAGCTTTTCCGGTTTTAGAGCTAGAAATAGC	This study
SET3-CRISPR-2-add-back	CGTAAACTATTTTTAATTTGGCTTCTTTACAACCTTAGCAGTTTTAGAGCTAGAAATAGC	This study
WOR1-2F	GCCTTTCCTGTATTGGTATTGGT	This study
WOR1-2R-overlap	GCCCCAAAATCCATCTTTTGATTCCCGACA	This study
WOR1-3F-overlap	CAAAAGATGGATTTTGGGGCTCATTGAGT	This study
WOR1-3R	CCTACAGATTGAGTTTAACCAAATC	This study
WOR1-CRISPR-1-deletion	CGTAAACTATTTTTAATTTGCGCGAAGATGACACGAACAAGTTTTAGAGCTAGAAATAGC	This study
WOR1-CRISPR-2-add-back	CGTAAACTATTTTTAATTTGGTATAGTCCCTACATATAATGTTTTAGAGCTAGAAATAGC	This study
AHO1096-ver2	GACGGCACGGCCACGCGTTTAAAC	Modified from Nguyen <i>et al.</i> (2017)
AHO1097	CCCGCCAGGCGCTGGGGTTTAAACACCG	Nguyen <i>et al.</i> (2017)
AHO1098-ver2	CGAGACTTGCCTAAACTATTTTTAATTTG	Modified from Nguyen <i>et al.</i> (2017)
AHO1236	TAAAGCTGCCACAAGAGGTATTTTC	Nguyen <i>et al.</i> (2017)
AHO1237	AGGTGATGCTGAAGCTATTGAAG	Nguyen <i>et al.</i> (2017)



**Figure 3.1. Schematic representation of the preparation of the CRISPR CAS9-gRNA cassette for integration into the *Candida albicans* genome from Nguyen *et al.* (2017).** **A** – The CAS9-cassette is liberated via digestion of pADH99 with PmeI. **B** – The 5' and 3' end of the gRNA-cassette is prepared through PCR of pADH110 and pADH147. **C** – The full gRNA-cassette is created through stitching PCR of amplicons created in B. **D** – The CAS9 and gRNA cassettes are co-transformed to create an intact CAS9-gRNA-cassette that integrates into the *HIS1* locus of the *Candida albicans* genome facilitated by homologous regions on each side of the cassette.

Donor DNA was designed to remove nearly the entire open reading frame of the target genes. A modification from the Nguyen and co-workers (2017) protocol entailed extending homologous regions to approximately 150 bp to 500 bp for deletion. In addition, homologous regions of approximately 500 bp were used for re-introduction of wild type genes (**Figure 3.2**).



**Figure 3.2.** Schematic of the method of homology directed repair (HDR) via addition of donor DNA (dDNA). **A** – donor DNA is designed to overlap with flanking regions of the gene of interest. After the formation of a double stranded break (DSB), dDNA is used to repair the DSB, facilitating the near entire removal of an open reading frame. **B** – a similar procedure is followed for the re-introduction of a gene, where the open reading frame is included in the

**Table 3.3** represents the information of the donor DNA constructed for the deletion of *SET3* and *WOR1*. Genomic DNA was extracted with the *Quick-DNA*<sup>TM</sup> Fungal/Bacterial Miniprep Kit (Zymo Research, USA) according to manufacturer's specifications. Two fragments were amplified from the *C. albicans* SC5314 genome with fragment 1 located at the 5' of the coding sequence fragment 2 at the 3'-end. Stitching PCR was performed to fuse fragment 1 and 2 using primer sets underlined in **Table 3.4** to produce complete donor DNA for deletion of the target genes.

**Table 3.3.** Unique CRISPR-site sequences without protospacer adjacent motif for deletion and add-back of *SET3* and *WOR1*. Site-characteristics obtained in *Geneious*.

Gene	Application	Sequence	On-site activity	Off-target activity
<i>SET3</i>	Deletion	ATACTTATGGAGCTTTTCCG	0.919	100.00%
<i>SET3</i>	Add-back	GCTTCTTTACAACCTTTAGCA	0.809	100.00%
<i>WOR1</i>	Deletion	CGCGAAGATGACACGAACAA	0.763	83.33%
<i>WOR1</i>	Add-back	GTATAGTCCCTACATATAAT	0.619	83.33%

### 3.3.2.2. Transformation

*Candida albicans* SC5314 was inoculated in 5 mL YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) and incubated overnight with shaking (30°C). The overnight culture was inoculated into fresh YPD (ratio of 1:50) and allowed to reach an optical density (600 nm) of 0.5 to 0.8 after which cells were washed two times with sterile water (Nguyen *et al.*, 2017). Cells were resuspended in 1/100 H<sub>2</sub>O of original volume. Un-purified digest of pADH99 and PCR products of gRNA cassette and dDNA were added to the washed cells together with 1 mL plate mix consisting of 875 µL 50% PEG 3350 (Sigma-Aldrich), 100 µL 10X TE buffer (100 mM Tris pH 7.4, 10 mM EDTA pH 8) and 25 µL 1M Lithium acetate (pH adjusted to 7 with acetic acid; Sigma-Aldrich) and incubated overnight at 30°C without shaking. Cells were heat-shocked at 44.6°C for 15 minutes, washed with sterile YPD and allowed to recover for 5 hours at 30°C with shaking. Recovered cells were plated onto YPD agar (15 g/L agar) plus 200 µg/mL nourseothricin (NTC, Jena Bioscience, Germany) and incubated for 2 to 3 days at 30°C to allow formation of colonies. Colony PCR was performed to amplify dDNA with primers SET3-2F and SET3-3R for *SET3*; and WOR1-2F and WOR1-3R for *WOR1*.

### 3.3.2.3. Removal of CAS9-gRNA cassette and knock-in of wild type gene

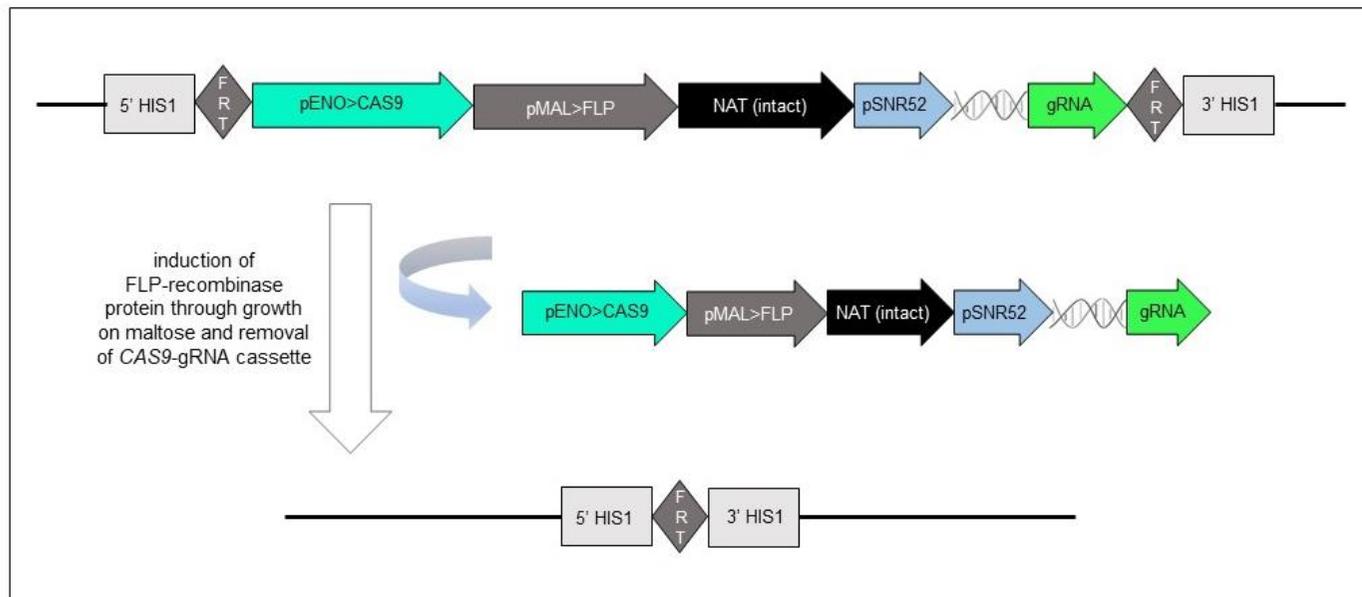
After confirmation of deletion of the target gene, the CAS9-gRNA cassette was removed by inducing expression of a FLP/FRT recombinase system (under control of maltose-inducible promoter (**Figure 3.3**) through growth in Yeast Peptone Maltose media (10 g/L yeast extract, 20 g/L peptone and 20 g/L maltose) for 24h @ 30°C with shaking (200 rpm) (Nguyen *et al.*, 2017). Cells were diluted in sterile H<sub>2</sub>O and plated on YPD agar for 48h @ 30°C to allow formation of single colonies. After formation of colonies, growth on NTC was evaluated by streaking on YPD agar with 300 µg/mL

**Table 3.4.** Summary of donor DNA characteristics for deletion and add-back of *SET3* and *WOR1*

Gene	Application	Fragment	Forward primer	Reverse primer	Start position*	End position*	Coordinates of deletion/add-back	Size
<i>SET3</i>	Deletion	1	<u>SET3-2F</u>	SET3-2R-overlap	-504	-33	-33 to 3039	3072 bp
		2	SET3-3F-overlap	<u>SET3-3R</u>	3039	3774		
	Add-back	N/A	SET3-2F	SET3-3R	-504	3774	-33 to 3039	4278 bp
<i>WOR1</i>	Deletion	1	<u>WOR1-2F</u>	WOR1-2R-overlap	-405	198	198 to 2385	2187 bp
		2	WOR1-3F-overlap	<u>WOR1-3R</u>	2385	2683		
	Add-back	N/A	WOR1-2F	WOR1-3R	-405	2683	198 to 2385	2187 bp

\* Position from first bp of coding sequence.

Underlined primers were used for stitching polymerase chain reaction to fuse fragment 1 and fragment 2 of each gene for deletion.



**Figure 3.3.** Schematic representation of the removal of the CRISPR CAS9-gRNA cassette from the *Candida albicans* genome.

NTC. The absence of growth in the presence of NTC indicates that the CAS9-gRNA cassette has been removed. After removal of the CAS9-gRNA cassette, a sequential round of transformation was performed as described above to re-introduce the wild type gene into its native locus (**Figure 3.2**).

### 3.3.3. Biofilm formation

#### 3.3.3.1. Monomicrobial biofilm formation by *C. albicans*

*Candida albicans* strains represented in **Table 3.1** were revived from glycerol stocks on YM agar for 24 h at 30 °C and inoculated into 5 mL yeast nitrogen base (YNB) broth (10 g/L glucose, 16 g/L YNB) and incubated at 30 °C for 24 h with shaking (150 rpm). Cells were harvested at 1878 g for 5 minutes and the supernatant removed. This was followed by washing the cells twice with phosphate buffered saline (PBS) (Oxoid, England). The cells were counted with a haemocytometer and diluted to  $1 \times 10^6$  cells/mL in filter sterilized (0.22 µm nitrocellulose filter, Merck Millipore, Ireland) RPMI-1640 medium (Sigma-Aldrich, USA).

#### 3.3.3.2. Polymicrobial biofilm formation by *C. albicans* and *P. aeruginosa*

*Candida albicans* strains represented in **Table 3.1** were revived from glycerol stocks on YM agar for 24 h at 30 °C and inoculated into 5 mL YNB broth and incubated at 30 °C for 24 h with shaking (150 rpm). Cells were harvested at 1878 g for 5 minutes and the supernatant removed. This was followed by washing the cells twice with phosphate buffered saline (PBS) (Oxoid, England). The cells were counted with a haemocytometer and diluted to  $1 \times 10^6$  cells/mL in filter sterilized RPMI-1640 medium. *Pseudomonas aeruginosa* PAO1 was revived on LB agar and inoculated into LB broth and incubated 24h at 37°C. Bacterial cells were washed with sterile PBS and diluted to an OD<sub>600</sub> of 0.05 in RPMI-1640 medium containing the *C. albicans* cells.

#### 3.3.3.3. Metabolic activity of mono- and polymicrobial biofilms

Cells were prepared as described above and 200 µL was dispensed into a 96-well plate (Corning Incorporated, Costar®, USA). The plate was incubated for 6h and 48h respectively at 37 °C to allow the formation of biofilms. Following incubation, the supernatant from each well was removed and the biofilms were washed twice with sterile PBS. Fifty microliter of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino) carbonyl]-2H tetrazolium hydroxide (XTT) (Sigma-Aldrich, USA) (1 g

XTT in 1L PBS, filter sterilized, aliquoted and stored at -20°C) containing 0.08 mM menadione (Fluka, USA) (a stock solution of 10 mM menadione in acetone) was added to each well and incubated for 3 h in the dark at 37°C (Fourie *et al.*, 2017; Kuhn *et al.*, 2003). Following incubation, the absorbance of each well was measured at 492 nm on an EZ Read 800 Microplate Reader (Biochrom, UK) and data was obtained. A cell free control (RPMI-1640) was included. This experiment was performed in triplicate with four technical replicates per biological replicate.

#### 3.3.3.4. *Biofilm biomass of mono- and polymicrobial biofilms*

Cells were prepared as described above and 200 µL was dispensed into a 96-well plate (Corning Incorporated, Costar®, USA). The plate was incubated for 6h and 48h respectively at 37°C to allow the formation of biofilms. The crystal violet assay was performed on biofilms according to Jin and co-workers (2003) with minor modifications. Briefly, the supernatant from each well was removed and the biofilms were washed twice with sterile PBS. Biofilms were then left to air dry for 45 minutes and stained with 110 µL crystal violet (0.4% w/v; Merck, Germany) for 45 min (Jin *et al.*, 2003). Biofilms were washed three times with 350 µL sterile H<sub>2</sub>O and de-stained with 200 µL 95% ethanol for 45 min. One hundred microliter of de-staining solution was then transferred to a clean 96-well plate and absorbance was measured at 595 nm. This experiment was performed in triplicate with four technical replicates per biological replicate.

#### 3.3.3.5. *Morphology of mono- and polymicrobial biofilms*

Cells for mono- and polymicrobial biofilms were prepared as described above in flat-bottom 6 well culture plates in 3 mL medium (Fourie *et al.*, 2017). After incubation, supernatant was removed and approximately 5 mm rectangular sections of the wells were cut and placed in PBS. Cells were fixed overnight in 3 % (v/v) glutardialdehyde (Merck, Germany) in phosphate buffer. This was followed by washing of biofilms with PBS and fixation with 1% osmium tetroxide (Merck, Germany) for 1h. Following fixation, biofilms were sequentially dehydrated with increasing concentration of ethanol (50% to 100%) and subjected to critical point drying (Samdri-795 Critical point dryer, Tousimis, USA). A gold layer was applied to the biofilms with a SEM coating system (Bio-Rad, UK) and examined using a JSM-7800F Extreme-resolution Analytical Field Emission SEM.

### 3.3.4. Survival of *C. albicans* in a polymicrobial biofilm with *P. aeruginosa*

Cells for mono- and polymicrobial biofilms were prepared as described above in flat-bottom 6 well culture plates (Corning Incorporated, USA) in 3 mL medium and incubated for 48h to allow biofilm formation to take place. After incubation, biofilms were washed twice with sterile PBS, scraped off and suspended in PBS. Biofilms were then vortexed 3 times for 1 minute to remove adherent cells from one another (adapted from Fourie *et al.*, 2017). For quantification of *C. albicans*, serially diluted cells were plated onto YM medium acidified with tartaric acid (final concentration 0.08%). For bacterial quantification, serially diluted cells were plated onto LB supplemented with 10 µg/mL amphotericin B (Sigma-Aldrich, USA) (Pires *et al.*, 2013). Plates were incubated overnight, to allow formation of colonies, and counted. This experiment was performed in triplicate.

### 3.3.5. Infection of A549-Dual™ lung epithelial cells by *C. albicans* and *P. aeruginosa*

#### 3.3.5.1. Initial culture from frozen stocks

A549-Dual™ lung epithelial cells (Invivogen, USA) were revived according to manufacturer's specifications with minor modifications. Briefly, frozen cells were thawed in a 37°C waterbath and washed with pre-warmed DMEM + GlutaMAX™ (Gibco, USA) with 20% gamma-irradiated fetal bovine serum (FBS, gibco). Cells were resuspended in 5 mL DMEM + GlutaMAX™ with 20% FBS and 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL Amphotericin B (Lonza, USA) in a T-25 cell culture flask (Nunc™ EasYFlask™, Thermo Scientific) and incubated at 37°C with 5% CO<sub>2</sub>.

#### 3.3.5.2. General cell maintenance

Cells, revived from frozen stocks, were maintained in growth medium (DMEM + GlutaMAX™ with 10% FBS and 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL Amphotericin B) with 10 µg/mL blasticidin (Gibco, USA) and 100 µg/mL Zeocin™ (Invitrogen) as selective antibiotics in a T-75 cell culture flask (Nunc™ EasYFlask™, Thermo Scientific) and passaged at 70-80% confluence, changing media every 3-4 days.

### 3.3.5.3. Infection

A549-Dual™ lung epithelial cells between 70-80% confluence were washed twice with sterile PBS and diluted to  $2 \times 10^5$  cells/ml in growth medium and 200  $\mu$ l of cell suspension was dispensed in a 96 well plate. Cells were incubated for 24h with 5% CO<sub>2</sub> to allow adherence to the well surface. After adherence of the A549-Dual™ lung epithelial cells, wells were washed three times with sterile PBS and 200  $\mu$ l of DMEM + Glutamax™ (serum- and antibiotic free) containing  $1 \times 10^6$  cells/mL *C. albicans* (alive) or  $5 \times 10^6$  cells/mL *C. albicans* (heat-killed, 30 min @ 70°C) were added to adhered cells. For co-infection, *P. aeruginosa* at 0.001 OD (alive) or 0.005 OD (heat-killed, 30 min @ 99°C) were added in addition to *C. albicans* cells. A549-Dual™ lung epithelial cells with *C. albicans* or *C. albicans* plus *P. aeruginosa* were incubated at 37°C with 5% CO<sub>2</sub>.

### 3.3.5.4. Innate immune induction of A549-Dual™ lung epithelial cells

To determine cytotoxicity of A549-Dual™ lung epithelial cells, CyQUANT™ LDH Cytotoxicity Assay (Invitrogen, USA) was utilised. To determine innate immune induction, QUANTI-Blue™ Solution (Invivogen, USA) for NF- $\kappa$ B induction, and QUANTI-Luc™ for interferon regulatory factor (IRF) pathway (Invivogen, USA) were used, according to manufacturer's specifications. For NF- $\kappa$ B and IRF induction, recombinant human tumor necrosis factor alpha (TNF- $\alpha$ ) (Invivogen, USA) was used as positive control.

## 3.3.6. Infection of *Caenorhabditis elegans* by *C. albicans* and *P. aeruginosa*

### 3.3.6.1. Propagation of *C. elegans*

*Caenorhabditis elegans* AU37 [glp-4(bn2) I; sek-1(km4 X)], obtained for the *Caenorhabditis* Genetic Centre (University of Minnesota), was used for all infections. The nematodes were propagated and maintained on Nematode Growth Medium (3 g/L NaCl, 2.5 g/L peptone, 5  $\mu$ g/mL cholesterol, 1 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 25 mM KPO<sub>4</sub>, 20 g/L agar) with *Escherichia coli* OP50 as a food source at 15°C (Brenner, 1974).

### 3.3.6.2. Infection with *C. albicans*

For infection by *C. albicans* alone, *C. albicans* strains were inoculated in YPD broth overnight at 30°C. Overnight cells were diluted to an OD<sub>600</sub> of 0.8 and 100 µL was plated onto brain-heart infusion (BHI)-agar plates and incubated overnight at 30°C. Synchronised L4-stage nematodes were washed with M9 buffer (3 g/L KH<sub>2</sub>PO<sub>4</sub>, 6 g/L Na<sub>2</sub>PO<sub>4</sub> and 1 mM MgSO<sub>4</sub>) and added to plates with *C. albicans*. Nematodes were incubated with *C. albicans* for 4 hours at 25°C and washed three times with M9 buffer to remove non-ingested *C. albicans* cells. Nematodes were then added at approximately 60 per well in a 6 well plate (Corning Incorporated, USA) containing 2 mL 80% M9 buffer and 20% BHI broth, with 90 µg/mL kanamycin and incubated at 25°C. Nematodes were monitored daily and dead nematodes (non-motile after mechanical stimulation with sterile pipette tip or penetration of *C. elegans* cuticle by *C. albicans* hyphae) were counted and removed.

### 3.3.6.3. Infection with *C. albicans* and *P. aeruginosa*

For dual-infection by both *C. albicans* and *P. aeruginosa*, *C. albicans* was prepared on BHI-agar plates as described above. *P. aeruginosa* was inoculated in LB broth overnight and diluted to OD<sub>600</sub> of 0.8. One hundred microliter of the *P. aeruginosa* suspension was plated on BHI-plates and incubated overnight at 37°C. Synchronised L4-stage nematodes were washed with M9 buffer and added to plates incubated with *C. albicans* for 2 hours at 25°C. Nematodes were washed three times with M9 buffer and added to BHI-plates with *P. aeruginosa* for 2 hours at 25°C. Nematodes were washed again with M9 buffer to remove non-ingested *P. aeruginosa* cells and placed in M9 buffer in 6 well plates (approximately 60 nematodes per well) and incubated at 25°C. Nematodes were monitored daily and dead worms (non-motile after mechanical stimulation with sterile pipette tip or penetration of *C. elegans* cuticle by *C. albicans* hyphae) were counted and removed.

Nematodes incubated with *E. coli* OP50 prior to survival assay was used as a control. Infection was performed in triplicate for a total of 180 nematodes per strain of *C. albicans*. Survival metrics including Kaplan-Meier statistics, median survival time and log-rank test were performed with online application for survival analysis 2 (OASIS 2; Han *et al.*, 2016).

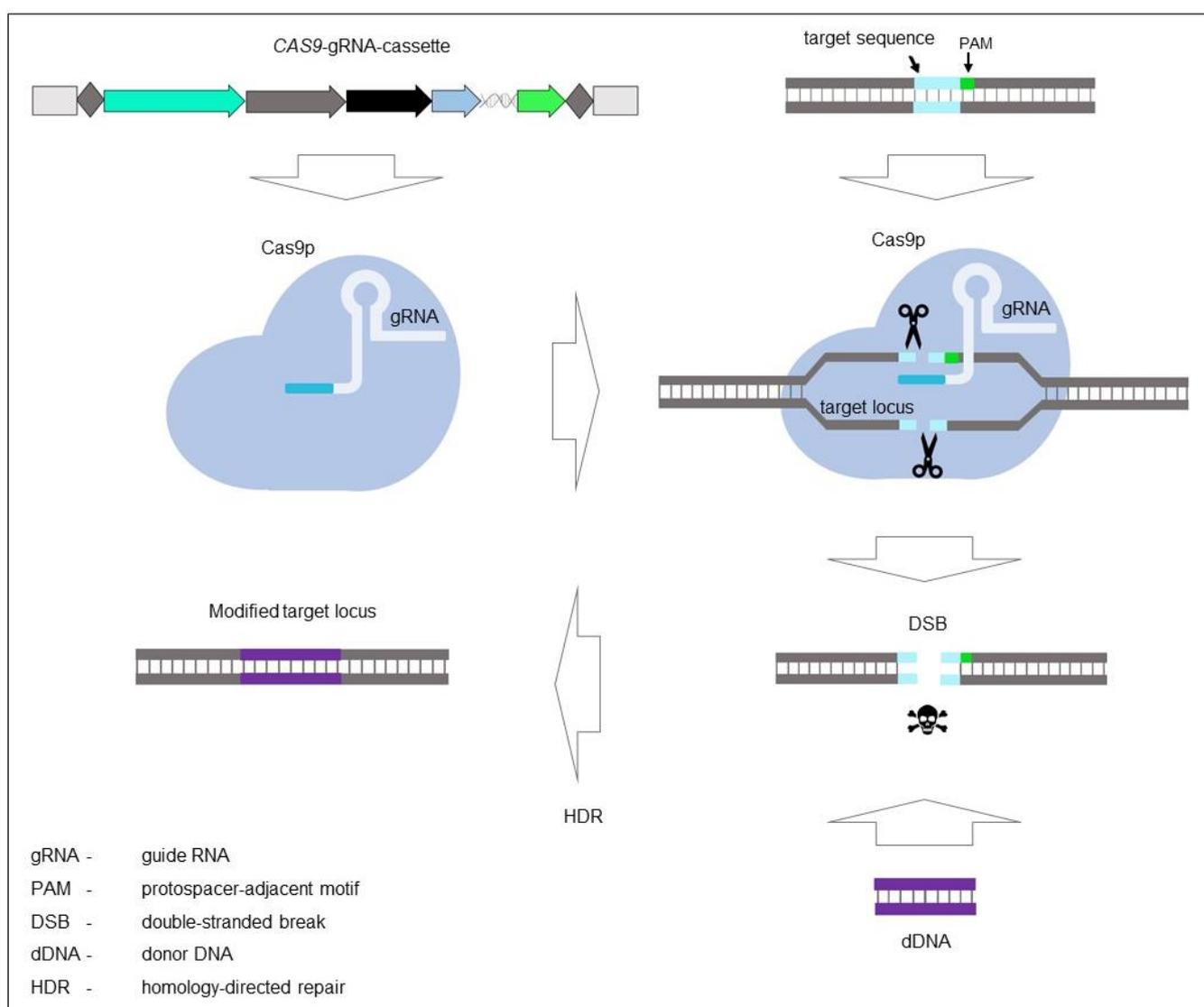
### 3.3.7. Statistical analysis

Differences between control and experimental, as well as between *C. albicans* strains were determined with *t*-test, performed with GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) unless otherwise stated.

## 3.4. RESULTS AND DISCUSSION

### 3.4.1. Homozygous knock-out and knock-in of selected genes

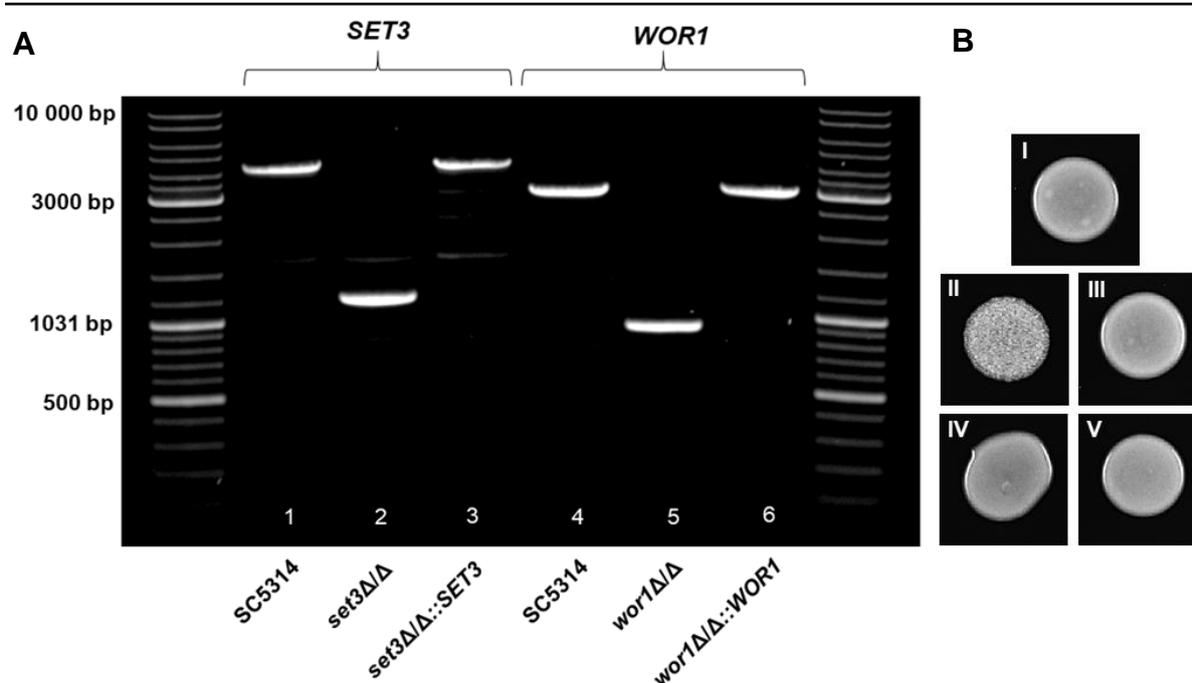
For deletion with the CRISPR/Cas9 system, three components are required, a Cas9, a site-specific guide RNA (gRNA) and donor DNA (dDNA) for homology directed repair (HDR). The system used in this study makes use of a *CAS9* (from *Streptococcus pyogenes*, recognising a protospacer adjacent motif, PAM, of NGG) under the control of an *ENO1* promoter and gRNA (under the control of a *SNR52* promoter), carried on two separate cassettes that are co-transformed to integrate into one allele of the *HIS1* locus (**Figure 3.1**). Successful transformants are NTC resistant, accomplished by the *N*-acetyltransferase (NAT) marker (Nguyen *et al.*, 2017). The *CAS9*-cassette contains the 5'-end of the NAT marker at its 3'-end, whereas the gRNA-cassette contains the 3'-portion on its 5'end. Successful integration of both cassettes yields a full NAT gene conferring resistance to nourseothricin. Expression of the Cas9p together with a unique guide RNA leads to a site-specific double stranded break. In addition, dDNA is co-transformed that is used for the repair of the double stranded break caused by the action of the Cas9. A graphical representation of this system can be seen in **Figure 3.4**.



**Figure 3.4.** Schematic representation of the action of the CRISPR-Cas9 system published by Nguyen and co-workers (2017) after integration into the *Candida albicans* genome. Expression of the Cas9p and gRNA leads to the formation of a Cas9-gRNA complex. A unique gRNA sequence guides the complex to a target in the genome where the Cas9-gRNA complex forms a double stranded break (DSB). This DSB is repaired via homology directed repair with donor DNA (dDNA) that contains homologous regions to the target site of the DSB.

After confirmation of deletion with PCR, the CAS9-gRNA cassette was removed by induction of a maltose-inducible FLP/FRT recombinase system that renders the homozygous deletion mutants ready for another round of transformation to add the wild type gene back (Nguyen *et al.*, 2017). By utilising this system, both copies of the wild-type gene can be reintroduced into the target locus. This second transformation event is also succeeded by the removal of the CAS9-gRNA cassette. The successful homozygous deletion and subsequent add-back of the wild type gene for *SET3* and

*WOR1* was achieved as indicated in **Figure 3.5A**. In addition, the hyperfilamentous phenotype of *set3Δ/Δ* can be seen in **Figure 3.5B** with wrinkled colony morphology on YPD agar incubated at 37°C, compared to smooth colony morphology of the wild type (SC5314).



**Figure 3.5. Confirmation of deletion and re-introduction of *SET3* and *WOR1* in *Candida albicans*.** **A** - Gel-electrophoresis of PCR products amplifying a region in the open reading frame of the gene of interest with primers as described in **Table 3.4** for stitching PCR (used for confirmation of modification). *SET3* – the wild type gene corresponds to a PCR product of 4278 bp, whereas a deletion is indicated by a band corresponding to 1206 bp. *WOR1* - wild type gene corresponds to a PCR product of 3088 bp, whereas a deletion is indicated by a band corresponding to 901 bp. First and last row indicate *Fermentas O'GeneRuler™ DNA Ladder*. **B** – Colony morphology of SC5314 (wild type) (I), *set3Δ/Δ* (II), *set3Δ/Δ::SET3* (III), *wor1Δ/Δ* (IV) and *wor1Δ/Δ::WOR1* (V) after 24

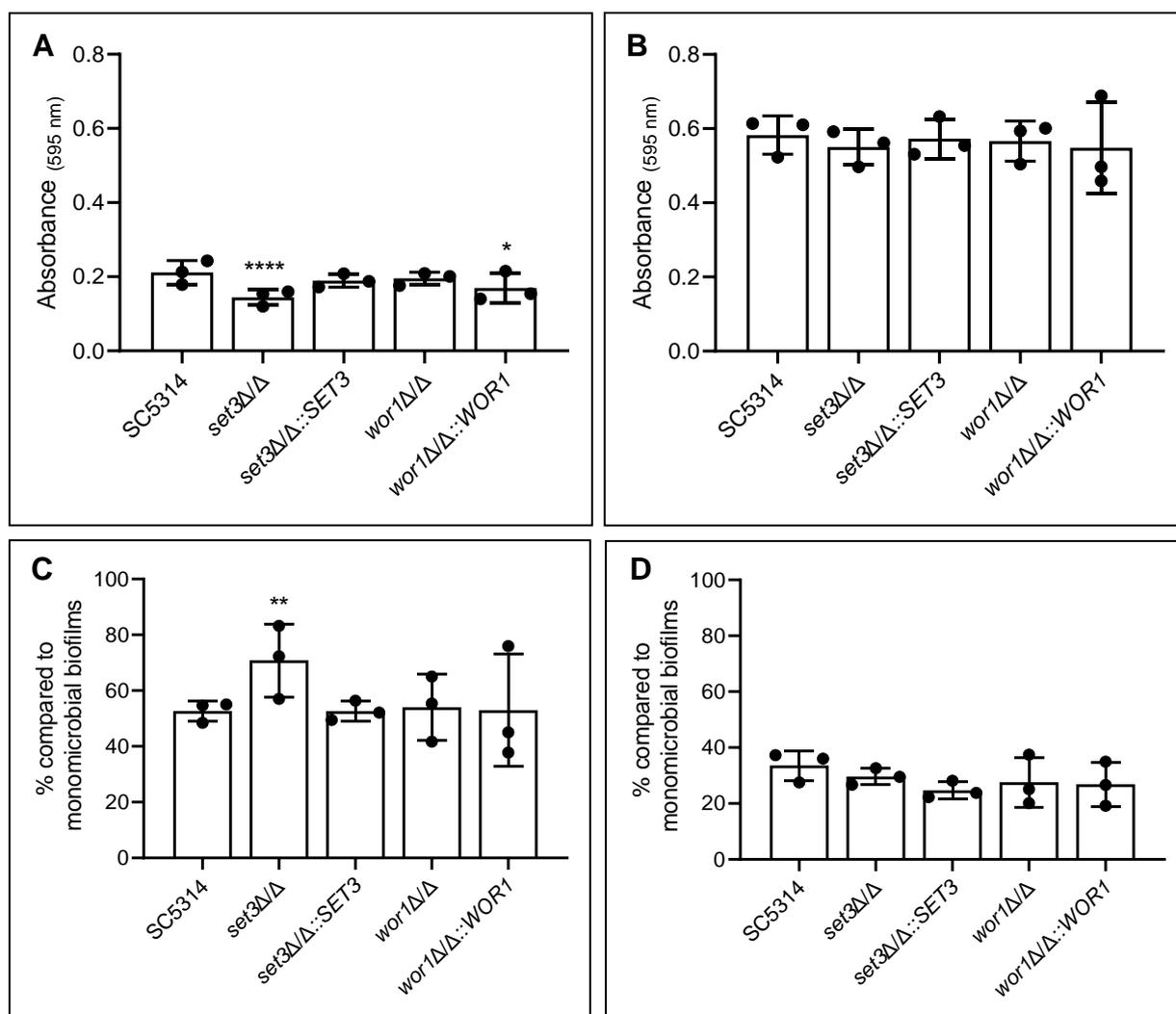
### 3.4.2. Mono- and polymicrobial biofilm growth of *set3Δ/Δ* and *wor1Δ/Δ*

To evaluate the effect of the deletion of *SET3* and *WOR1* on *C. albicans* biofilm formation capability, biomass and metabolic activity during early biofilm development (6h) as well as after 48 hours (mature biofilms) in both mono- and polymicrobial biofilms was quantified as presented in **Figure 3.6** and **Figure 3.7**.

#### 3.4.2.1. Monomicrobial biofilms

**Figure 3.6A** indicates that a significant ( $P < 0.0001$ ) reduction in early (6h) monomicrobial biofilm biomass (approximately 33.6%) of *set3Δ/Δ* compared to the wild type. This was restored to wild type values with the homozygous re-introduction of the *SET3* gene. This phenomenon is unexpected as deletion of *SET3* is known to increase filamentous growth in the presence of hyphae-inducing conditions (Nobile *et al.*, 2014). However, to measure biomass, biofilms are subjected to rigorous mechanical stress during washing to remove non-adherent cells and to remove crystal violet that could have led to loss of biomass due to mechanical shearing. Nobile and co-workers (2014) reported the contrary, with deletion of *SET3* conferring “rubbery” biofilms that are recalcitrant to mechanical shearing. Therefore, reason for the decreased biofilm biomass of *set3Δ/Δ* in the present study is unknown. However, this effect is lost during maturation of biofilms (48h; **Figure 3.6B**). When evaluating metabolic activity of monomicrobial biofilms, no significant decrease in *set3Δ/Δ* is seen after 6h (**Figure 3.7A**). After 48h a significant decrease (approximately 27.4%) in *set3Δ/Δ* is observed that is restored to wild type levels with the re-introduction of the wild type gene (**Figure 3.7B**). The reason for this phenomenon is unknown.

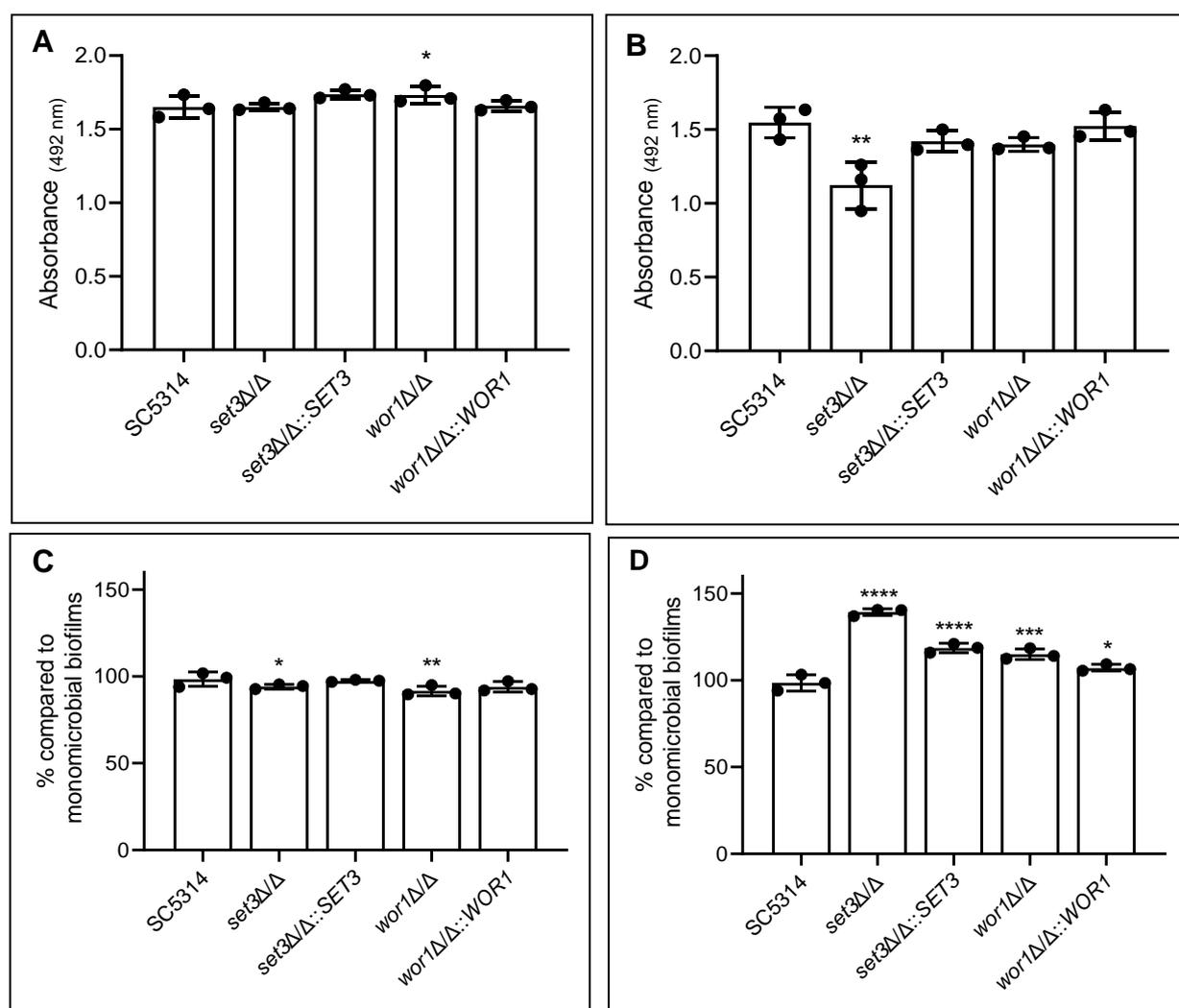
The loss of *WOR1* did not significantly affect monomicrobial biofilm biomass after early or mature biofilm formation (**Figure 3.6A**). This is expected as *WOR1* transcript counts were low during monomicrobial biofilm formation and may not affect monomicrobial biofilm development (**Supplementary Table S2.2**). A statistically significant ( $P = 0.0435$ ) decrease in biofilm biomass (approximately 22.0%) after 6h is observed with the re-introduction of *WOR1* into its native locus, indicating that a growth defect-inducing modification may have taken place during strain construction. However, this effect is lost after 48h.



**Figure 3.6. Biomass, as measured with the crystal violet assay, of selected *Candida albicans* mutants as mono- and polyeicrobial biofilms with *Pseudomonas aeruginosa*.** Monomicrobial biofilm biomass after 6 h (A) and 48 h (B) of homozygous mutants of *SET3* (*set3Δ/Δ*) and *WOR1* (*wor1Δ/Δ*). Homozygous mutants with add-back of wild-type genes are included (*set3Δ/Δ::SET3* and *wor1Δ/Δ::WOR1* respectively). In addition, percentage biomass of polyeicrobial biofilms, of 6h (C) and 48h (D), compared to monomicrobial biofilms are indicated. \*Significantly different from wild type (SC5314) (\*  $P < 0.05$ ; \*\*  $P < 0.005$ ; \*\*\*  $P < 0.0005$ ; \*\*\*\*  $P < 0.0001$ ).

### 3.4.2.2. Polyeicrobial biofilms

When *set3Δ/Δ* is exposed to *P. aeruginosa* in a polyeicrobial biofilm, a significant increase (approximately 34.4%) in biomass is seen after 6h (Figure 3.6C), however, this is accompanied by a minor decrease (approximately 4.2%) in metabolic activity



**Figure 3.7. Metabolic activity of selected *Candida albicans* mutants as mono- and polymicrobial biofilms with *Pseudomonas aeruginosa*.** Monomicrobial biofilm metabolic activity after 6 h (A) and 48 h (B) of homozygous mutants of *SET3* (*set3Δ/Δ*) and *WOR1* (*wor1Δ/Δ*). Homozygous mutants with homozygous add-back of wild-type genes are included (*set3Δ/Δ::SET3* and *wor1Δ/Δ::WOR1* respectively). In addition, percentage metabolic activity of polymicrobial biofilms, of 6h (C) and 48h (D), compared to monomicrobial biofilms are indicated. \*Significantly different from wild type (SC5314) (\*  $P < 0.05$ ; \*\*  $P < 0.005$ ; \*\*\*  $P < 0.0005$ ; \*\*\*\*  $P < 0.0001$ ).

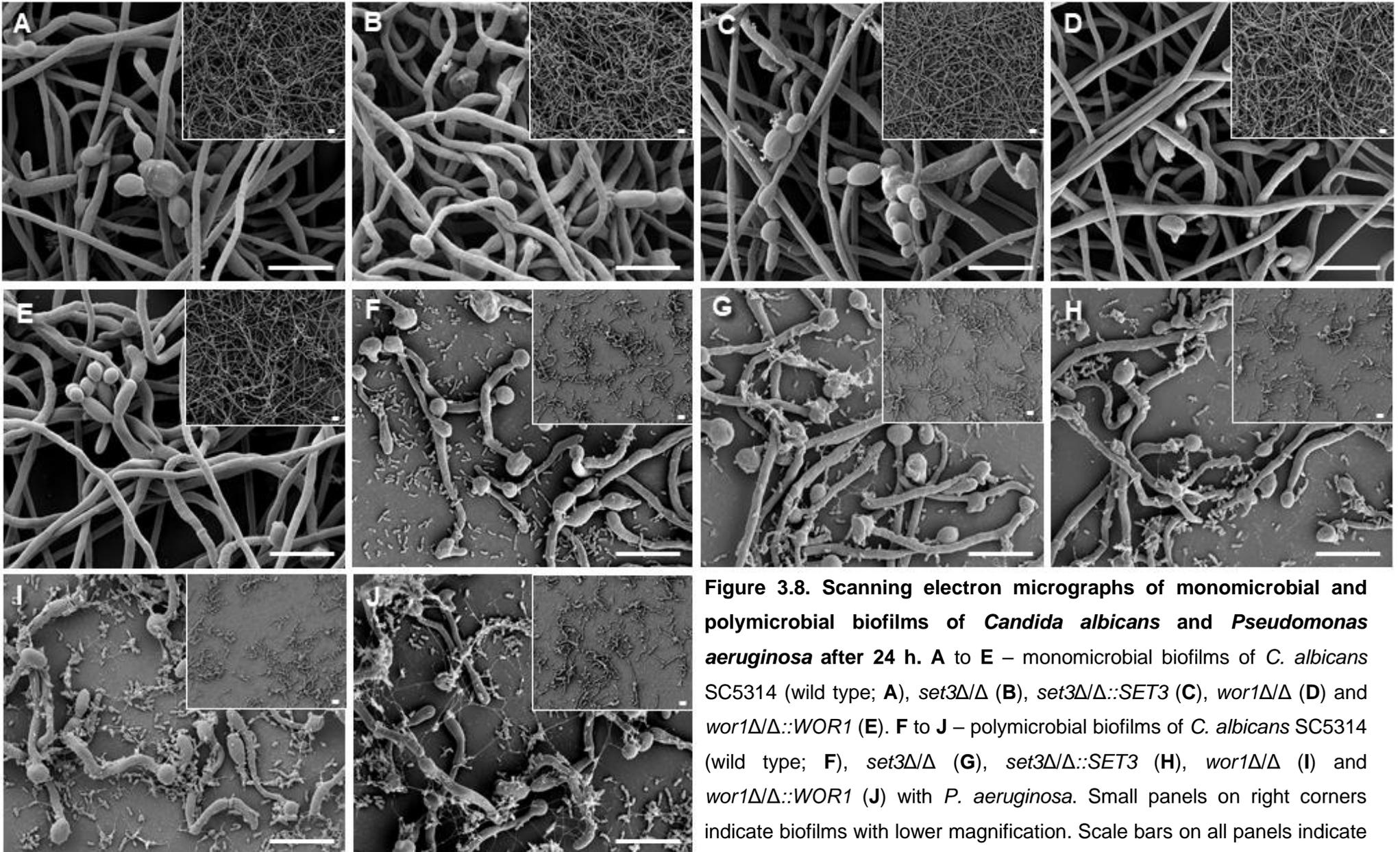
(Figure 5C). Due to the decrease in monomicrobial biomass, the increase in biomass may be due to an increase in *P. aeruginosa*. This effect on biomass is lost during biofilm maturation (Figure 3.6D), however, an increase in metabolic activity is seen (Figure 3.7D). This phenomenon is not fully rescued by the re-introduction of the wild-

type *SET3* gene. In addition, a small but significant increase in metabolic activity in *wor1Δ/Δ* is seen, however, the effect is not fully rescued by the add-back of the wild-type gene. The inability of the re-introduction of the wild type genes to rescue to observed differences in phenotypes may indicate off target events during CRISPR-Cas9 mediated gene editing. However, these differences are not consistent across experiments.

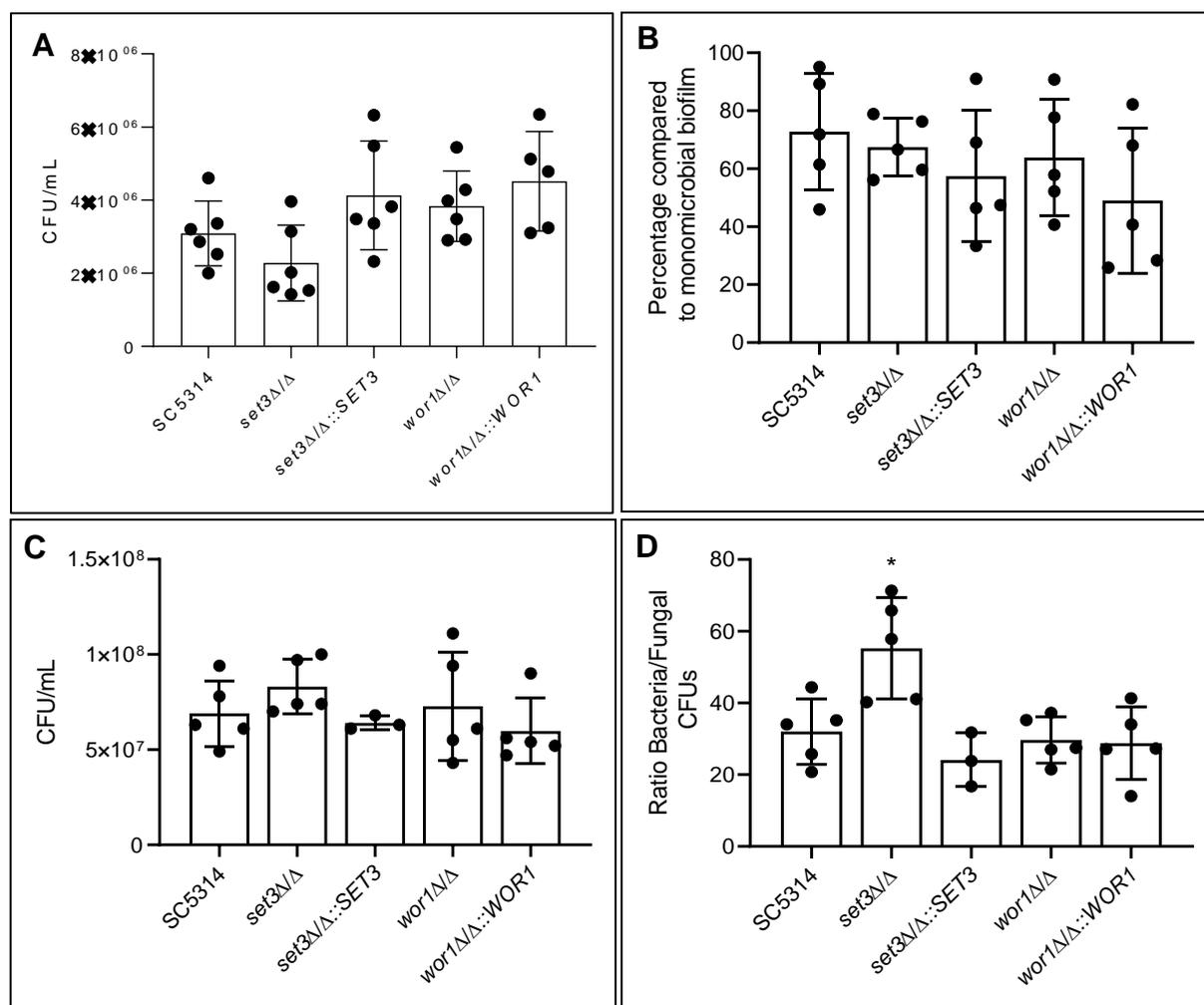
### 3.4.3. Deletion of *SET3* does not influence morphogenesis of *C. albicans* in response to *P. aeruginosa*

In *S. cerevisiae*, the Set3C binds to transcriptional regions in the genome, where it deacetylates various residues of histone H3 and H4 (Hnisz *et al.*, 2012; Wang *et al.*, 2002). In *C. albicans*, the Set3C acts as a transcriptional co-factor of metabolic and morphogenesis-related genes. Morphogenesis involves transcriptional changes that affect approximately 600 genes, corresponding to roughly 10 % of the genome of *C. albicans* (Hnisz *et al.*, 2012; Kadosh and Johnson, 2005; Nantel *et al.*, 2002). The process of morphogenesis is regulated by a set of transcriptional factors (Brg1p, Efg1p, Nrg1p and Tec1p) that form an interwoven transcriptional circuit that regulates hyphal morphogenesis. The Set3C then acts as an additional layer of chromatin remodelling, which superimposes on the core transcriptional circuit. It delays the formation of a hyphae-specific gene program upon stimulation of hyphae-inducing signals through modulating levels of the core transcription factors involved in morphogenesis. Furthermore, the Set3C binds to 5 out of 6 major biofilm regulators (gene products of *BRG1*, *TEC1*, *EFG1*, *NDT80* and *ROB1*, but not *BCR1*) implicating the complex in biofilm formation (Hnisz *et al.*, 2012). This allows *C. albicans* to “buffer” various contrasting stimuli to effectively adapt to an environment, specifically regarding morphogenesis and biofilm formation. As *SET3*, a core component of the Set3C, was induced upon exposure to *P. aeruginosa*, it prompted us to investigate the role of the Set3C in response to *P. aeruginosa*. Deletion of *SET3* did not affect the inhibition of morphogenesis by *P. aeruginosa* (**Figure 3.8**). However, Hnisz and co-workers (2012) reported that although binding of the Set3C correlates with gene expression during morphogenesis, deletion of *SET3* leaves the expression of most genes unaffected, whereby it may only transiently affect expression levels of key morphogenesis-related genes. Therefore, *set3Δ/Δ* is still able to efficiently initiate filamentation as well as inhibition thereof. With regards to this information, it seems that expression of target

genes and the core circuit of transcription factors involved in morphogenesis may play a predominant role in the inhibition of hyphal-formation by *P. aeruginosa* and that the Set3C may provide additional regulation, however, deletion of a core component of the complex is unable to modify the response of *C. albicans* to *P. aeruginosa* in terms of morphology. Notably, increased killing of *C. albicans* containing a deletion of the Tup1p repressor, exhibiting a constitutively filamentous phenotype, is observed (Hogan and Kolter, 2002). This indicates that the inability to revert to yeast form is detrimental in the presence of *P. aeruginosa*. To evaluate if *set3Δ/Δ* alters population dynamics of *C. albicans* in polymicrobial biofilms with *P. aeruginosa* and if increased killing of *C. albicans* is observed with deletion of *SET3*, colony forming units (CFU) of both species were determined. Interestingly, no significant effect on *C. albicans* (**Figure 3.9B**) or *P. aeruginosa* (**Figure 3.9C**) CFUs is seen in the polymicrobial biofilms. However, large variation between samples is seen. Unexpectedly, an increase in bacterial CFU is seen when expressed as a ratio of *P. aeruginosa* CFU over *C. albicans* CFU with polymicrobial biofilms of *set3Δ/Δ* (**Figure 3.9D**). We speculated that this may be due to decreased filamentation of *set3Δ/Δ* that may have led to a reduction in *C. albicans* CFUs compared to yeast-form cells, as a small but significant difference in *set3Δ/Δ* CFUs is seen. However, as can be seen in **Figure 3.8G**, no observable difference in filamentation of *set3Δ/Δ* is seen.



**Figure 3.8. Scanning electron micrographs of monomicrobial and polymicrobial biofilms of *Candida albicans* and *Pseudomonas aeruginosa* after 24 h. A to E – monomicrobial biofilms of *C. albicans* SC5314 (wild type; **A**), *set3Δ/Δ* (**B**), *set3Δ/Δ::SET3* (**C**), *wor1Δ/Δ* (**D**) and *wor1Δ/Δ::WOR1* (**E**). F to J – polymicrobial biofilms of *C. albicans* SC5314 (wild type; **F**), *set3Δ/Δ* (**G**), *set3Δ/Δ::SET3* (**H**), *wor1Δ/Δ* (**I**) and *wor1Δ/Δ::WOR1* (**J**) with *P. aeruginosa*. Small panels on right corners indicate biofilms with lower magnification. Scale bars on all panels indicate 10  $\mu$ m.**



**Figure 3.9.** Colony forming units (CFUs) of selected *Candida albicans* mutants in polymicrobial biofilms with *Pseudomonas aeruginosa*. **Figure 3.9A** represents the CFU of *C. albicans* homozygous mutants of *SET3* (*set3Δ/Δ*) and *WOR1* (*wor1Δ/Δ*) in monomicrobial biofilms. Homozygous mutants with homozygous add-back of wild-type genes are included (*set3Δ/Δ::SET3* and *wor1Δ/Δ::WOR1* respectively). **Figure 3.9B** - percentage CFUs of *C. albicans* in polymicrobial biofilms. **Figure 3.9C** represents the CFU/mL of *P. aeruginosa* in polymicrobial biofilms with *C. albicans*. **Figure 3.9D** represents the ration of bacterial over fungal cells in polymicrobial biofilms. \*Significantly different from wild type (SC5314) (\*  $P < 0.05$ ; \*\*  $P < 0.005$ ; \*\*\*  $P < 0.0005$ ; \*\*\*\*  $P < 0.0001$ ).

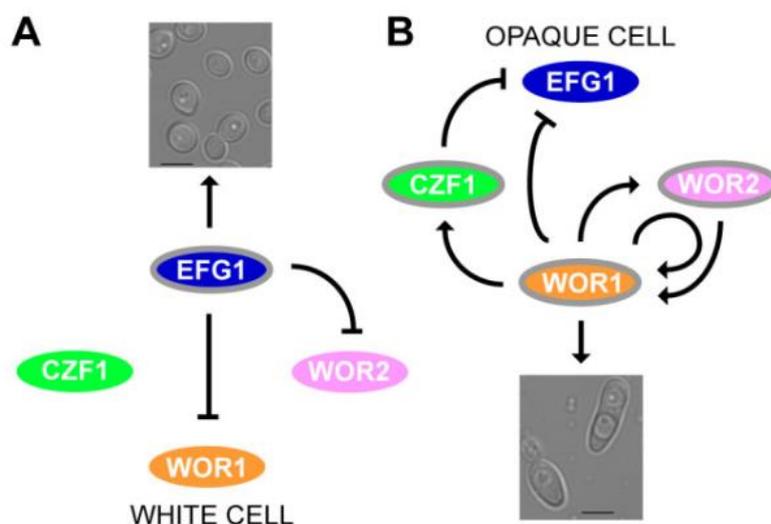
#### 3.4.4. *WOR1* deletion does not alter survival of *C. albicans* in polymicrobial biofilms with *P. aeruginosa*

*WOR1* regulates white-opaque switching in an all or nothing manner (Huang *et al.*, 2006). Whereas the white phenotype is associated with standard laboratory conditions (forming white, round colonies) and systemic infection, the opaque phenotype is characterised by elongated cells with pimples forming flat opaque colonies (Huang *et*

*al.*, 2006; Xie *et al.*, 2013). The opaque phenotype is associated with cutaneous infection and is mating competent with homozygosity at the mating type locus (MTL). *WOR1* is repressed by the heterodimer  $\alpha1$ - $\alpha2$  encoded by the alleles at MTL. The switching between white and opaque phenotypes can occur spontaneously, however, the frequency of this is low ( $10^{-4}$  generations). Due to the low expression associated with single species growth in standard cultivation conditions, such as those used in this study, it is not surprising that we did not observe alterations in morphology and biofilm formation ability in biofilms consisting only of *wor1* $\Delta/\Delta$  (**Figure 3.6, 3.7, 3.8**). The frequency of switching to the opaque phenotype can be increased by the presence of various stimuli, such as the presence of bacterial cell wall components (*N*-acetylglucosamine), anaerobic conditions, as well as high CO<sub>2</sub> (Dumitru *et al.*, 2007; Hnisz *et al.*, 2009). These stimuli have the ability to cause the overexpression of *WOR1* in individual cells that can initiate a feedback loop when a quorum is reached. (Huang *et al.*, 2006, Zordan *et al.*, 2007). Several transcription factors (*WOR1*, *WOR2*, *CZF1*, and *EFG1*) form a white-opaque regulatory circuit (Hernday *et al.*, 2013). The interplay in this circuit can be seen in **Figure 3.10** (Hernday *et al.*, 2013). Lohse and co-workers (2013) identified an additional transcription factor that could cause mass switching to the opaque state if ectopically expressed, named *WOR3*.

Notably, Fox and co-workers (2014) determined the differential expression of several transcription factors (*CZF1*, *WOR1*, *WOR2* and *WOR3*) with co-cultivation with *Klebsiella pneumoniae*, *Escherichia coli*, *Enterococcus faecalis*, *Clostridium perfringens* and *Bacillus fragilis*. They reported overexpression of *WOR1* in the presence of *K. pneumoniae*, *E. coli* and *E. faecalis*. Furthermore, they compared their results to the differential expression of these genes in opaque cells (Hernday *et al.*, 2013). They indicated that these transcriptional regulators are differentially expressed in opaque cells, and that a unique profile of differential expression is seen for co-cultivation with each bacterium (**Figure 3.11**). They found that *CZF1*, *WOR2* and *WOR3* were induced independent of *WOR1*, and that deletion had no change on morphology of polymicrobial biofilms with *K. pneumoniae*. This result is similar to our observations with *P. aeruginosa*, as deletion of *WOR1* did not notably alter polymicrobial biofilm formation (**Figure 13.6 and 13.7**), with only small differences in metabolic activity of polymicrobial biofilms. Additionally, morphology (**Figure 3.8**) and

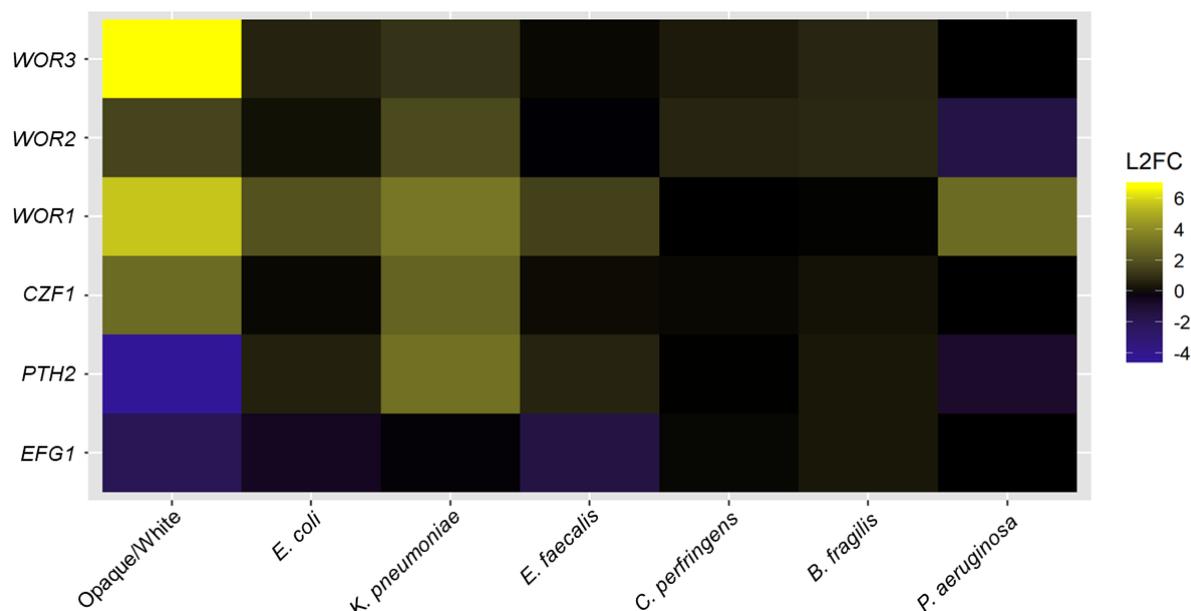
population dynamics (**Figure 3.9**) of *wor1* $\Delta/\Delta$  was similar to SC5314 in polymicrobial biofilms.



**Figure 3.10. White-opaque regulatory circuit in *Candida albicans* (Hernday *et al.*, 2013).**

**A** – In *C. albicans* white cells, Efg1p repressed *WOR1*. In addition, *WOR2* is repressed by Efg1p. **B** – In *C. albicans* opaque cells, a positive feedback loop is established through Wor1p, Wor2p and Czf1p to maintain the opaque phenotype. In addition, *EFG1* is repressed. Induced genes are bordered in grey and those without a border are repressed. Arrows represent activation and bars represent repression.

We compared the differential expression of these transcriptional regulators in response to *P. aeruginosa* (**Supplementary Table S2.2**) with the transcriptomic data obtained by Fox and co-workers (2014) and Hernday and co-workers (2013) (**Figure 3.11**). Whereas *EFG1* is repressed in opaque cells, transcription is not significantly affected in the presence of *K. pneumoniae*. Again, this is similar to what we observed. *Klebsiella pneumoniae* caused an induction of *PTH2*, a homologue of *WOR1*, although it is repressed in opaque cells (Fox *et al.*, 2014). Interestingly, in the presence of *P. aeruginosa* we observed an induction of *PTH2*. In addition, whereas *WOR2* is slightly induced in response to *K. pneumoniae* as well as in opaque cells, it is repressed in response to *P. aeruginosa*. *WOR3* is unaffected by co-cultivation with *P. aeruginosa*, although it is slightly induced during exposure to *K. pneumoniae*. Given the differential expression results presented in **Figure 3.11**, it is evident that different bacteria may



**Figure 3.11. Heat map of differential expression of transcriptional regulators involved in white-opaque switching in *Candida albicans* in response to co-incubation with bacteria.**

Opaque/White – differential expression (Log<sub>2</sub> fold change; L2FC) of transcriptional regulators (named on left of panel) in *C. albicans* opaque cells compared to white cells as determined by Hernday *et al.* (2013). Furthermore, the differential expression of these regulators is given for *C. albicans* in the presence of *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Enterococcus faecalis* (*E. faecalis*), *Clostridium perfringens* (*C. perfringens*) and *Bacillus fragilis* (*B. fragilis*) as determined by Fox *et al.* (2014). Lastly, the differential expression of *C. albicans* exposed to *P. aeruginosa* is included from this study (**Supplementary Table S2.2**).

elicit a unique transcriptional response in *C. albicans*, possibly aiding in its adaption in the presence of various bacteria. Interestingly, Fox and co-workers (2014) reported that a full opaque program was not initiated in response to *E. coli*, *K. pneumoniae* or *E. faecalis*. Similarly, transcriptomic evaluation in *Chapter 2*, indicated that the transcriptional response of *C. albicans* towards *P. aeruginosa* did not represent the full transcriptional profile of opaque cells, or an intermediate between white and opaque phenotypes (gray phenotype). Fox and co-workers (2014) speculated that additional signals may be required for the display of the heritable opaque phenotype. Furthermore, no morphologies reminiscent of opaque cells were visible in the presence of *P. aeruginosa*, further indicating that the full opaque phenotype is not induced. Therefore, it seems probable that although *WOR1* may play significant roles

in the interaction of *C. albicans* with bacterial species such as *K. pneumoniae*, *E. coli* and *E. faecalis*, driving synergism and increased commensalism, it is not crucial for polymicrobial biofilm formation with *P. aeruginosa*. At least under the conditions tested.

Ramirez-Zavala and co-workers (2008) showed that switching could be induced in *C. albicans* by an anaerobic environment in the presence of high temperature (37°C), such as those found in the mammalian gastrointestinal tract. However, this only occurred in the WO-1 and CAI-4 strains, whereas this could not be replicated in the SC5314 strain that is heterozygous at the mating type locus ( $a1/\alpha2$ ). This is since WO-1 and CAI-4 experienced a duplication of chromosome one, therefore containing three copies of the *WOR1* gene (Ramirez-Zavala *et al.*, 2008). In addition to this, other chromosomal events, such as homozygosity of the mating type locus, enabled the induction of these strains to undergo phenotypic switching under these circumstances and display the full opaque program. The authors further speculated that these chromosomal alterations are required for switching to take place during hypoxia at high temperature. A feature that is not own to SC5314. Due to this, the strain of *C. albicans* (SC5314) may experience an induction of *WOR1* in the presence of *P. aeruginosa* (such as with *E. coli*, *K. pneumoniae* and *E. faecalis*) in a polymicrobial biofilm, however, the full opaque program may not be induced as a trisomy of chromosome one and homozygosity of the MTL or further stimuli to induce *WOR1* may be needed. It would be interesting to evaluate these bacteria in the presence of a *WOR1*-overexpressing strain to determine the effect of the full opaque program in the interaction with bacteria such as *P. aeruginosa*.

#### **3.4.5. Infection of A549-Dual™ cells by *C. albicans* and *P. aeruginosa***

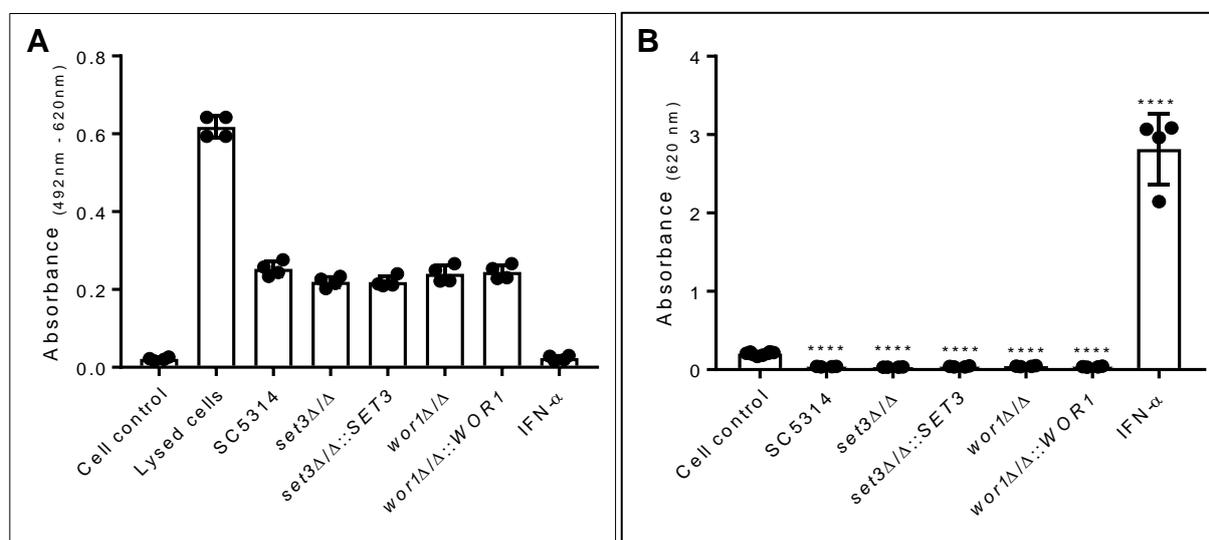
In order to determine if deletion of *SET3* and *WOR1* affects both cell death and innate immune response of single species infection by *C. albicans* as well as dual species infection by *C. albicans* and *P. aeruginosa*, the A549 Dual™ lung epithelial cell line was used. The A549 Dual™ lung epithelial cell line is adherent epithelial cells that are derived from an explant culture of adenocarcinoma lung tissue of a 58-year-old Caucasian male (Giard *et al.*, 1973). This cell line was chosen as it may represent host cells that *C. albicans* and *P. aeruginosa* may encounter during co-infection, as both species have been co-isolated from respiratory infections (Azoulay *et al.*, 2006;

Chotirmall *et al.*, 2010; Haiko *et al.*, 2019; Hamet *et al.*, 2012; Kerr, 1994; Kim *et al.*, 2015; Nseir *et al.*, 2007). Furthermore, the airway epithelial cells play a role in initiating both innate defence and inflammatory responses to activate immune cells in the lung (Hawdon *et al.*, 2010; Martin and Frevert, 2005).

In addition to providing information regarding cytotoxicity imposed by infection, the A549-Dual™ lung epithelial cell line contains two reporters for innate immune induction pathways, namely, the nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway, as well as the interferon regulatory factor (IRF) pathway (<https://www.invivogen.com/a549-dual>, accessed 12 December 2019). For NF- $\kappa$ B, a secreted embryonic alkaline phosphatase reporter gene, under the control of the IFN- $\beta$  minimal promoter fused to five NF- $\kappa$ B sites, is used. This reporter protein is then quantifiable with a detection reagent (QUANTI-Blue™). For the purpose of this study, cytotoxicity and NF- $\kappa$ B induction of A549 Dual™ lung epithelial cells infected with *C. albicans* alone or in combination with *P. aeruginosa* was measured. To determine if deletion of *SET3* and *WOR1* shows differences in the ability to kill A549-Dual™ lung epithelial cell line, cytotoxicity was measured after 24 hours of infection (**Figure 3.12A**). Infection by *C. albicans* wild type (SC5314) alone causes approximately 40.9% cell death compared to lysed A549-Dual™ lung epithelial cells alone. No significant differences were observed with *set3* $\Delta/\Delta$  or *wor1* $\Delta/\Delta$ . Therefore, deletion of *SET3* and *WOR1* does not affect the virulence of *C. albicans* with A549-Dual™ lung epithelial cells. Importantly, deletion of *SET3* shows strongly attenuated virulence in a murine model of systemic infection (Hnisz *et al.*, 2010), indicating that although we did not observe a difference in cytotoxicity of A549-Dual™ lung epithelial cells, virulence may still be affected during systemic infection.

To determine if deletion of these genes affect the ability of *C. albicans* to elicit an innate immune response, induction of NF- $\kappa$ B was also measured, as NF- $\kappa$ B induction due to *C. albicans* has been reported before (Kiyoura and Tamai, 2015). *Candida albicans* is recognised by a variety of pattern recognition receptors, such as toll-like receptors (TLRs) and C-type lectin receptors such as dectin-1 to dectin-3 (Kiyoura and Tamai, 2015; Kondo *et al.*, 2012). *Candida albicans* is recognised through cell wall components, such as mannans and  $\beta$ -glucan. Mannans can be recognised by TLR-4, TLR-2, dectin-2, dectin-3 and mannose receptor (Gantner *et al.*, 2003; Kiyoura and

Tamai, 2015; Netea *et al.*, 2006).  $\beta$ -glucans can be recognised by TLR-2 and dectin-1.



**Figure 3.12. Cytotoxicity and nuclear factor- $\kappa$ B (NF- $\kappa$ B) induction of A549 Dual™ Cells infected with selected *Candida albicans* mutants. **A** represents the cytotoxicity of A549 Dual™ Cells as measured by the CyQUANT™ LDH Cytotoxicity Assay when infected with *C. albicans* homozygous mutants of *SET3* (*set3Δ/Δ*) and *WOR1* (*wor1Δ/Δ*) for 24 hours. Homozygous mutants with homozygous add-back of wild-type genes are included (*set3Δ/Δ::SET3* and *wor1Δ/Δ::WOR1* respectively). Cell control consists of A549 cells without infection. Lysed cells represent the total LDH assay activity after lysis of A549 cells. Interferon- $\alpha$  (IFN- $\alpha$ ) is used as positive control for induction of NF- $\kappa$ B. \*Significantly different from wild type *C. albicans* (SC5314). **B** represents the induction of the innate immune NF- $\kappa$ B pathway as measured by the QUANTI-Blue™ assay. \*Significantly different from the cell control calculated with *t*-test.**

Furthermore, the cell wall also contains chitin, which may be recognised by dectin-1 and mannose receptors, however this still needs to be confirmed (Kiyoura and Tamai, 2015; Mora-montes *et al.*, 2011; Mullin *et al.*, 1997). In addition, fungal DNA can bind to TLR-9 (Bellocchio *et al.*, 2004; Kiyoura and Tamai, 2015; Miyazato *et al.*, 2009). Simultaneous activation of these pattern recognition receptors is frequently required to elicit an adequate response and activate the nuclear factor- $\kappa$ B (NF- $\kappa$ B) transcription factor. The NF- $\kappa$ B heterodimer is composed of two subunits, RelA and p50, existing in the cytoplasm bound to the I $\kappa$ B inhibitor (Chen *et al.*, 2008; Mercurio and Manning, 1999a, 1999b). Activation of the I $\kappa$ B kinase complex, phosphorylates I $\kappa$ B, leading to ubiquitination and degradation. After this, NF- $\kappa$ B translocates to the nucleus and

initiates transcription of inflammatory mediators such as chemokines and cytokines and subsequent recruitment of neutrophils.

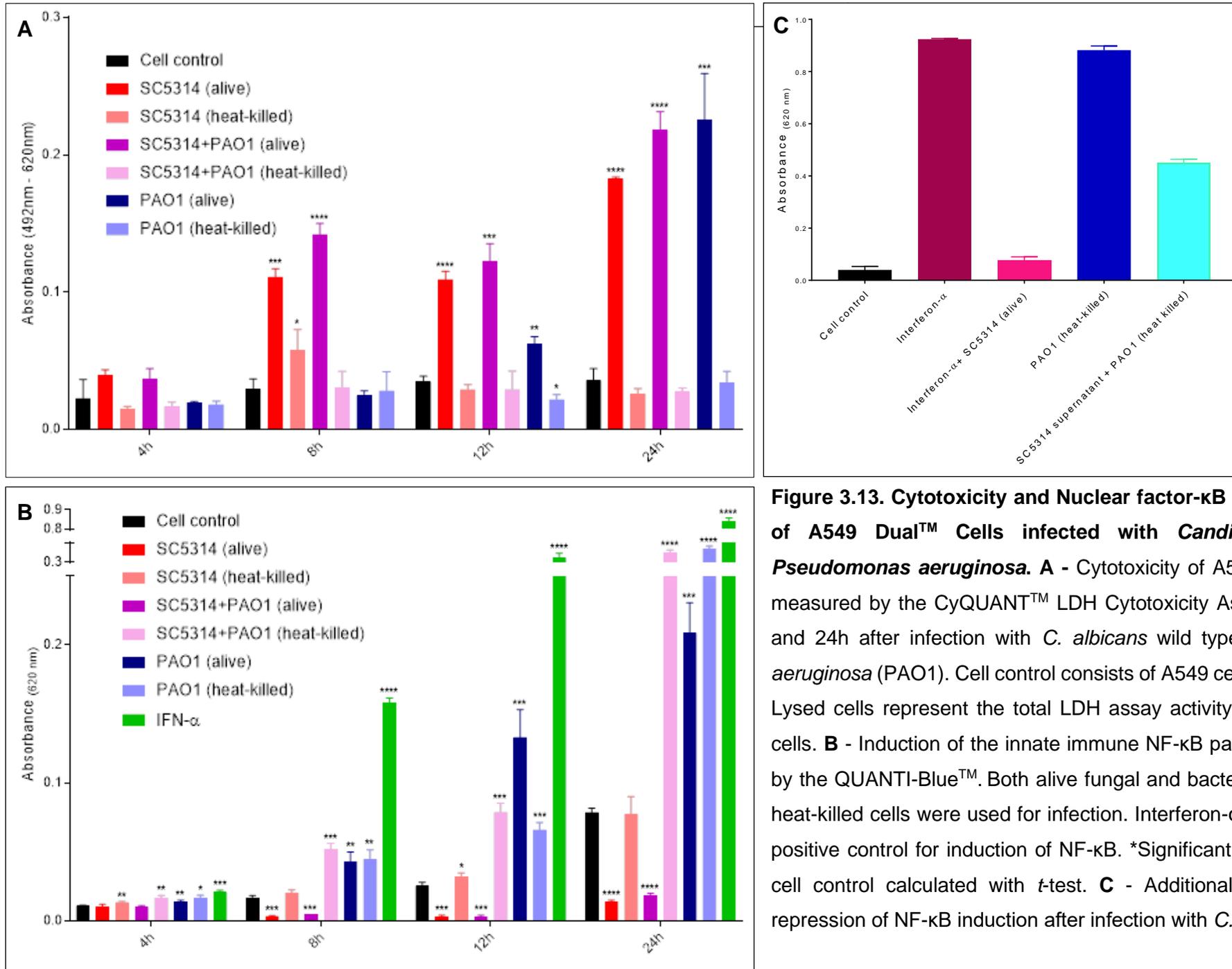
Strikingly, a significant decrease in NF- $\kappa$ B induction was observed compared to A549-Dual™ lung epithelial cells in the absence of infection (**Figure 3.12B**). To determine if the lack in NF- $\kappa$ B induction was due to an abrogation of induction after 24h of infection, earlier time points (4h, 8h, 12h and 24h) was also included. Additionally, live *P. aeruginosa* was also included, reported to cause NF- $\kappa$ B induction in A549 cells (Hawdon *et al.*, 2010). Lastly, *C. albicans* in combination with *P. aeruginosa* was also used to infect A549 cells. As can be seen in **Figure 3.13A**, a time dependent increase in *C. albicans*-mediated cytotoxicity was observed after 8h of infection. Infection by *P. aeruginosa* exhibited an increase in cytotoxicity after 12 to 24 hours of infection. Similar to *C. albicans* alone, co-infection increased cytotoxicity after 8 hours of infection. Notably, live *C. albicans* cells significantly repressed NF- $\kappa$ B induction after just 8 hours of infection (**Figure 3.13B**). However, *P. aeruginosa* was able to elicit NF- $\kappa$ B induction after just 4 hours with significant increase over time to 24h. *Pseudomonas aeruginosa* is able to elicit activation of the NF- $\kappa$ B pathway through recognition of ligands such as lipopolysaccharide (LPS), pili and flagella by TLRs (Chen *et al.*, 2008). TLR-2 can recognise peptidoglycan, LPS, ExoS, mannuronic acid polymers, slime glycolipoprotein and flagellin (reviewed by Cigana *et al.*, 2011). LTR-4 can recognise LPS ExoS and mannuronic acid polymers whereas LTR-5 and TLR-9 recognise flagellin and bacterial DNA respectively. Furthermore, nucleotide-binding oligomerization domain-like receptors (NLRs) can recognise peptidoglycan, flagellin and the type-III-secretion system.

Importantly, *C. albicans* repressed the induction of NF- $\kappa$ B by *P. aeruginosa*, indicating that this effect is not just an absence of induction of NF- $\kappa$ B, but a repression of the pathway. Furthermore, inhibition of NF- $\kappa$ B activation in airway epithelium impairs the clearance of *P. aeruginosa*, primary due to impaired neutrophil recruitment. Due to this, further research is required to determine if this inhibition of NF- $\kappa$ B by *C. albicans* in the presence of *P. aeruginosa* occurs *in vivo*. Importantly, although lung epithelial cells express TLR-4 (MacRedmond *et al.*, 2005), A549 Dual™ lung epithelial cells do not express it (<https://www.invivogen.com/a549-dual>, accessed 12 December 2019). This prompts the question if the inhibition of NF- $\kappa$ B induction may be due to the lack of TLR-4. Importantly, TLR-4-mediated pro-inflammatory signals are lost during the

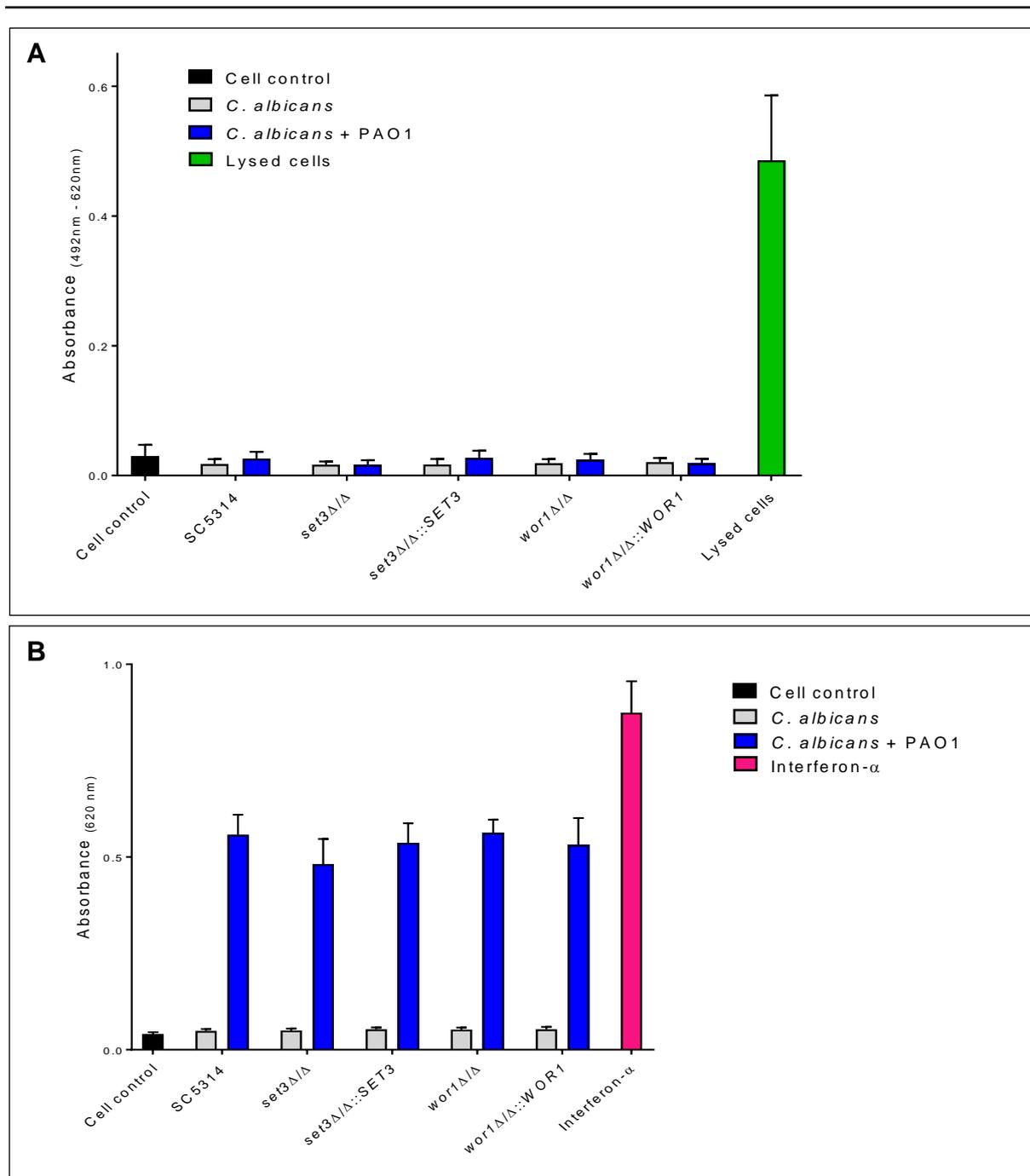
yeast-to-hyphal transition. As *C. albicans* may exhibit hyphal morphology in the incubation conditions used, such as incubation at 37°C (Whiteway and Bachewich, 2007), the role of TLR-4 during infection of A549 Dual™ lung epithelial cells may be questionable. However, A549 Dual™ lung epithelial cells still express TLR-2, TLR-3 and TLR-5. Notably, *C. albicans* cells can stimulate the production of IL-10, an anti-inflammatory cytokine through TLR-2-dependent mechanisms (Van der Graaf *et al.*, 2005).

To determine if the inhibitory effect is due to physical or secreted factors of *C. albicans*, heat-killed *C. albicans* was included (**Figure 3.13B**). Heat inactivation of *C. albicans* removed the inhibitory effect of *C. albicans*, confirming that this effect may be due to secreted factors or requires live *C. albicans* cells. Importantly, heat-killed *C. albicans* has increased surface expression of 1,3-β-glucan (found within mannans), indicating that this effect is independent of cell-wall-associated mechanisms alone. Interestingly, heat inactivation of *P. aeruginosa* was still able to induce the NF-κB pathway in A549-Dual™ lung epithelial cells, both in the presence or absence of heat-killed *C. albicans* cells. To further elucidate the effect of *C. albicans*, A549-Dual™ lung epithelial cells were infected with live *C. albicans* together with a positive control (interferon-α) for NF-κB induction. Strikingly, *C. albicans* was able to inhibit induction of NF-κB by the positive control (**Figure 3.13C**). To determine if the inhibitory effect was due to secreted factors released by *C. albicans*, supernatant from *C. albicans* live cells, in combination with heat-killed *P. aeruginosa*, was added to A549-Dual™ lung epithelial cell line. Interestingly, a 51% decrease in NF-κB induction was observed in the presence of *C. albicans* supernatant compared to heat-killed *P. aeruginosa* alone. This indicates that an extracellular factor produced by *C. albicans* may be responsible, in part, for the repression of the NF-κB pathway. However, an interaction of A549-Dual™ lung epithelial cell line with live *C. albicans* may be required to obtain the full inhibitory effect. It is possible that secreted proteinases may degrade the protein reporter of the NF-κB pathway. Further research is required to investigate this phenomenon.

Due to the inhibition of NF-κB pathway by live *C. albicans* cells, we proceeded to evaluate the effect of deletion of *SET3* and *WOR1* on the innate immune induction of A549 cells with heat-killed *C. albicans* and *P. aeruginosa*. This was done to determine if the immunogenicity of the cell wall is affected by the deletion of *SET3* or *WOR1*. As can be seen in **Figure 3.14A**, neither heat-killed *C. albicans* nor *P. aeruginosa*



**Figure 3.13. Cytotoxicity and Nuclear factor- $\kappa$ B (NF- $\kappa$ B) induction of A549 Dual™ Cells infected with *Candida albicans* and *Pseudomonas aeruginosa*. **A** - Cytotoxicity of A549 Dual™ Cells as measured by the CyQUANT™ LDH Cytotoxicity Assay at 4h, 8h, 12h and 24h after infection with *C. albicans* wild type (SC5314) and *P. aeruginosa* (PAO1). Cell control consists of A549 cells without infection. Lysed cells represent the total LDH assay activity after lysis of A549 cells. **B** - Induction of the innate immune NF- $\kappa$ B pathway as measured by the QUANTI-Blue™. Both alive fungal and bacterial cells as well as heat-killed cells were used for infection. Interferon- $\alpha$  (IFN- $\alpha$ ) is used as positive control for induction of NF- $\kappa$ B. \*Significantly different from the cell control calculated with *t*-test. **C** - Additional data showing the repression of NF- $\kappa$ B induction after infection with *C. albicans*.**



**Figure 3.14. Cytotoxicity and nuclear factor- $\kappa$ B (NF- $\kappa$ B) induction of A549 Dual™ Cells infected with *Candida albicans* mutants and *Pseudomonas aeruginosa*. Figure 3.14A - Cytotoxicity of A549 Dual™ Cells as measured by the CyQUANT™ LDH Cytotoxicity Assay when infected with *C. albicans* wild type (SC5314) or homozygous mutants of *SET3* (*set3Δ/Δ*) and *WOR1* (*wor1Δ/Δ*) alone or in combination with *P. aeruginosa* (PAO1). Add-backs of wild-type genes are included (*set3Δ/Δ::SET3* and *wor1Δ/Δ::WOR1* respectively). Cell control consists of A549 cells without infection. Lysed cells represent the total LDH assay activity after lysis of A549 cells. Interferon- $\alpha$  (IFN- $\alpha$ ) is used as positive control for induction of NF- $\kappa$ B. Figure 3.14B - Induction of the innate immune NF- $\kappa$ B pathway as measured by the QUANTI-Blue™ assay. \*Significantly different from wild type *C. albicans* (SC5314)**

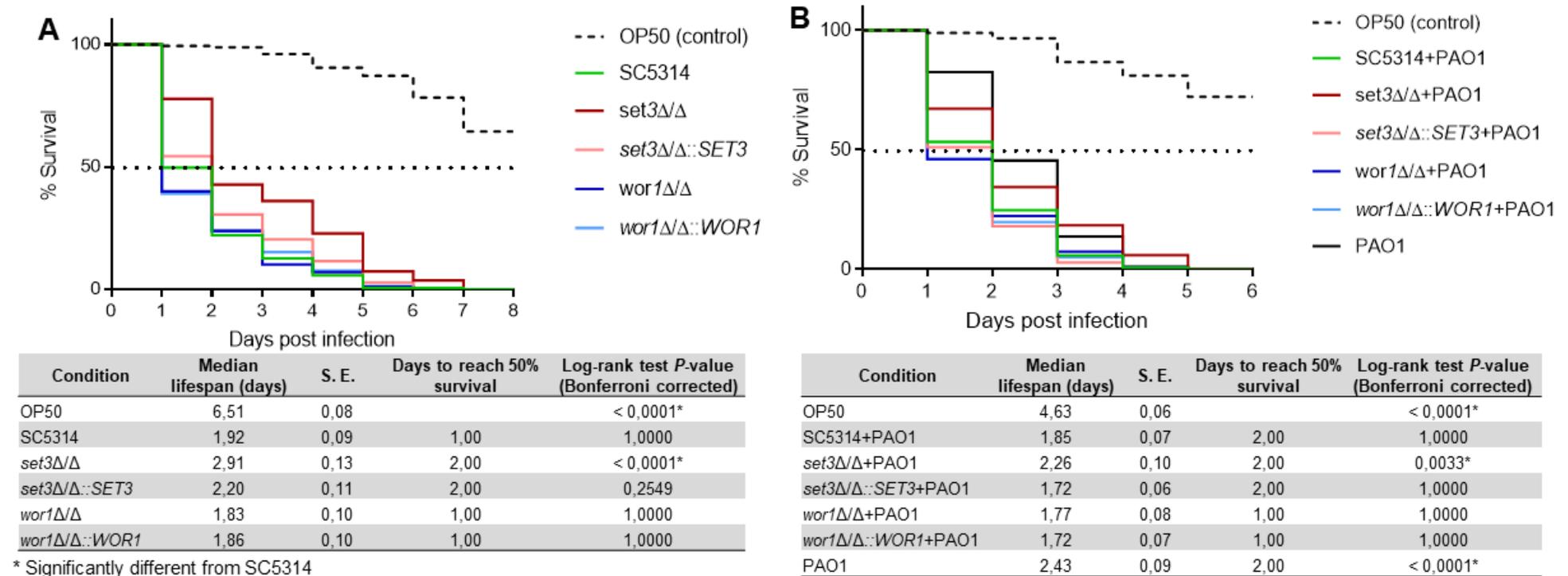
significantly caused cytotoxicity of A549 Dual<sup>TM</sup> lung epithelial cells. Furthermore, although heat-killed *P. aeruginosa* induced NF- $\kappa$ B induction by A549 Dual<sup>TM</sup> lung epithelial cells (**Figure 3.14B**), even in the presence of heat-killed *C. albicans*, no significant effect was observed with deletion of *SET3* and *WOR1* in combination with *P. aeruginosa*.

#### 3.4.6. Infection of *C. elegans* by *C. albicans* and *P. aeruginosa*

In addition to infection of A549-Dual<sup>TM</sup>, the role of *SET3* and *WOR1* was further investigated using *C. elegans* as an infection model. *Caenorhabditis elegans* is a genetically tractable model frequently used in studies of microbial pathogenesis, focused on host-pathogen interactions and innate immunity (Alper *et al.*, 2008; Marsh and May, 2012). Importantly, it is susceptible to *C. albicans* and *P. aeruginosa* (Pukkila-Worley *et al.*, 2009; Tan *et al.*, 1999). *Caenorhabditis elegans* is known to share a similar innate immune response with humans (Pukkila-Worley *et al.*, 2011). However, this model does present several drawbacks. Firstly, infection of *C. elegans* needs to take place at a temperature below 37°C (25°C in this study), as physiological temperature is lethal to *C. elegans* (Zevian and Yanowitz, 2014; Sulston and Hodgkin, 1998). Furthermore, although infection by pathogens frequently lead to dissemination inside the host, this does not occur in *C. elegans*, making it unsuitable to study systemic infections. *Caenorhabditis elegans* is capable of producing many progenies, which could interfere with survival assays. Furthermore, larvae may hatch inside the nematode, killing *C. elegans* (McCulloch and Gems, 2003; Tan *et al.*, 1999). Therefore, a *glp-4* mutant, unable to propagate at 25°C, was used (Miyata *et al.*, 2008). In addition, the strain used is devoid of *SEK-1*, rendering it immunocompromised to facilitate infection. *SEK-1* encodes a conserved mitogen-activated protein kinase involved in innate immunity (Kim *et al.*, 2002). It functions upstream of a homolog of mammalian p38 mitogen-activated protein kinase (Breger *et al.*, 2007). In mammals, p38 is involved in the response to candidiasis (Breger *et al.*, 2007; Deva *et al.*, 2003). Breger and co-workers (2007) suggest that the use of immunocompromised *C. elegans* (*sek-1*) may be comparable to the frequently immunocompromised nature of human hosts.

To accomplish infection, *C. elegans* was allowed to ingest *C. albicans* yeast cells and were monitored to determine their survival. Nematodes allowed to feed on *E. coli*

OP50 were used as control. **Figure 3.15** indicates the percentage survival of *C. elegans* with survival statistics of *C. elegans* infected with *C. albicans* alone, or co-infected with *P. aeruginosa*. Survival statistics and significant differences in survival, compared to SC5314 (wild type) was determined with the Log-rank test using Online Tool for Survival Analysis 2 (OASIS 2) (Han *et al.*, 2016). Deletion of *WOR1* did not impact survival of *C. elegans* during both single (**Figure 3.15A**) and co-infection (**Figure 3.15B**), indicating that *WOR1* is not required for virulence in a *C. elegans* infection model and does not affect the ability of *P. aeruginosa* to cause infection. In contrast, a significant ( $P < 0.0001$ ) increase in survival of *C. elegans* infected with *set3Δ/Δ* compared to SC5314 was found (**Figure 3.15A**). Therefore, this corroborates findings of reduced virulence of *set3Δ/Δ* as found previously in a murine model of systemic candidiasis (Hnisz *et al.*, 2010). The primary method for killing of *C. elegans* by *C. albicans* relies on the production of hyphae (Pukkila-Worley *et al.*, 2009). Notably, the deletion of *SET3* did not abrogate the ability of *C. albicans* to form hyphae and pierce the cuticle of *C. elegans*. Virulence of *set3Δ/Δ* in *C. elegans* was restored through re-introduction of the wild-type gene (*set3Δ/Δ::SET3*). Similar to single-species infection, co-infection by *C. albicans* and *P. aeruginosa* also exhibited decreased virulence of *set3Δ/Δ* (**Figure 3.15B**), indicating that Set3C contributes to virulence of *C. albicans* in *C. elegans*, even in the presence of *P. aeruginosa*.



**Figure 3.15. Survival of *Caenorhabditis elegans* infected with *Candida albicans* mutants and *Pseudomonas aeruginosa*.** **A** - Percentage survival of *C. elegans* infected with *C. albicans* wild type (SC5314) or homozygous mutants of *SET3* (*set3Δ/Δ*) and *WOR1* (*wor1Δ/Δ*). Add-backs of wild-type genes are included (*set3Δ/Δ::SET3* and *wor1Δ/Δ::WOR1* respectively). **B** - Percentage survival of *C. elegans* co-infected with *C. albicans* mutants and *P. aeruginosa* (PAO1). Controls consists of *C. elegans* allowed to feed on *Escherichia coli* OP50 (OP50). The tables represent median lifespan with standard error (S. E.) along with days to reach 50% mortality. *P*-values are included for the Log-rank test for overall differences in survival. \*Significantly different from wild type *C. albicans* (SC5314).

### 3.5. CONCLUSIONS

The present study evaluated the role of *C. albicans* *SET3* and *WOR1* on the polymicrobial interaction with *P. aeruginosa*, as they were both induced upon exposure of *C. albicans* to *P. aeruginosa* in polymicrobial biofilms (*Chapter 2*). Deletion of *SET3* did not impact the morphology of *C. albicans* in the presence of *P. aeruginosa*. Neither did it notably impact the ability to form polymicrobial biofilms. Therefore, deletion of *SET3* did not significantly impact the ability of *C. albicans* to form polymicrobial biofilms with *P. aeruginosa*. Furthermore, although the white-opaque regulator, *WOR1*, is induced during exposure to *P. aeruginosa*, it did not affect single or polymicrobial biofilm formation, morphology or population dynamics in polymicrobial biofilms with *P. aeruginosa*. In addition, although overexpression of *WOR1* is an indication for switching to the opaque phenotype, a full opaque transcriptional program was not found, similar to results previously obtained when *C. albicans* was exposed to other bacteria such as *K. pneumoniae*.

In addition, we evaluated A549 Dual™ Cells as an *in vitro* model for *C. albicans* single and polymicrobial infection. This was done by evaluating cytotoxicity and innate immune induction via the NF-κB pathway. We found that *SET3* and *WOR1* was unable to affect cytotoxicity of *C. albicans* towards these cells. In addition, we observed a significant decrease in NF-κB induction with live *C. albicans* cells, even in the presence of *P. aeruginosa*. This may be attributed to secreted factors produced by *C. albicans*. Additionally, we evaluated the effect of deletion of *SET3* and *WOR1* in *C. albicans* on virulence in a *C. elegans* infection model. A decrease in virulence was found when *C. elegans* was infected with *set3Δ/Δ* alone, corresponding to previous studies in a murine model. Furthermore, deletion of *SET3* reduced to virulence of *C. albicans* in *C. elegans*, even in the presence of *P. aeruginosa*. In addition, deletion of *WOR1* did not affect the virulence of *C. albicans*.

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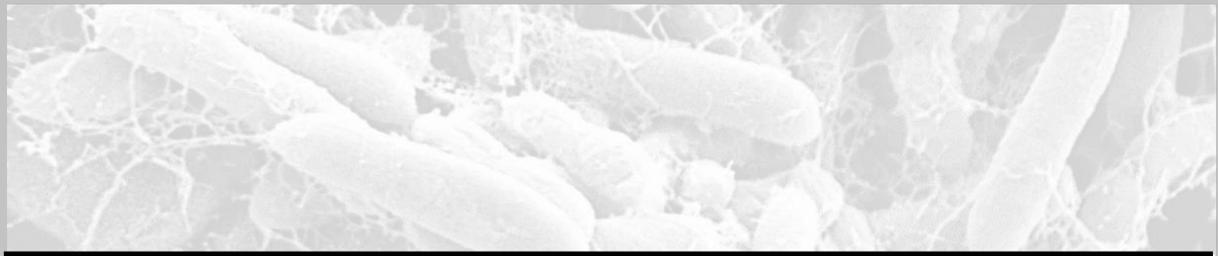
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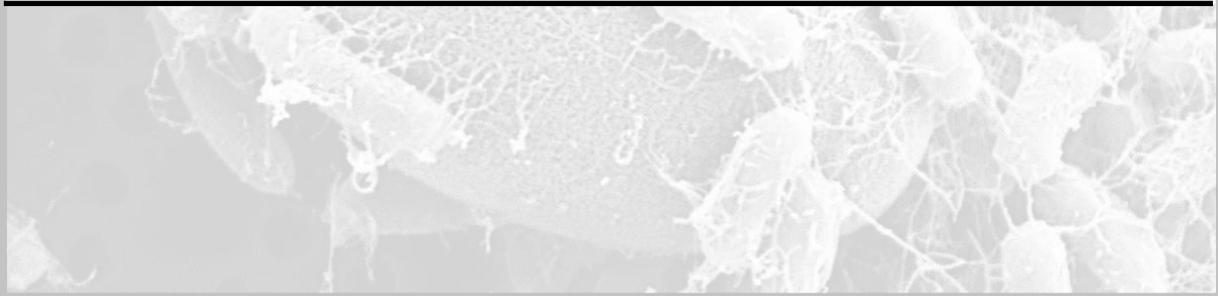
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**CHAPTER 4**

**Arachidonic acid alters Cdr1p transcription and activity in a dose dependent manner**



#### 4.1. ABSTRACT

*Candida albicans* is a polymorphic yeast that can cause superficial to systemic infection in immunocompromised hosts. Eradication of infection is hampered by multiple methods of antifungal resistance. These include the formation of antifungal and mechanical resistant biofilms, expression of drug efflux pumps, modification of target proteins and alterations in drug target pathways. Polyunsaturated fatty acids (PUFAs) have shown potential as an alternative or adjuvant therapy treatment of infections due to their antibiotic and antifungal properties. Arachidonic acid (AA), is a C20 PUFA released by host cells during infection. In addition to acting as the precursor for an important immunomodulatory class of lipids, it has been shown to inhibit morphogenesis in *C. albicans*, as well as reduce its resistance to antifungal agents. However, the mechanism of this action is still unknown.

In *Chapter 2* an increase expression of genes involved in membrane organisation, was found in response to 0.1 mM AA. Among these, *IPT1*, *RTA3* and *CDR1* showed increased expression in the presence of AA, as well as in response to co-incubation with *Pseudomonas aeruginosa*. Importantly, all three of these genes are associated with antifungal resistance, with *CDR1* overexpression known as an important mechanism in fluconazole-resistant isolates. In this chapter the role of these genes in biofilm formation in the presence of AA was further evaluated through the use of homozygous deletion mutants. This led to identification of a primary role for *CDR1* among these genes in response to AA. *CDR1* was not only overexpressed, but AA was able to increase Cdr1p in a dose dependent manner. However, the activity was diminished by the addition of AA. Further evaluation indicated that this may be due to a combination of factors, including mislocalisation, reduced functionality of the mitochondria, as well as competitive inhibition. Interestingly, deletion of *CDR1* caused a minor but significant decrease in virulence in a *Caenorhabditis elegans* infection model, whereas a minor increase in virulence was observed when *IPT1* is deleted. This study then not only provides possible mechanisms for the reduced resistance of *C. albicans* in the presence of PUFAs, but highlights the importance of research into PUFAs in antifungal therapy.

## 4.2. INTRODUCTION

*Candida albicans* is a polymorphic fungus, exhibiting an impressive array of virulence factors (Calderone and Fonzi, 2001). It is frequently isolated as a commensal organism in the gastro-intestinal tract, skin and other mucosal surfaces of humans (Cauchie *et al.*, 2017; Kashem and Kaplan, 2016; Nash *et al.*, 2017; Ruhnke *et al.*, 1994; Shao *et al.*, 2019). During a state of compromised immunity of the host, such as with HIV infection, cancer treatment, antibiotic treatment-linked dysbiosis, *C. albicans* can become pathogenic, responsible for both superficial and systemic infections (Anwar *et al.*, 2012; Gutierrez *et al.*, 2020; Koh *et al.*, 2008; Teoh and Pavelka, 2016). The latter is linked to high mortality rates (Pfaller and Diekema, 2007). Furthermore, *C. albicans* can reversibly switch between yeast and hyphal morphologies and form biofilms (Andes *et al.*, 2004; Ramage *et al.*, 2001; Tsui *et al.*, 2016). This form of growth exhibits increased resistance to mechanical, immune and antifungal stresses.

Polyunsaturated fatty acids (PUFAs) have known antifungal and antibacterial properties and can reduce biofilm formation and virulence (Beavers *et al.*, 2019; Chanda *et al.*, 2018; Thibane *et al.*, 2012). Furthermore, they can increase susceptibility to antifungal agents (Ells *et al.*, 2009). Arachidonic acid (AA), a PUFA released by host as well as pathogen-derived phospholipases during infection, inhibits morphogenesis in *C. albicans* and increases susceptibility to antifungal agents (Castro *et al.*, 1994; Ells *et al.*, 2009; Shareck and Belhumeur, 2011; Smeekens *et al.*, 2010). However, the reason for inhibition of morphogenesis and increased susceptibility to antifungals is unknown. Furthermore, the effect of AA on the interaction between *C. albicans* and *P. aeruginosa* is not known.

We attempted to address this lack of knowledge through evaluation of the transcriptional response of *C. albicans* in response to AA as single species biofilms, as well as in combination with *P. aeruginosa*, with the use of RNAseq discussed in Chapter 2. We found that genes associated with membrane organisation, such as *IPT1* and *RTA3*, are induced upon exposure of *C. albicans* biofilms to AA. *IPT1* encodes for inositol phosphoryl transferase, catalysing the synthesis of the most abundant sphingolipid, mannose-(inositol-P)<sub>2</sub>-ceramide (M(IP)<sub>2</sub>C) (Prasad *et al.*, 2005). Deletion of *IPT1* is associated with decreased resistance to various antifungal agents (Pasrija *et al.*, 2005; Prasad *et al.*, 2005). Rta3p is a 7-transmembrane receptor

protein, involved in regulation of asymmetric lipid distribution in the plasma membrane (Srivastava *et al.*, 2017; Whaley *et al.*, 2016). Deletion of this gene is also associated with decreased resistance towards antifungal agents. Cdr1p is known as a promiscuous drug exporter that is overexpressed in fluconazole-resistant isolates (Chen *et al.*, 2010; Sanglard *et al.*, 1995; Tsao *et al.*, 2009). Furthermore, it is overexpressed during treatment with antifungal compounds such as azole antifungals and plays a predominant role in extrusion of xenobiotic compounds from the cell, including antifungal compounds (Prasad *et al.*, 1995). This would suggest that AA may increase the drug resistance of *C. albicans* due to overexpression of these three genes, especially *CDR1*.

In addition to exposure to AA, *CDR1*, *IPT1* and *RTA3* were induced during co-incubation with *P. aeruginosa*. Therefore, homozygous deletion mutants of these genes were used to evaluate their role in survival in the presence of *P. aeruginosa*, as well as their role in the biofilm formation and fluconazole resistance of *C. albicans* in the presence of AA, with special attention to Cdr1p.

### 4.3. MATERIALS AND METHODS

#### 4.3.1. Strains used in this study

The strains used in this study are summarized in **Table 4.1**. Strain ASCa1 (Szczepaniak *et al.*, 2015) was a kind gift from Prof. Anna Krasowska from the Faculty of Biotechnology at the University of Wroclaw. *Candida albicans* strains were stored at -80°C with 15% glycerol. Yeast strains were revived and maintained on yeast malt (YM) agar (3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone, 10 g/L glucose, 16 g/L agar) at 30°C. *Pseudomonas aeruginosa* PAO1 was stored at -80°C with 25% glycerol and revived/maintained on Luria-Bertani (LB) agar (5 g/L yeast extract, 10 g/L tryptone, 10 g/L sodium chloride and 15 g/L agar).

Table 4.1. Strains used in this study

Name	Species	Genotype	Parental strain	Description	Origin
SC5314	<i>Candida albicans</i>	Wild type	N/A	Reference strain	N/A
<i>cdr1Δ/Δ</i>	<i>Candida albicans</i>	<i>cdr1Δ;cdr1Δ</i>	SC5314	Homozygous deletion of <i>CDR1</i>	This study
<i>cdr1Δ/Δ::CDR1</i>	<i>Candida albicans</i>	<i>cdr1Δ::CDR1;cdr1Δ::CDR1</i>	<i>cdr1Δ/Δ</i>	Homozygous add-back of <i>CDR1</i> gene into <i>cdr1Δ/Δ</i> to restore wild type	This study
<i>ipt1Δ/Δ</i>	<i>Candida albicans</i>	<i>ipt1Δ;ipt1Δ</i>	SC5314	Homozygous deletion of <i>IPT1</i>	This study
<i>ipt1Δ/Δ::IPT1</i>	<i>Candida albicans</i>	<i>ipt1Δ::IPT1;ipt1Δ::IPT1</i>	<i>ipt1Δ/Δ</i>	Homozygous add-back of <i>IPT1</i> gene into <i>ipt1Δ/Δ</i> to restore wild type	This study
<i>ipt1Δ/Δcdr1Δ/Δ</i>	<i>Candida albicans</i>	<i>ipt1Δ;ipt1Δ;cdr1Δ;cdr1Δ</i>	SC5314	Double homozygous deletion of <i>IPT1</i> and <i>CDR1</i>	This study
<i>rta3Δ/Δ</i>	<i>Candida albicans</i>	<i>rta3Δ;rta3Δ</i>	SC5314	Homozygous deletion of <i>RTA3</i>	This study
<i>rta3Δ/Δ::RTA3</i>	<i>Candida albicans</i>	<i>rta3Δ::RTA3;rta3Δ::RTA3</i>	<i>rta3Δ/Δ</i>	Homozygous add-back of <i>RTA3</i> gene into <i>rta3Δ/Δ</i> to restore wild type	This study
ASCa1	<i>Candida albicans</i>	<i>ura3Δ::imm434/URA3 CDR1-GFP</i>	CAF4-2	Fusion of GFP to Cdr1p	Szczepaniak <i>et al.</i> , 2015
PMA1-GFP	<i>Candida albicans</i>	<i>ura3 Δ::λ imm434, RP10-URA3-PMA1-GFP</i>	CAI1-4	Fusion of GFP to <i>PMA1</i> and insertion at <i>RP10</i> locus	This study
PMA1-CRISPR-GFP	<i>Candida albicans</i>	<i>PMA1::GFP;PMA1::GFP</i>	SC5314	Homozygous insertion of GFP at 3'-end of <i>PMA1</i> separated by linker (Janke <i>et al.</i> , 2004)	This study
CDR1-HIS	<i>Candida albicans</i>	<i>CDR1::6XHIS; CDR1::6XHIS</i>	SC5314	Homozygous insertion of polyhistidine sequence at 3'-end of <i>CDR1</i>	This study
PAO1	<i>Pseudomonas aeruginosa</i>	Wild type	N/A	Reference strain	N/A

### 4.3.2. Construction of homozygous deletion mutants with CRISPR/Cas9

A CRISPR-Cas9 system published by the group of Aaron Hernday (Nguyen *et al.*, 2017) was used for the formation of homozygous mutants for *CDR1*, *IPT1* and *RTA3*. The plasmids used in this study were obtained from Addgene ([www.addgene.org](http://www.addgene.org)). All primers were purchased from Integrated DNA Technologies and are listed in **Table 4.2**. All Polymerase chain reactions (PCRs) were performed with KAPA Hifi Hotstart PCR Kit (KAPA Biosystems Inc.) and colony PCR was performed with KAPA Taq PCR Kit (KAPA Biosystems Inc.), unless otherwise stated.

#### 4.3.2.1. Plasmids and cassettes for deletion

Two microgram pADH99 (Addgene plasmid # 90979) was digested with PmeI (New England Biolabs, US) to release the CAS9 cassette (**Chapter 3 Figure 3.1A**). For the gRNA cassette, the 5' of the gRNA cassette was amplified from pADH110 (Addgene plasmid # 90982) with primers AHO1096-ver2 and AHO1098-ver2 (**Chapter 3 Figure 3.1B**). To introduce target-specific CRISPR-sites into the gRNA cassette, gene sequences (for *CDR1*, *IPT1* and *RTA3*) with approximately 500 bp upstream and downstream were obtained from *Candida* Genome Database (CGD) and CRISPR-sites (20 bp sequence followed by a protospacer adjacent motif, PAM) for *CDR1*, *IPT1* and *RTA3* respectively were retrieved with *Geneious* (version 10.2.6; <https://www.geneious.com>). CRISPR-sites were chosen as to have the highest on-site activity score (Doench *et al.*, 2014) and off-target score (Hsu *et al.*, 2013) compared to the diploid genome of *C. albicans* SC5314 (Muzzey *et al.*, 2013; Skrzypek *et al.*, 2017) to minimize off target events in the *C. albicans* genome. In addition, CRISPR sites were chosen only if they were identical on both alleles of the diploid genome according to Assembly 22 of the *C. albicans* SC5314 genome (Muzzey *et al.*, 2013; Skrzypek *et al.*, 2017). The flanking sequences, 5'-CGTAAACTATTTTAAATTTG-3' and 5'-GTTTTAGAGCTAGAAATAGC-3' were added to the 5' and 3' sides of the 20bp CRISPR site without the PAM sequence respectively (**Table 4.3**). These two regions are complementary to *SNR52* promoter on pADH110 (Addgene plasmid # 90982) and structural gRNA sequence on pADH147 (Addgene plasmid # 90991) respectively. The 3' of the gRNA cassette was constructed through amplification of pADH147 with the oligos with flanking sequences described above as forward primer (**Chapter 3 Figure 3.1B**) and AHO1097 as reverse primer. The 3' of the gRNA cassette (product size of



<b>IPT1-3R</b>	GGATCGAAAAACCATTGAATTTT	This study
<b>IPT1-CRISPR-1-deletion</b>	CGTAAACTATTTTTAATTTGCCATTCATATGAATAAACAGTTTTAGAGCTAGAAATAGC	This study
<b>IPT1-CRISPR-2-add-back</b>	CGTAAACTATTTTTAATTTGTTAGAAATACTAAAATTCAAGTTTTAGAGCTAGAAATAGC	This study
<b>PMA1-1F</b>	CCAACCAACCAAAACAAA	This study
<b>PMA1-1R</b>	CAAACAATGAAATTCAAAATGG	This study
<b>PMA1-2F</b>	TTGATGATATCGTTGTTGATGTGAA	This study
<b>PMA1-2R</b>	TTCGTGTTGAGTAGATACTCTTGC	This study
<b>PMA1-GFP-F</b>	GAGTATCTACTCAACACGAAATGTCTAAAGGTGAAGAATTATTC	This study
<b>PMA1-GFP-R</b>	ATCAACAACGATATCATCAATTATTTGTACAATTCATCCATAC	This study
<b>PMA1-GFP-1F</b>	TGCTCCATACGATCCAAAAC	This study
<b>PMA1-GFP-1R</b>	TGTAAGGATGGGGATATAATTATCA	This study
<b>PMA1-GFP-CRISPR-1-KI</b>	CGTAAACTATTTTTAATTTGGTTGAAAGAAGGTTTAAGGGGTTTTAGAGCTAGAAATAGC	This study
<b>RTA3-2F</b>	GCGGAGATAAGCTCAGTCGAATT	This study
<b>RTA3-2R-overlap</b>	CATACCACCCCTGTAATTACCGCAAGATCCA	This study
<b>RTA3-3F-overlap</b>	GTAATTACAGGGTGGTATGATTCCTAGCAA	This study
<b>RTA3-3R</b>	CCAACAAAACCTTTGGGTCTCT	This study
<b>RTA3-CRISPR-1-deletion</b>	CGTAAACTATTTTTAATTTGTTATATTCGTTGTATTTACAGTTTTAGAGCTAGAAATAGC	This study
<b>RTA3-CRISPR-2-add-back</b>	CGTAAACTATTTTTAATTTGCTGCACTAATTATAACCAAAGTTTTAGAGCTAGAAATAGC	This study
<b>SPACER-GFP-F</b>	GCAAAGAGTATCTACTCAACACGAATCTGGTGCCGGTGCCGGTGCCGGTGCCATTTTG	This study
<b>SPACER-GFP-R</b>	TGAATAATTCTTCACCTTTAGACATCAAATGGCACCGGCACCGGCACCGGCACCGGCACCAGA	This study
<b>S-PMA1-F</b>	GACTGACATTGTCACTGT	This study
<b>S-PMA1-R</b>	ACCATAACCGAAAGTAGT	This study

704 bp) was fused with the amplification product (1066 bp) of pADH110 with a stitching polymerase chain reaction with primers AHO1237 and AHO1236 (*Chapter 3 Figure 3.1C*). The cassettes containing the *CAS9* as well as the gRNA is co-transformed into *C. albicans* to form an intact *CAS9*-gRNA cassette for integration into the *HIS1* locus (*Chapter 3 Figure 3.1D*).

Donor DNA was designed to remove nearly the entire open reading frames of the target genes. A modification from the Nguyen co-workers (2017) protocol entailed extending homologous regions to approximately 150 bp to 500 bp for deletion. In addition, homologous regions of approximately 500 bp were used for re-introduction of wild type genes (*Chapter 3 Figure 3.2*).

**Table 4.4** represents the information of the donor DNA constructed for the deletion of *CDR1*, *IPT1* and *RTA3*. Genomic DNA was extracted with the *Quick-DNA<sup>TM</sup>* Fungal/Bacterial Miniprep Kit (Zymo Research, USA) according to manufacturer's specifications. Two fragments were amplified from the *C. albicans* SC5314 genome with fragment 1 located at the 5' of the coding sequence fragment 2 at the 3'-end. Stitching PCR was performed to fuse fragment 1 and fragment 2 using primer sets underlined in **Table 4.4** to produce complete donor DNA for deletion of the target genes.

**Table 4.3.** Unique CRISPR-site sequences without protospacer adjacent motif for deletion and add-back of *CDR1*, *IPT1* and *RTA3*. Site-characteristics obtained in *Geneious*.

Gene	Application	Sequence	On-site activity score	Off-target activity score
<i>CDR1</i>	Deletion	TTTCACCACCGGAAACACCA	0.876	83.33%
<i>CDR1</i>	Add-back	GATATCCGTCTCAACTACAA	0.802	83.33%
<i>IPT1</i>	Deletion	CCATTCATATGAATAAACCA	0.784	83.33%
<i>IPT1</i>	Add-back	TTAGAAATACTAAAATTCAA	0.721	83.33%
<i>RTA3</i>	Deletion	TTATATTCGTTGTATTTACA	0.781	83.33%
<i>RTA3</i>	Add-back	CTGCACTAATTATAACCAAA	0.732	83.33%

#### 4.3.2.2. Transformation

*Candida albicans* SC5314 was inoculated in 5 mL YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) and incubated overnight with shaking (30°C). The overnight culture was inoculated into fresh YPD (ration of 1:50) and allowed to reach an optical density (600 nm) of 0.5 to 0.8 after which cells were washed two times with sterile

**Table 4.4.** Specifications of construction of donor DNA for deletion and re-introduction (add-back) of wild type genes.

Gene	Application	Fragment	Forward primer	Reverse primer	Start position*	End position*	Coordinates of deletion/insertion*	Size of deletion/insertion
<i>CDR1</i>	Deletion	1 of 2	<u>CDR1-2F</u>	CDR1-2R-overlap	48	199	199 to 4301	4102 bp
		2 of 2	CDR1-3F-overlap	<u>CDR1-3R</u>	4301	4484		
	Add-back	1 of 1	CDR1-1F	CDR1-1R	-687	4710		
<i>IPT1</i>	Deletion	1 of 2	<u>IPT1-2F</u>	IPT1-2R-overlap	12	170	170 to 1346	1176 bp
		2 of 2	IPT1-3F-overlap	<u>IPT1-3R</u>	1346	1535		
	Add-back	1 of 1	IPT1-1F	IPT1-1R	-491	1937		
<i>RTA3</i>	Deletion	1 of 2	<u>RTA3-2F</u>	RTA3-2R-overlap	-254	31	31 to 1412	1381 bp
		2 of 2	RTA3-3F-overlap	<u>RTA3-3R</u>	1412	1647		
	Add-back	1 of 1	RTA3-2F	RTA3-3R	-254	1647		

\* Position from first bp of coding sequence.

Underlined primers were used for stitching polymerase chain reaction to fuse fragment 1 and fragment 2 of each gene to construct donor DNA for deletion

water (Nguyen *et al.*, 2017). Cells were resuspended in 1/100 H<sub>2</sub>O of original volume. The digest of pADH99 and PCR products of gRNA cassette, dDNA and 10 µL (10 mg/mL) denatured salmon sperm were added to the washed cells together with 1 mL plate mix consisting of 875 µL 50% PEG 3350 (Sigma-Aldrich), 100 µL 10X TE buffer (100 mM Tris pH 7.4, 10 mM EDTA pH 8) and 25 µL 1M Lithium acetate (pH adjusted to 7 with acetic acid; Sigma-Aldrich) and incubated overnight at 30°C without shaking. Cells were heat-shocked at 44.6°C (myBlock™ Mini Dry Bath, Benchmark Scientific) for 15 minutes, washed with sterile YPD and allowed to recover for 5 hours at 30°C with shaking. Recovered cells were plated onto YPD agar (15 g/L agar) plus 200 µg/mL nourseothricin (NTC, Jena Bioscience, Germany) and incubated for 2 days at 30°C to allow formation of colonies. Colony PCR was performed to amplify dDNA with primers CDR1-2F and CDR1-3R for *CDR1*; IPT1-2F and IPT1-3R for *IPT1*; and RTA3-2F and RTA3-3R for *RTA3*.

#### 4.3.2.3. Removal of CAS9-gRNA cassette and knock-in of wild type gene

After confirmation of deletion, the CAS9-gRNA cassette was removed by inducing expression of a FLP/FRT recombinase system (under control of maltose-inducible promoter; *Chapter 3 Figure 3.3*) through growth in Yeast Peptone Maltose media (10 g/L yeast extract, 20 g/L peptone and 20 g/L maltose) for 24h @ 30°C with shaking (200 rpm). Cells were diluted in sterile H<sub>2</sub>O and plated on YPD agar for 48h at 30°C to allow formation of single colonies. After formation of colonies, growth on NTC was evaluated by streaking on YPD agar with 300 µg/mL nourseothricin. The absence of growth in the presence of NTC indicates that the CAS9-gRNA cassette has been removed. After removal of the CAS9-gRNA cassette, a sequential round of transformation was performed to re-introduce the wild type gene into its native locus the use of dDNA containing the ORF for the gene of interest (*Chapter 3 Figure 2*).

### 4.3.3. Biofilm formation

#### 4.3.3.1. Monomicrobial biofilm formation by *C. albicans*

*Candida albicans* strains represented in **Table 4.1** were revived from glycerol stock on YM agar for 24 h at 30 °C and inoculated into 5 mL yeast nitrogen base (YNB) broth (10 g/L glucose, 16 g/L YNB) and incubated at 30 °C for 24 h with shaking (150 rpm). Cells were harvested at 1878 g for 5 minutes and the supernatant removed. This was

followed by washing the cells twice with phosphate buffered saline (PBS) (Oxoid, England). The cells were counted with a haemocytometer and diluted to  $1 \times 10^6$  cells/mL in filter sterilized (0.22  $\mu\text{m}$  nitrocellulose filter, Merck Millipore, Ireland) RPMI-1640 medium (Sigma-Aldrich, USA).

#### 4.3.3.2. Polymicrobial biofilm formation by *C. albicans* and *P. aeruginosa*

*Candida albicans* strains represented in **Table 4.1** were revived from glycerol stock on YM agar for 24 h at 30 °C and inoculated into 5 mL YNB broth and incubated at 30 °C for 24 h with shaking (150 rpm). Cells were harvested at 1878 g for 5 minutes and the supernatant removed. This was followed by washing the cells twice with phosphate buffered saline (PBS) (Oxoid, England). The cells were counted with a haemocytometer and diluted to  $1 \times 10^6$  cells/mL in filter sterilized RPMI-1640 medium. *Pseudomonas aeruginosa* PAO1 was revived on LB agar and inoculated into LB broth and incubated 24h at 37°C. Bacterial cells were washed with sterile PBS and diluted to an OD<sub>600</sub> of 0.05 in RPMI-1640 medium containing the *C. albicans* cells.

#### 4.3.4. Survival of *C. albicans* in a polymicrobial biofilm with *P. aeruginosa*

Cells for mono- and polymicrobial biofilms were prepared as described above in flat-bottom 6 well culture plates (Corning Incorporated, USA) in 3 mL medium and incubated for 48h to allow biofilm formation to take place. After incubation, biofilms were washed twice with sterile PBS, scraped off and suspended in PBS. Biofilms were then vortexed 3 times for 1 minute to remove adherent cells from one another (adapted from Fourie *et al.*, 2017). For quantification of *C. albicans*, serially diluted cells were plated onto YM medium acidified with tartaric acid (final concentration 0.08%). For bacterial quantification, serially diluted cells were plated onto LB supplemented with 10  $\mu\text{g/mL}$  amphotericin B (Sigma-Aldrich, USA) (Pires *et al.*, 2013). Plates were incubated overnight, to allow formation of colonies, and counted. This experiment was performed in triplicate.

#### 4.3.5. Metabolic activity of biofilms

Cells were prepared as described above with the addition of 0.1 mM AA (or equivalent ethanol control – 0.076% v/v) and 200  $\mu\text{L}$  was dispensed into a 96-well plate (Corning Incorporated, Costar®, USA). The plate was incubated for 6h and 48h respectively at 37 °C to allow the formation of biofilms. Following incubation, the supernatant from

each well was removed and the biofilms were washed twice with sterile PBS. Fifty microliters of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino) carbonyl]-2H tetrazolium hydroxide (XTT) (Sigma-Aldrich, USA) (1 g XTT in 1L PBS, filter sterilized, aliquoted and stored at -20 °C) containing 0.08 mM menadione (Fluka, USA) (a stock solution of 10 mM menadione in acetone) was added to each well and incubated for 3 h in the dark at 37°C (Fourie *et al.*, 2017; Kuhn *et al.*, 2003). Following incubation, the absorbance of each well was measured at 492 nm on an EZ Read 800 Microplate Reader (Biochrom, UK) and data was obtained. This experiment was performed in triplicate with four technical replicates per biological replicate.

#### 4.3.6. Biofilm biomass

Cells were prepared as described above with the addition of 0.1 mM AA (or equivalent ethanol control – 0.076% v/v) and 200 µL was dispensed into a 96-well plate (Corning Incorporated, Costar®, USA). The plate was incubated for 6h and 48h respectively at 37°C to allow the formation of biofilms. The crystal violet assay was performed on biofilms according to Jin and co-workers (2003) with minor modification. Briefly, the supernatant from each well was removed and the biofilms were washed twice with sterile PBS. Biofilms were then left to air dry for 45 minutes and stained with 110 µL crystal violet (0.4 % w/v; Merck, Germany) for 45 min (Jin *et al.*, 2003). Biofilms were washed three times with 350 µL sterile H<sub>2</sub>O and de-stained with 200 µL 95 % ethanol for 45 min. one hundred microliter of de-staining solution was then transferred to a clean 96-well plate and absorbance was measured at 595 nm. This experiment was performed in triplicate with four technical replicates per biological replicate.

#### 4.3.7. Influence of antioxidants on *cdr1Δ/Δ* biofilm biomass

Biofilms of *cdr1Δ/Δ* were prepared in 96-well plates as described above. In addition, 0.5 mM α-tocopherol or 0.045 mM butylated hydroxytoluene (Sigma-Aldrich, USA), were added in the absence or presence of 0.1 mM AA. Biofilm biomass was quantified with the crystal violet assay as described above.

#### 4.3.8. Infection of *Caenorhabditis elegans* by *C. albicans*

##### 4.3.8.1. Propagation of *C. elegans*

*Caenorhabditis elegans* AU37 [glp-4(bn2) I; sek-1(km4 X)], obtained from the *Caenorhabditis* Genetic Centre (University of Minnesota), was used for all infections.

The nematodes were propagated and maintained on Nematode Growth Medium (3 g/L NaCl, 2.5 g/L peptone, 5 µg/mL cholesterol, 1 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 25 mM KPO<sub>4</sub>, 20 g/L agar) with *Escherichia coli* OP50 as a food source at 15°C (Brenner, 1974).

#### 4.3.8.2. Infection with *C. albicans*

For infection by *C. albicans*, strains were inoculated in YPD broth overnight at 30°C. Overnight cells were diluted to an OD<sub>600</sub> of 0.8 and 100 µL was plated onto brain-heart infusion (BHI)-agar plates and incubated overnight at 30°C. Synchronised L4-stage nematodes were washed with M9 buffer (3 g/L KH<sub>2</sub>PO<sub>4</sub>, 6 g/L Na<sub>2</sub>PO<sub>4</sub> and 1 mM MgSO<sub>4</sub>) and added to plates with *C. albicans*. Nematodes were incubated with *C. albicans* for 4 hours at 25°C and washed three times with M9 buffer to remove non-ingested *C. albicans* cells. Nematodes were then added at approximately 60 worms per well in a 6 well culture plate containing 2 mL 80% M9 buffer and 20% BHI broth, with 90 µg/mL kanamycin and incubated at 25°C. Nematodes were monitored daily and dead nematodes (non-motile after mechanical stimulation with sterile pipette tip or penetration of *C. elegans* cuticle by *C. albicans* hyphae) were counted and removed. Nematodes incubated with *E. coli* OP50 prior to survival assay, was used as a control. Infection was performed in triplicate for a total of 180 nematodes per strain of *C. albicans*. Survival metrics including Kaplan-Meier statistics, median survival time and log-rank test were performed with online application for survival analysis 2 (OASIS 2; Han *et al.*, 2016).

#### 4.3.9. Fluorescence of GFP-tagged Cdr1p

For fluorescence microscopy, a Cdr1-GFP fusion was utilized (AsCa1). Biofilms were prepared as above in 6 well culture plates in 3 mL medium with AA (0.1 mM or 1 mM) or equivalent ethanol control and incubated for 6 hours @ 37°C. After biofilm formation, cells were scraped off and re-suspended in paraformaldehyde (4 g/L) and incubated at room temperature for 15 minutes followed by washing of cells in with KPO<sub>4</sub>/sorbitol mixture (1.2 M sorbitol with 0.1 M potassium phosphate) (Biggins, "GFP Fixation in Yeast"). Fluorescence was visualized with a confocal laser scanning microscope (CLSM) (Nikon TE 2000, Japan).

#### 4.3.10. Rhodamine 6G efflux assay

Biofilms were prepared as above (in the absence or presence of 0.1 mM AA) in black 96-well microtiter plates (Thermo Fisher Scientific, USA) and incubated for 6h at 37°C. After biofilm formation, medium was removed, and cells were washed twice with sterile PBS. Thereafter, biofilms were incubated in PBS for 1h at 37°C to de-energize cells (Ells *et al.*, 2013; Maesaki *et al.*, 1999, Szczepaniak *et al.*, 2017). Rhodamine 6G (10 µM) was added to each well, incubated at 37°C and the fluorescence was measured every 10 minutes for 1 hour at an excitation of 530 nm and emission of 590 nm to evaluate rhodamine 6G uptake. After rhodamine 6G uptake, AA (0.1 mM or 1 mM in PBS) was added for 1 hour 37°C. After treatment, cells were washed 3X with sterile PBS and 2 mM glucose (in PBS) were added to each well to induce efflux of rhodamine 6G. Efflux was measured every 10 minutes for 1 hour.

#### 4.3.11. Effect of 0.1 mM AA on susceptibility to fluconazole

Biofilms were prepared in 96-well plates as described above. Fluconazole (1 µg/mL) (Sigma-Aldrich, USA) in the absence or presence of 0.1 mM AA was added prior to biofilm formation and incubated for 6 hours or added after biofilm formation for 1 hour. Biofilm biomass and metabolic activity of biofilms were quantified as described above.

#### 4.3.12. Filipin staining

Biofilms were prepared as described above in 6 well culture plates. After 6 hours of biofilm formation, biofilms were scraped off and resuspended in PBS. Ten micrograms per millilitre filipin (Merck, USA) were used to stain cells for 10 minutes (Martin and Konopka, 2004) and fluorescence was visualised using a CKX53 Microscope with U-HGLGPS Fluorescence Light Source and SC50 camera (Olympus, Japan). Blue fluorescence was visualised using a UV filter and processed using Olympus cellSens standard software.

#### 4.3.13. Insertion of Green Fluorescent Protein at 3'-end of *PMA1*

To determine localisation of Pma1p in *C. albicans* exposed to AA, the protein was tagged with green fluorescent protein (GFP) at the C-terminal end. This was attempted in two ways. Firstly, the Clp10 integration plasmid (Murad *et al.*, 2000) was used to construct the PMA1-GFP fusion and integrated at the *RP10* locus in the *C. albicans* genome. The second method entailed the use of the CRISPR-Cas9 system (Nguyen

*et al.*, 2017) to introduce a double stranded break in the *PMA1* gene (constructed as described above for deletions and add-backs of *CDR1*, *IPT1* and *RTA3*). Donor DNA was modified to introduce the sequence of GFP at the 3'-end of the native *PMA1* gene with a linker sequence between the *PMA1* gene and GFP sequence. These two methods will be described separately.

#### 4.3.13.1. Integration of *PMA1*-GFP in the *RP10* locus of *C. albicans* using *Clp10* (Murad *et al.*, 2000)

The open reading frame of *PMA1*, with 990bp upstream and 475bp downstream, was amplified from the *C. albicans* genome, using primers PMA1-1F and PMA1-1R. The PCR product was ligated into the pMiniT 2.0 plasmid using the NEB® PCR Cloning Kit (New England Biolabs, USA). Restriction digestion with *XhoI* (Thermo Fisher Scientific, USA) was used to introduce the *PMA1* gene with flanking regions into the *Clp10* plasmid (Murad *et al.*, 2000) yielding *Clp10-URA3-PMA1*. Primers PMA1-2F and PMA1-2R were used to linearise *Clp10-URA3-PMA1* and remove the stop codon at the 3'-end of *PMA1*.

pGEN-GFP-URA-GFP (Addgene plasmid # 72606), containing the *C. albicans* codon-optimised gene for GFP, was obtained from Bruce Granger via Addgene ([www.addgene.org](http://www.addgene.org)). The codon optimised GFP gene (with intact stop codon) (Granger, 2018) was amplified from the plasmid, using primers PMA1-GFP-F and PMA1-GFP-R and introduced into *Clp10-PMA1* (without the stop codon), using the NEBuilder® HiFi DNA Assembly Cloning Kit (New England Biolabs, USA) according to manufacturer's specifications, yielding *Clp10-URA3-PMA1-GFP*. To accomplish insertion via NEBuilder® HiFi DNA Assembly Cloning Kit, 20bp overlap with *Clp10-URA3-PMA1* was added to PMA1-GFP-F and PMA1-GFP-R, complementary to the 3'-end and 5'-end of linearised *Clp10-URA3-PMA1* respectively. Confirmation of in-frame insertion of the GFP was done with Sanger sequencing (Hitachi 3130xl Genetic Analyzer, Applied Biosystems, USA) using primers S-PMA1-F and S-PMA1-R with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA).

The plasmid *Clp10-URA3-PMA1-GFP* was linearised with unique *StuI* sites (Thermo Fisher Scientific, USA) on the *Clp10* plasmid. Transformation into the *URA3*-deficient *C. albicans* strain, CAI-4, was done using a modified protocol from Nguyen and co-workers (2017). Briefly, *Candida albicans* CAI-4 was inoculated in 5 mL YPD and

incubated overnight with shaking (30°C). The overnight culture was inoculated into fresh YPD (ration of 1:50) and allowed to reach an optical density (600 nm) of 0.5 to 0.8 after which cells were washed two times with sterile water. Cells were resuspended in 1/100 H<sub>2</sub>O of original volume. Approximately 500 ng of digested Clp10-*URA3-PMA1-GFP* together with 10 µL (10 mg/mL) of salmon sperm was added to washed cells together with 1 mL plate mix consisting of 875 µL 50% PEG 3350, 100 µL 10X TE buffer (100 mM Tris pH 7.4, 10 mM EDTA pH 8) and 25 µL 1M Lithium acetate (pH adjusted to 7 with acetic acid) and incubated overnight at 30°C without shaking. Cells were heat-shocked at 44°C for 15 minutes, washed with sterile YPD and allowed to recover for 5 hours at 30°C with shaking. Selective pressure of transformation is conferred by the introduction of *URA3* (carried on Clp10) into the CAI-4 strain and plating onto complete synthetic dropout medium without uracil (6.7 g/L YNB, 20 g/L glucose, 20 mg/L histidine, 40 mg/L tryptophan, 60 mg/L leucine, 0.6 g/L CSM, 15 g/L agar). The *PMA1-GFP* fusion is integrated at the *RP10* locus via homologous recombination (Murad *et al.*, 2000). After transformation and identification of successful transformants (via Sanger sequencing with primers S-PMA1-F and S-PMA1-R), *PMA1-GFP* was evaluated for fluorescence using CKX53 Microscope with U-HGLGPS Fluorescence Light Source and SC50 camera. Green fluorescence was visualised using a blue filter and processed using Olympus cellSens standard software.

#### 4.3.13.2. Introduction of GFP into native *PMA1*-locus with linker using CRISPR-Cas9 (Nguyen *et al.*, 2017)

Clp10-*URA3-PMA1-GFP* was linearised with primers GFP-1-F and PMA1-2R. Primers SPACER-GFP-F and SPACER-GFP-R were used to generate a 89bp PCR product with a 39bp sequence for a 13 amino acid linker (amino acid sequence of SGAGAGAGAGAIL) (Janke *et al.*, 2004). In addition to the linker sequence, 25bp overlaps were included complementary to the 3'-end of *PMA1* (carried on SPACER-GFP-F) and 5'-end of GFP (carried on SPACER-GFP-R). The linker sequence was inserted into Clp10-*URA3-PMA1-GFP* using the NEBuilder<sup>®</sup> HiFi DNA Assembly Cloning Kit yielding Clp10-*URA3-PMA1-S-GFP*. In-frame insertion of the linker was confirmed with Sanger sequencing. Primers PMA1-GFP-1F and PMA1-GFP-1R were used to amplify the construct (containing the 3'-end of *PMA1*, linker, GFP and downstream sequence of *PMA1*) of 1718bp to be used as dDNA. To construct gRNA

for a Cas9-mediated DSB in the *C. albicans* genome, oligo PMA1-GFP-CRISPR-1-KI (with CRISPR-site) was used as described in Section 4.3.2. This CRISPR site (5'-GTTGAAAGAAGGTTTAAGGG-3') (on-site activity score of 0.550 and off-target score of 83.3%) is located the downstream sequence following the *PMA1* gene in the *C. albicans* genome and the construct described above was used to modify the locus yielding a homozygous insertion of GFP at the 3'-end of *PMA1* (*PMA1*-CRISPR-GFP). Successful transformants (generated as discussed in Section 3.4.2) were confirmed with Sanger sequencing using primers S-PMA1-F and S-PMA1-R and evaluated for fluorescence (as discussed in Section 4.3.10.1).

#### 4.3.14. Adenosine triphosphate (ATP) quantification

Biofilms were prepared in 90 mm polystyrene petri dishes (20 mL volume) as described above (in the absence of AA) and incubated at 37°C for 6 hours to allow biofilm formation. After 6 hours, 0.1 mM AA (or equivalent ethanol control) was added to biofilms and incubated for 1 hour at 37°C. After treatment, biofilms were scraped off and biomass was pooled (3 biofilms per condition). Cells were resuspended in 400 µL 50 mM HEPES (pH 7.75) and 100 µL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) and disrupted with acid-washed glass beads, using a BeadBug™ microtube homogenizer (Benchmark Scientific, USA) with 5 cycles of 30 second pulses separated with 30 seconds on ice (adapted from Garcia *et al.*, 2008). Cell debris was pelleted by centrifugation (2500g for 10 minutes at 4°C) and the supernatant was used for ATP quantification (ATP Determination Kit, Molecular Probes™).

#### 4.3.15. Evaluation of mitochondrial membrane potential

Biofilms were prepared in 6-well plates as described above (in the absence of AA) and incubated for 6 hours at 37°C. After 6 hours, 0.1 mM AA (or equivalent ethanol control) was added to biofilms and incubated for 1 hour at 37°C. Biofilms were scraped off and suspended in HEPES buffer (pH 7.4). 3,3'-dihexyloxycarbocyanide iodide (DiOC<sub>6</sub>(3), Molecular Probes Yeast Mitochondrial Stain Sampler Kit) was added to cells at a final concentration of 175 nM and incubated at room temperature for 30 minutes (Koning *et al.*, 1993). Fluorescence was measured with a CKX53 Microscope with U-HGLGPS Fluorescence Light Source and SC50 camera. Green fluorescence was visualised using a blue filter and processed using Olympus cellSens standard software.

#### 4.3.16. Protein extraction and visualisation of Cdr1p on SDS-PAGE

Biofilms of *C. albicans* SC5314 and *cdr1Δ/Δ* were prepared in 6-well plates as described above and incubated for 6 hours at 37°C. After biofilm formation, cells were degermised with PBS for 1 hour, after which biofilms were treated with 0.1 mM AA, 1 mM AA or equivalent ethanol control for 1 hour. Cells in the biofilm were reenergised with 2 mM glucose in PBS for 15 min and biofilms were scraped off and suspended in 1 mL ice cold dH<sub>2</sub>O. Protein extraction was done according to Szczepaniak and co-workers (2015). Briefly, 150 μL of 1.85 M NaOH with 7.5% β-mercaptoethanol was added to the cell suspension and incubated on ice for 10 minutes. One hundred and fifty μL of 50% trichloroacetic acid was added and incubated on ice for 10 minutes to precipitate proteins. The solution was centrifuged (10 000g, 5 min, 4°C) and washed with 1 mL of 1 M Tris-HCl. Proteins were resuspended in 50 μL sample buffer (40 mM Tris-HCl, 8 M urea, 5% SDS, 0.1 mM EDTA, 1% β-mercaptoethanol and 0.1 mg/mL bromophenol blue) and incubated at 37°C for 30 minutes. Ten microliters of sample were loaded onto a 7.5% acrylamide/bis-acrylamide SDS-PAGE gel and proteins were separated for approximately 1 hour at 125 V. The SDS-PAGE gel was stained with Coomassie blue and destained as described previously (Fairbanks *et al.*, 1971).

#### 4.3.17. Introduction of a polyhistidine sequence at the C-terminal of *CDR1*

To aid in purification of Cdr1p and visualisation of Cdr1p with Western blot, we attempted to introduce a C-terminal polyhistidine tag. Primers CDR1-HIS-1F and CDR1-HIS-1R were used to amplify the 3'-end (760bp) together with 433bp of sequence downstream of *CDR1* in the genome. The PCR product (1193bp) was ligated into the pMiniT 2.0 plasmid using the NEB<sup>®</sup> PCR Cloning Kit yielding pMini-*CDR1*. Primers CDR1-HIS-2F and CDR1-HIS-2R were used to linearise the plasmid containing the *CDR1* insert. Primers CDR1-HIS-3F and CDR1-HIS-3R were used to construct a 97bp insert with the coding sequence for 6 consecutive histidine residues (5'-CATCACCATCACCATCAC-3'). The polyhistidine sequence is flanked with 37bp on the 5'-end complementary to the 3'-end of the linearised plasmid. The 3'-end of the polyhistidine sequence is flanked by a 42bp sequence complementary to the 5'-end of the linearised plasmid. The polyhistidine sequence with complementary regions was ligated into pMini-*CDR1* with NEBuilder<sup>®</sup> HiFi DNA Assembly Cloning Kit yielding pMini-*CDR1*-HIS. Notably, the polyhistidine sequence was inserted before the stop

codon of *CDR1*. In-frame insertion of the polyhistidine sequence was confirmed with Sanger sequencing using primers CDR1-HIS-S-1F and CDR1-HIS-S-1R. Primers CDR1-HIS-1F and CDR1-HIS-1R were used to amplify the construct from pMini-*CDR1*-HIS to yield dDNA for transformation (1211bp). Two separate CRISPR-sites were used to integrate the polyhistidine sequence using the dDNA described above. The first CRISPR-site (carried on CDR1-HIS-CRSIPR-1 oligo; 5'-ACTGATTCTGTCAATCCTCG-3') (on-site activity score of 0.802 and off-target score of 83.3%) is located 585bp upstream from the *CDR1* stop codon and is therefore located inside the coding sequence of *CDR1*. A second CRISPR-site (carried on CDR1-HIS-CRSIPR-2 oligo; 5'-TCATTGTTATGTAGACGTGG-3') (on-site activity score of 0.569 and off-target score of 83.3%) is located 177bp downstream of the *CDR1* stop codon (outside coding sequence of *CDR1*). Donor DNA was integrated using both CRISPR-sites separately with the transformation protocol described in Section 4.3.2.2. In-frame integration of the polyhistidine sequence in the genome of *C. albicans* was confirmed with primers CDR1-HIS-S-1F and CDR1-HIS-S-1R.

#### 4.3.18. Immunodetection of polyhistidine sequence

##### 4.3.18.1. Protein extraction and SDS-PAGE

To determine if the polyhistidine tag was successfully introduced in Cdr1p, several methods were utilised for protein extraction. Overnight cultures of SC5314 and strain CDR1-HIS were inoculated in 5 mL YPD, containing 0, 1 or 5 µg/mL fluconazole, and allowed to reach an OD<sub>600</sub> of 0.4. Cells were collected and frozen at -80°C or proteins were extracted immediately.

###### 4.3.18.1.1. Method 1

Proteins were extracted as described in Section 4.3.16 (Szczepaniak *et al.*, 2015). A aliquot (10 µL) of the sample was loaded on an SDS-PAGE gel, prepared according to the 10% TGX™ FastCast™ Acrylamide Kit (Bio-Rad, USA) specifications. Protein separation was performed for approximately 1 hour at 125 V.

###### 4.3.18.1.2. Method 2

Cells were washed with ice cold dH<sub>2</sub>O and resuspended in 50 mM Tris-HCl (pH 8.0) with 5 mM EDTA. Cells were disrupted with a BeadBug™ microtube homogenizer with 5 cycles of 30 second pulses separated with 30 seconds on ice. Twenty-five microliters

of homogenised cells were mixed with 25  $\mu$ L Laemmli buffer (Bio-Rad, USA) with 5%  $\beta$ -mercaptoethanol and incubated at 95°C for 10 minutes. After centrifugation (3000g for 5 minutes), 10  $\mu$ L of sample was loaded on a 10% TGX™ FastCast™ Acrylamide SDS-PAGE gel and proteins were separated for approximately 1 hour at 125 V.

#### 4.3.18.1.3. Method 3

Cells were washed with ice cold dH<sub>2</sub>O and resuspended in 50 mM Tris-HCl (pH 8.0) with 5 mM EDTA. Cells were disrupted with a BeadBug™ microtube homogenizer (Benchmark Scientific, USA) with 5 cycles of 30 second pulses separated with 30 seconds on ice. The homogenised cell solution was centrifuged two times at 1000g (5 minutes at 4°C) and one time at 3000g (5 minutes at 4°C), followed by centrifugation at 20 000g for 40 minutes (4°C) to isolate plasma membranes (Wagner *et al.*, 2019). Proteins were precipitated with 6% trichloroacetic acid, centrifuged (10 000g, 5 min, 4°C) and washed with 1 mL of 1 M Tris-HCl (Szczepaniak *et al.*, 2015). Proteins were resuspended in 50  $\mu$ L sample buffer (40 mM Tris-HCl, 8 M urea, 5% SDS, 0.1 mM EDTA, 1%  $\beta$ -mercaptoethanol and 0.1 mg/mL bromophenol blue) and incubated at 37°C for 30 minutes. Ten microliters of sample were loaded on a 10% TGX™ FastCast™ Acrylamide SDS-PAGE gel and protein separation was performed for approximately 1 hour at 125 V.

#### 4.3.18.2. Western blot analysis

Following separation of proteins by SDS-PAGE, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using the Trans-Blot Turbo Transfer System (Bio-Rad, USA) according to manufacturer's specifications. The presence of a polyhistidine sequence added to Cdr1p was evaluated with SuperSignal® West HisProbe™ Kit (Thermo Fisher Scientific, USA) according to manufacturer's specifications.

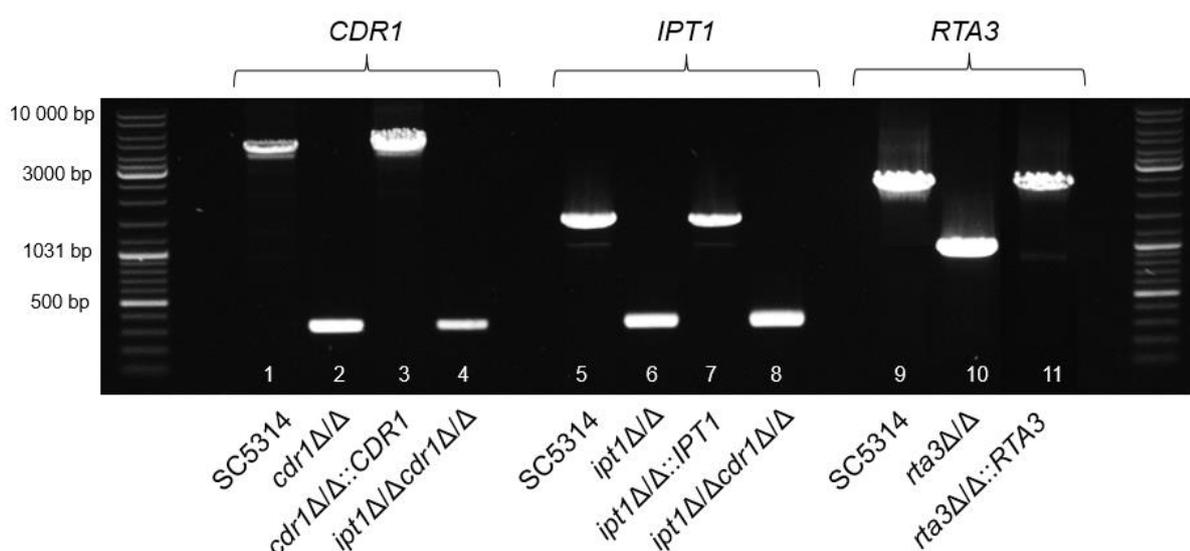
#### 4.3.19. Statistical analysis

Differences between control and experimental, as well as between *C. albicans* strains were determined with *t*-test, performed with GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA, [www.graphpad.com](http://www.graphpad.com)) unless otherwise stated.

## 4.4. RESULTS AND DISCUSSION

### 4.4.1. Construction of *CDR1*, *IPT1* and *RTA3* homozygous deletion mutants and re-introduction of wild-type genes

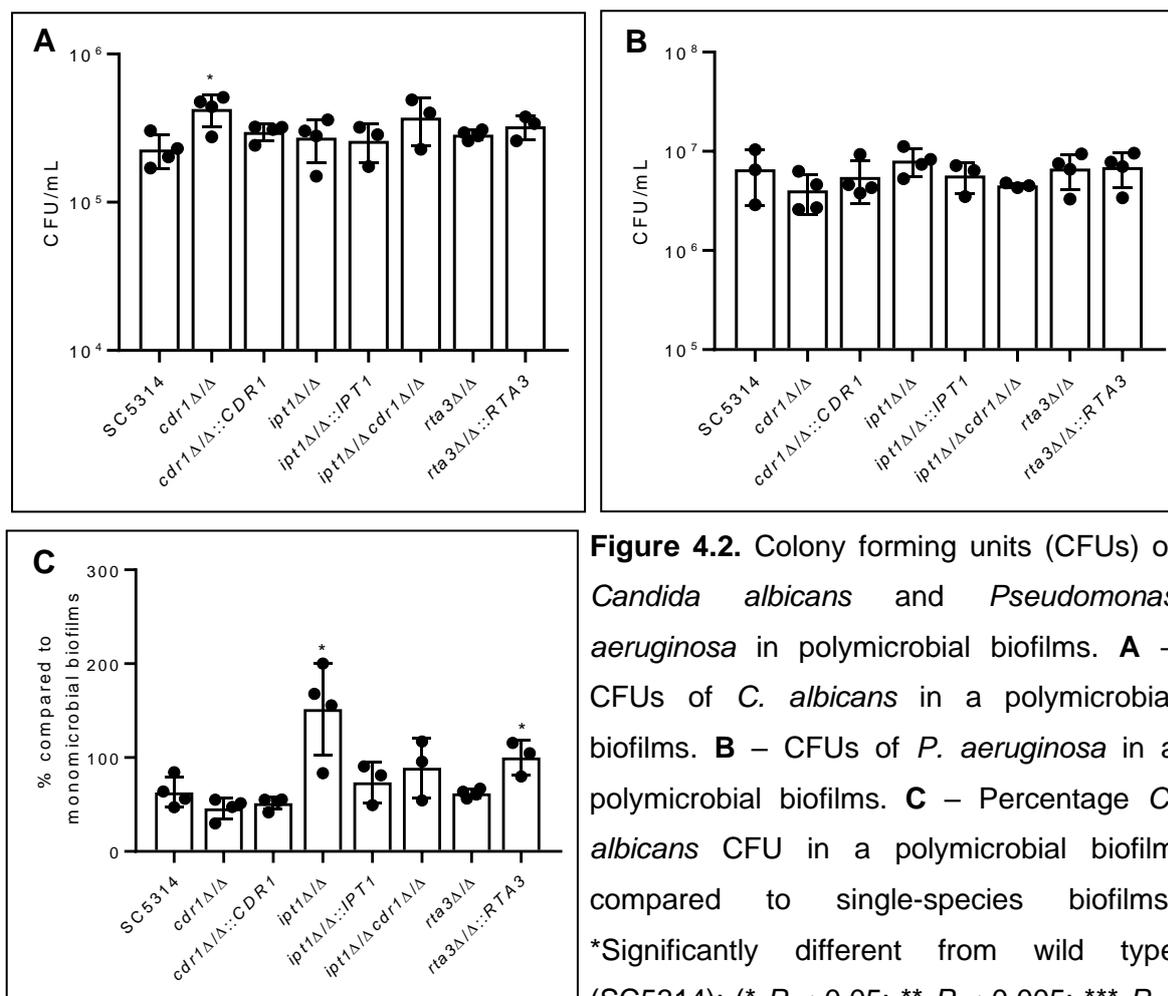
To determine the role of selected genes (*CDR1*, *IPT1* and *RTA3*) in biofilm formation of *C. albicans* in the presence of AA and during co-incubation with *P. aeruginosa*, homozygous deletion mutants of the respective genes were constructed with a CRISPR-Cas9 system published by Nguyen and co-workers (2017). The selected mutants were also subjected to a second round of transformation to reintroduce wild type genes. These add-backs were also included in subsequent experiments to ensure that the phenotypes obtained were due to the deletion of the target genes, and not off-target events. In addition, a double deletion mutant of both *CDR1* and *IPT1* was constructed. **Figure 4.1** indicates the successful deletion and add-back of selected genes through amplification of segments of the target site.



**Figure 4.1.** Confirmation of deletion and re-introduction of *CDR1*, *IPT1* and *RTA3* in *Candida albicans*. Gel-electrophoresis of PCR products amplifying a region in the open reading frame of the gene of interest with primers as described in **Table 4.4** for stitching PCR. *CDR1* – the wild type gene corresponds to a PCR product of 4436bp, whereas a deletion is indicated by a band corresponding to 334bp. *IPT1* - wild type gene corresponds to a PCR product of 1523bp, whereas a deletion is indicated by a band corresponding to 347bp. *RTA3* - wild type gene corresponds to a PCR product of 2428bp, whereas a deletion is indicated by a band corresponding to 1046bp.

#### 4.4.2. Role of *CDR1*, *IPT1* and *RTA3* in the interaction of *C. albicans* with *P. aeruginosa*

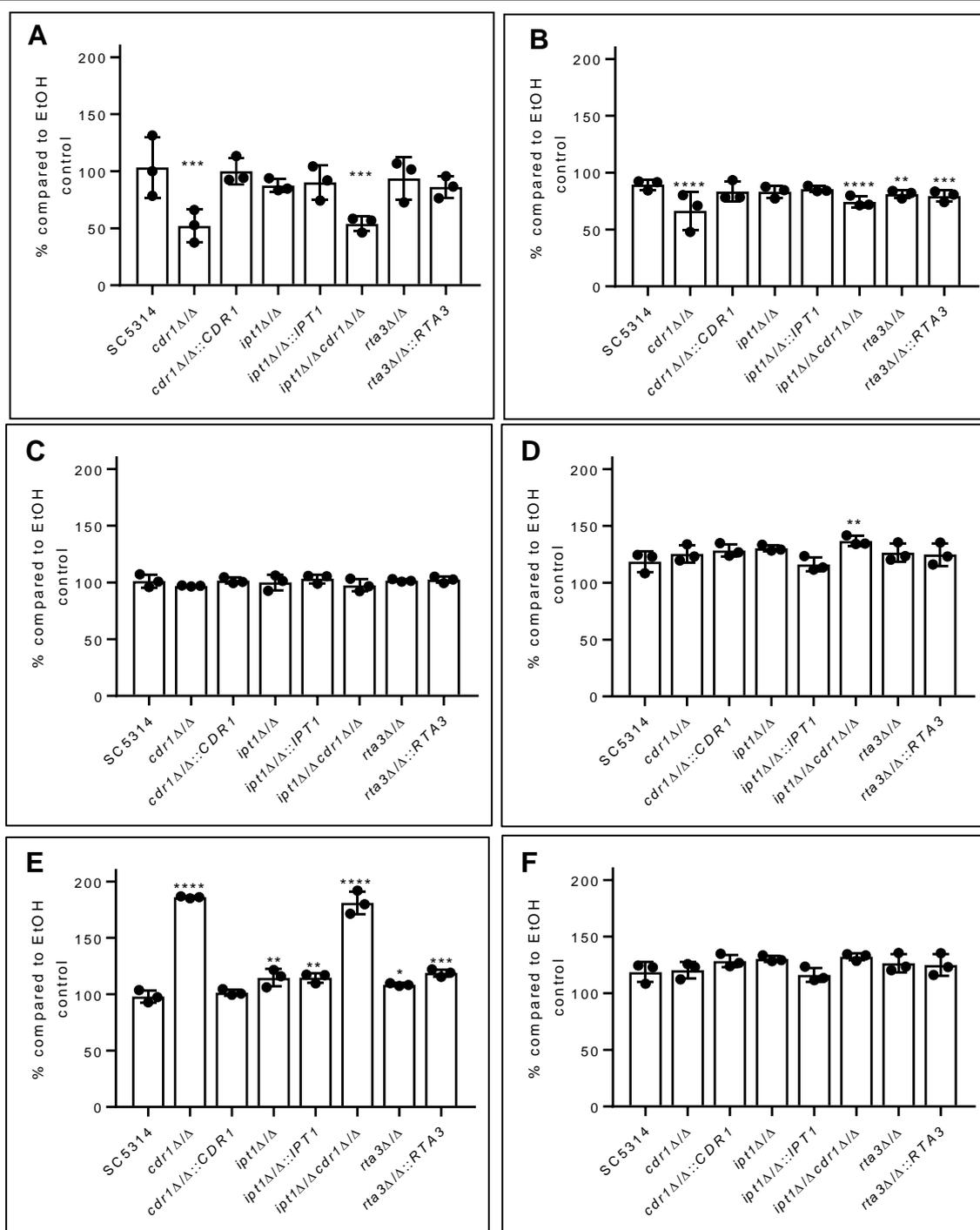
Selected genes were not only induced by AA, but also during co-incubation with *P. aeruginosa*, therefore, their role in the interaction of *C. albicans* with *P. aeruginosa* was evaluated. CFUs of *C. albicans*, determined in the presence of *P. aeruginosa*, indicated a small increase in *cdr1* $\Delta/\Delta$  compared to SC5314 (**Figure 4.2A**). However, although small variations in the CFUs of *P. aeruginosa* was seen, they were not significant (**Figure 4.2B**). When CFUs in polymicrobial biofilms were compared to single species biofilms (alleviating differences due to growth of mutants), an increase in *ipt1* $\Delta/\Delta$  (141,1%) compared to SC5314 was seen (**Figure 2C**). Deletion of *IPT1* has previously been reported to cause a deficit in filamentation (Prasad *et al.*, 2005). *Pseudomonas aeruginosa* preferentially binds to hyphal forms and cause physical cell



death (Hogan and Kolter, 2002), deletion of *IPT1* may provide a protective effect against physical killing of *P. aeruginosa*. Furthermore, *rta3Δ/Δ::RTA3* indicated a slight but significant increase in *C. albicans* CFU compared to SC5314 during co-incubation, indicating a possible off-target event during strain construction.

#### 4.4.3. Effect of deletion of *CDR1*, *IPT1* and *RTA3* on *C. albicans* biofilm formation in the presence of arachidonic acid

The effect of deletion of selected genes regarding biofilm formation in the presence of AA, including biofilm biomass and metabolic activity, was evaluated (**Figure 4.3**). A significant decrease (49.6 %) in biofilm biomass is observed for *cdr1Δ/Δ* in the presence of 0.1 mM AA compared to biofilms exposed to the ethanol control during early biofilm development (6 hours) (**Figure 4.3A**). This is confirmed with reduction in biofilm biomass with the double deletion of *CDR1* and *IPT1* (*ipt1Δ/Δcdr1Δ/Δ*, 47.9 %). Interestingly, this reduction is not found during evaluation of metabolic activity via the XTT assay (**Figure 4.3C**). Although the XTT assay is frequently used to evaluate differences across biofilms according to growth, metabolic activity is not equal to biofilm biomass and different strains may exhibit altered metabolic activity (Kuhn *et al.*, 2003). Therefore, metabolic activity was normalised against the biofilm biomass values obtained, to produce a ratio. With this procedure, a significant increase (89.9 %) in metabolic activity, normalized against biomass was seen for *cdr1Δ/Δ*, exposed to AA, (**Figure 4.3E**). This same phenomenon is observed for *ipt1Δ/Δcdr1Δ/Δ* (84.7 %).

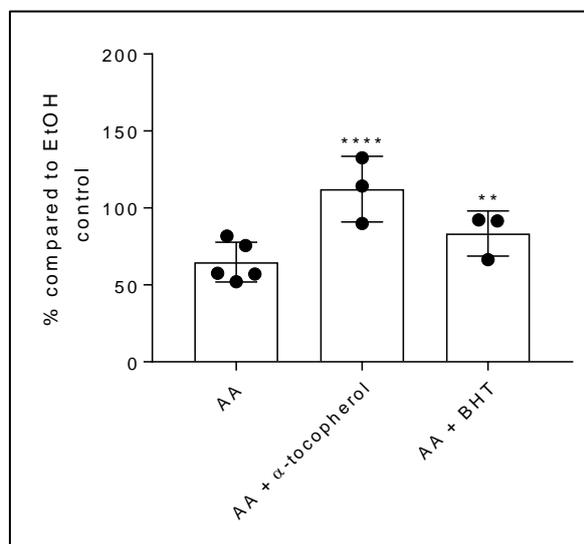


**Figure 4.3. Biomass (A and B) and metabolic activity (C - F) of selected *Candida albicans* mutants in the presence of 0.1 mM arachidonic acid (AA).** Percentage biofilm biomass after 6 h (A) and 48 h (B) of *C. albicans* selected mutants in the presence of 0.1 mM AA compared to ethanol control (0.076%). Percentage metabolic activity of 6h (C) and 48h (D) biofilms exposed to AA, compared to ethanol control. Ratio of metabolic activity / biomass of 6h (E) and 48h (F) biofilms exposed to AA, compared to ethanol control \*Significantly different from wild type (SC5314) (\*  $P < 0.05$ ; \*\*  $P < 0.005$ ; \*\*\*  $P < 0.0005$ ; \*\*\*\*  $P < 0.0001$ ).

The XTT assay relies on the reduction of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) to formazan (a coloured compound) by metabolic activity (Kuhn *et al.*, 2003). This reduction process is reliant on the action of mitochondrial succinoxidase, cytochrome P450 systems and flavoprotein oxidase. Furthermore, XTT may also be reduced by pro-oxidant-induced cellular responses (Berridge *et al.*, 1996). Naoi and co-workers (2010) observed increased XTT reduction by Madin-Darby bovine kidney cells, when challenged with a pro-oxidant (causing oxidative stress), linked to the activation of NADPH-regenerating enzymes. Polyunsaturated fatty acids, such as AA, are prone to peroxidation (Naseem *et al.*, 2015; Catalá, 2010; Li *et al.*, 2015; Yin *et al.*, 2011), forming peroxy radicals, promoting oxidative stress and damage within cells. Therefore, the reduced biomass and increased XTT values obtained from *cdr1Δ/Δ* exposed to AA, may indicate that increased lipid peroxidation may be taking place, causing oxidative stress (which may hinder growth and biomass production) and as well as increased reduction of XTT. This increased lipid peroxidation may imply that more AA is present in *cdr1Δ/Δ* cells compared to wild type *C. albicans*, as this increase in XTT reduction was not observed for SC5314 in the presence of AA (**Figure 4.3E**).

Cdr1p is a promiscuous transporter with proclivity for hydrophobic compounds (Prasad *et al.*, 2015; 2018). This may indicate that Cdr1p may export AA or AA containing lipids. The absence of Cdr1p in *cdr1Δ/Δ* may abrogate the ability of cells to export AA, increasing lipid peroxidation and consequently, sensitivity to AA. To test this, *cdr1Δ/Δ* was exposed to 0.1 mM AA as well as  $\alpha$ -tocopherol (vitamin E), a lipophilic reducing agent that localises to membranes and prevents lipid peroxidation (Li *et al.*, 2015; Yehye *et al.*, 2015; Yin *et al.*, 2011). In addition, the synthetic antioxidant, butylated hydroxytoluene (BHT), acting as an inhibitor of free radical-chain reactions, was also included. Notably, due to the reducing capabilities of  $\alpha$ -tocopherol, it is reported to interfere with tetrazolium salt assays such as the XTT assay, by reducing XTT to formazan in the absence of metabolically active cells (Lim *et al.*, 2015). Therefore, the effect of supplementation with antioxidants was only measured with the crystal violet assay (biofilm biomass) to determine if  $\alpha$ -tocopherol and BHT could rescue the reduction in biofilm biomass of *cdr1Δ/Δ* exposed to AA (**Figure 4.4**). The addition of both  $\alpha$ -tocopherol and BHT reduced the toxic effect of *cdr1Δ/Δ* exposed to AA, indicating that the reduction in biomass of *cdr1Δ/Δ* may be due to oxidative stress. The

effect of  $\alpha$ -tocopherol was more pronounced. As  $\alpha$ -tocopherol localises to membranes, it may indicate that the oxidative stress of *cdr1* $\Delta/\Delta$  is due to lipid peroxidation in the membranes, compared to the more general antioxidant properties of BHT (Li *et al.*, 2015; Yehye *et al.*, 2015; Yin *et al.*, 2011).



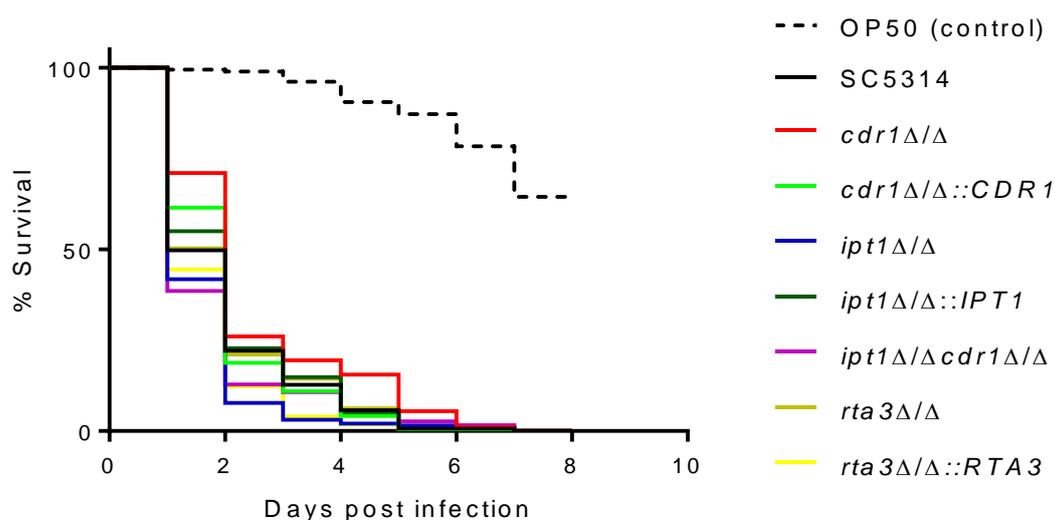
**Figure 4.4. Biomass of *Candida albicans* *cdr1* $\Delta/\Delta$  in the presence of 0.1 mM arachidonic acid (AA) and antioxidants.**

Percentage biofilm biomass after 6 h of *cdr1* $\Delta/\Delta$  in the presence of 0.1 mM AA compared to ethanol control (0.076%). In addition, 0.5 mM  $\alpha$ -tocopherol and 0.045 mM butylated hydroxytoluene (BHT) were added respectively. \*Significantly different from *cdr1* $\Delta/\Delta$  with only AA (\*  $P < 0.05$ ; \*\*  $P < 0.005$ ; \*\*\*  $P < 0.0005$ ; \*\*\*\*  $P < 0.0001$ ).

It is of note to mention that the effects observed for *cdr1* $\Delta/\Delta$  exposed to AA was observed after 6 hours, whereas these effects dissipated and were less pronounced after 48 hours (Figure 4.3 B, D and F). This suggests that 0.1 mM may have an inhibitory effect at early biofilm development, but prolonged incubation may enable the metabolism of AA and consequently, the relief of its toxic effects. Furthermore, deletion of *IPT1* or *RTA3* did not produce notable effects on *C. albicans* biomass or metabolic activity in the presence of AA at early (6 hours) or later (48 hours) time points.

#### 4.4.4. Effect of deletion of *CDR1*, *IPT1* and *RTA3* on virulence of *C. albicans*

To determine the effect of *CDR1*, *IPT1* and *RTA3* on the virulence of *C. albicans*, a *Caenorhabditis elegans* infection model was also used. Infection of *C. elegans* indicated small but significant reduction in virulence for *cdr1* $\Delta/\Delta$  and an increase in virulence for *ipt1* $\Delta/\Delta$  (Figure 4.5). Importantly, deletion of *IPT1* has previously been shown to reduce innate immune induction (Rouabhia *et al.*, 2011). This increase may be due to decreased immune induction by deletion of *IPT1* via decreased antigen



Condition	Median lifespan (days)	S. E.	Days to reach 50% survival	Log-rank test <i>P</i> -value (Bonferroni corrected)
OP50	6,51	0,08		0,0000*
SC5314	1,92	0,09	1,00	1,0000
<i>cdr1Δ/Δ</i>	2,39	0,11	2,00	0,0014*
<i>cdr1Δ/Δ::CDR1</i>	1,96	0,08	2,00	1,0000
<i>ipt1Δ/Δ</i>	1,57	0,07	1,00	0,0274*
<i>ipt1Δ/Δ::IPT1</i>	1,98	0,09	2,00	1,0000
<i>ipt1Δ/Δcdr1Δ/Δ</i>	1,72	0,09	1,00	1,0000
<i>rta3Δ/Δ</i>	1,93	0,09	2,00	1,0000
<i>rta3Δ/Δ::RTA3</i>	1,63	0,06	2,00	0,0728

\* Significantly different from SC5314

**Figure 4.5. Survival of *Caenorhabditis elegans* infected with *Candida albicans* mutants.**

Percentage survival of *C. elegans* infected with *C. albicans* wild type (SC5314) or homozygous mutants of *CDR1* (*cdr1Δ/Δ*), *IPT1* (*ipt1Δ/Δ*), *RTA3* (*rta3Δ/Δ*) as well as a double deletion mutant of *IPT1* and *CDR1*. Add-backs of wild-type genes are included (*cdr1Δ/Δ::CDR1*, *ipt1Δ/Δ::IPT1* and *rta3Δ/Δ::RTA3* respectively). Control consists of *C. elegans* allowed to feed on *Escherichia coli* OP50 (OP50). The table represents median lifespan with standard error (S. E.) along with days to reach 50% mortality. *P*-values are included for the Log-rank test for overall differences in survival. \*Significantly different from wild type *C. albicans* (SC5314).

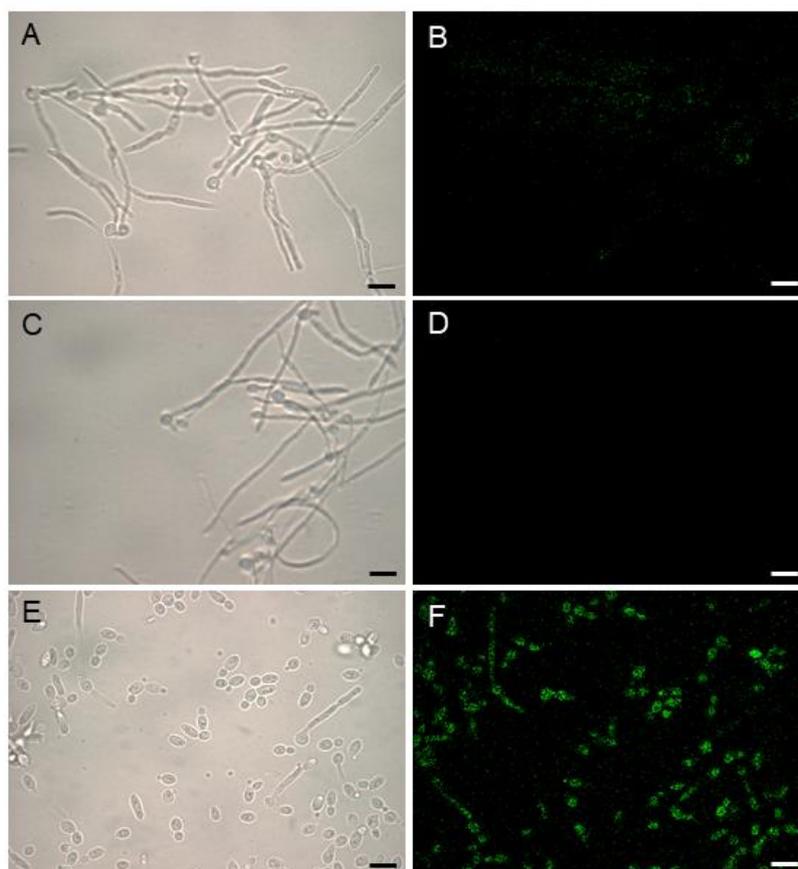
presentation. This may hamper the ability of *C. elegans* to recognise *C. albicans* and clear infection. Rouabhia and co-workers (2011) indicated that *ipt1Δ/Δ* exhibits defective toll like receptor (TLR) activation and expression of TLR genes in engineered human oral mucosa tissue. In addition, a reduction in the production of antimicrobial peptides is observed. As discussed in *Chapter 3*, the strain of *C. elegans* used is immunocompromised, with a deletion of *SEK-1*, a mitogen-activated protein kinase

(MAPK) kinase (Breger *et al.*, 2007). This cascade regulates activation of antimicrobial peptides and expression of pro-inflammatory cytokines (Elkabti *et al.*, 2018). In addition to MAPK, *C. elegans* relies on transforming growth factor- $\beta$  pathway, insulin signalling pathway and toll pathway (TLR pathway) for protection from pathogens. The current results may indicate that the deletion of *IPT1* may hamper innate immune recognition of *C. albicans* by *C. elegans*, even in the absence of MAPK. Furthermore, double deletion of *IPT1* and *CDR1* resulted in wild type virulence, which may be an additive effect. In addition, deletion of *RTA3* decreases biofilm formation *in vivo* in a rat catheter model (Srivastava *et al.*, 2017), however no influence on virulence is seen during *C. elegans* infection. To the author's knowledge, the effect of deletion of *CDR1* on virulence has not been evaluated previously. Due to the decrease in *cdr1* $\Delta/\Delta$  in the *C. elegans* infection model used, further research is required if *CDR1* may participate in virulence mammalian models.

#### 4.4.5. Arachidonic acid increases Cdr1p in a dose dependent manner

The data obtained regarding the effect of deletion of *CDR1* on biofilm formation and virulence indicate that, out of the three genes investigated, it may play the biggest role in the interaction of *C. albicans* with AA. This prompted further investigation into *CDR1*. Cdr1p is an ATP-binding cassette (ABC) transporter that transports phospholipids in an in-to-out direction (Prasad *et al.*, 1995; Smitri *et al.*, 2002). It is a ~170kDa protein consisting of two halves, each consisting of a transmembrane domain preceded by a nucleotide binding domain (Prasad *et al.*, 2015). The transmembrane domains are responsible for the binding of substrates, whereas the nucleotide binding domains are responsible for ATP hydrolysis, powering the efflux of these substrates. The substrates of Cdr1p is not limited to phospholipids and generally include substrates with high hydrophobicity index, including lipids, antifungals antibiotics and fluorescent dyes.

Although there is an observed increase in *CDR1* expression in the presence of AA, this may not reflect in an increase in Cdr1p. To evaluate this, we obtained a GFP-fused *CDR1* strain (AsCa1; Szczepaniak *et al.*, 2015) and evaluated the fluorescence of Cdr1-GFP in the presence of AA. As can be seen in **Figure 4.6**, low fluorescence is observed with control biofilms exposed only to ethanol (0.076 %). No observable difference in fluorescence is observed when 0.1 mM AA is added. Therefore, although *CDR1* mRNA is increased with exposure to 0.1 mM AA, Cdr1p is not increased at the



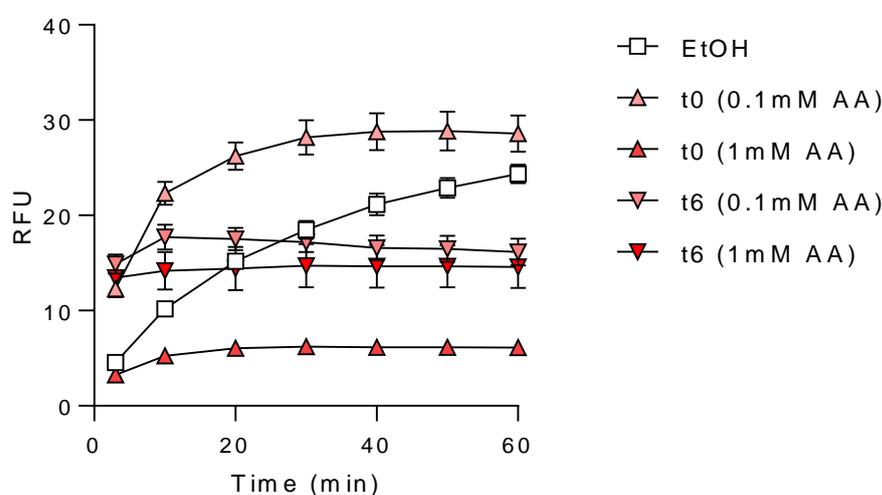
**Figure 4.6.** Fluorescence of *Candida albicans* CDR1-GFP (strain AsCa1) with exposure to ethanol control, 0.1 mM and 1 mM arachidonic acid (AA). **A**, **C** and **E** represent light micrographs, whereas **B**, **D** and **F** are corresponding fluorescent micrographs. **A** and **B** – AsCa1 exposed to ethanol; **C** and **D** – AsCa1 exposed to 0.1 mM AA; **E** and **F** – AsCa1 exposed to 1 mM AA. Scale bars represent 10  $\mu$ m.

time of measurement (6 hours). When the concentration is increased to 1 mM, an observable increase in fluorescence is seen, indicating an increase in Cdr1p. The lack of increased fluorescence of AsCa1 in the presence of 0.1 mM may indicate that AA may be metabolised, and the effect lost after 6 hours, whereas an adequate amount of AA is still present after 6 hours when treated with 1 mM AA.

#### 4.4.6. Arachidonic reduces ABC efflux activity in a dose dependent manner

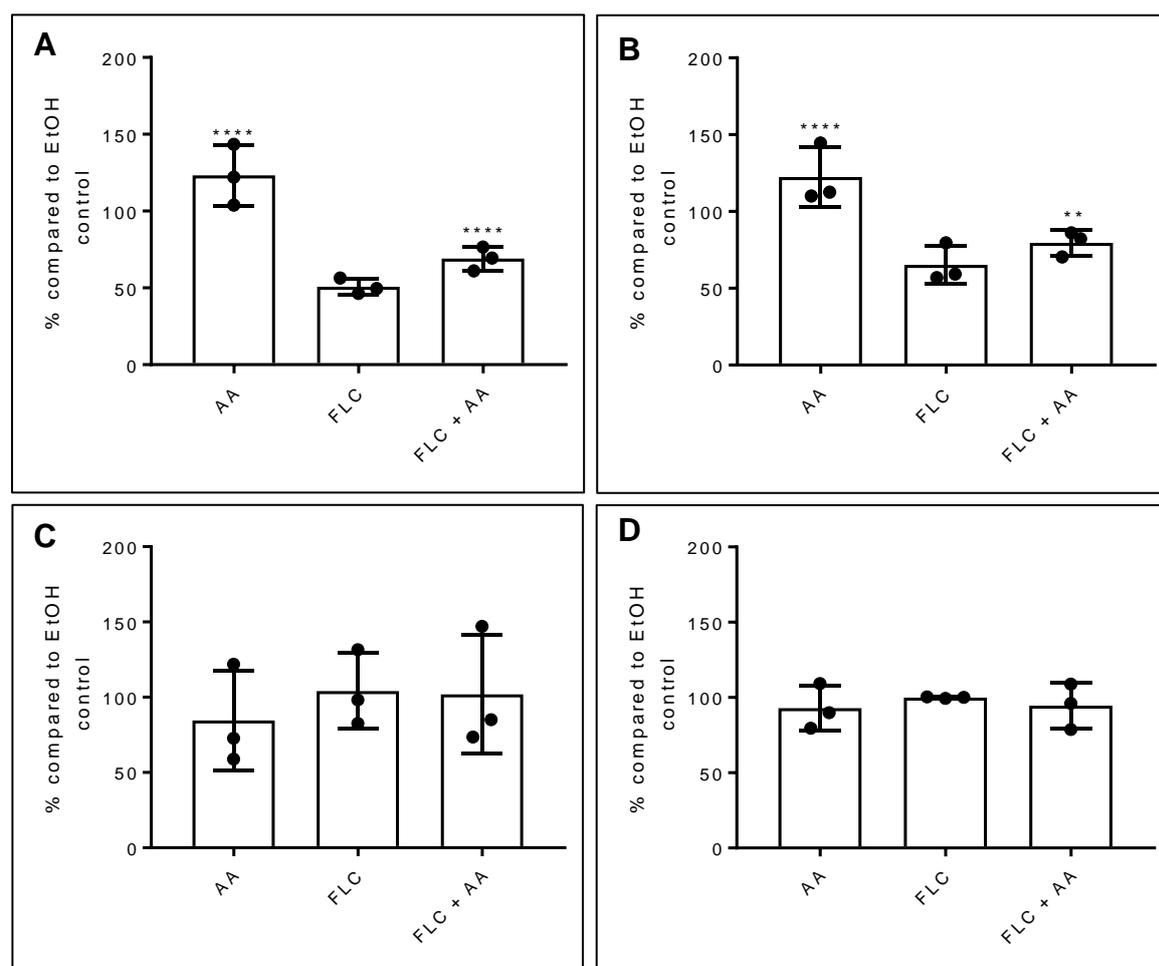
The increase in *CDR1* expression and increase in Cdr1p was surprising, as 1 mM AA has been reported to increase the susceptibility to clotrimazole (Ells *et al.*, 2009). The increased susceptibility would suggest a possible decrease in *CDR1* expression and Cdr1p. Therefore, we determined if the activity of Cdr1p is affected. To evaluate this,

the efflux of rhodamine 6G (R6G) was followed in the presence of AA (Maesaki *et al.*, 1999). This process involves passive diffusion of R6G into de-energized cells, followed by extrusion of R6G via the energy-dependent efflux activity facilitated by exposure of de-energized cells to glucose (Kundu *et al.*, 2019). Efflux of R6G is mediated by ABC transporters, of which Cdr1p is the main transporter (Tsao *et al.*, 2009). Therefore, this assay can be used to determine the function of Cdr1p. The influence of AA on R6G-efflux was evaluated in two ways. Firstly, six-hour biofilms were allowed to form in the presence of AA (added at time zero) and secondly, biofilms (6h) were allowed to form (in the absence of AA) and were treated with AA for 1 hour prior to measurement of R6G-efflux. This would indicate if AA is required during biofilm formation to elicit the effect on Cdr1p, or if short-term treatment could elicit this effect. As can be seen in **Figure 4.7**, the addition of 0.1 mM AA at time-zero (t0) caused an increase in efflux compared to control biofilms. However, when 0.1 mM AA was added after biofilm formation (for 1 hour), a significant inhibition of efflux was observed. Strikingly, the addition of 1 mM AA at t0 or 6h both resulted in significant inhibition of drug efflux activity. This indicates that AA is capable of reducing Cdr1p activity.



**Figure 4.7. Rhodamine 6G-efflux of *Candida albicans* in the presence of ethanol, 0.1 mM or 1 mM arachidonic acid (AA).** Y-axis represents relative fluorescent units (excitation/emission: 530/590 nm). Control – biofilms exposed to ethanol vector; t0 0.1mM – 0.1 mM AA added prior to biofilm formation; t0 1mM – 1 mM AA added prior to biofilm formation; t6 0.1mM – 0.1 mM AA added after biofilm formation (6 hours); t6 1mM – 1 mM AA added after biofilm formation (6 hours).

At high concentrations of AA (1 mM), an increase in antifungal susceptibility is observed (Ells *et al.*, 2009). To determine if 0.1 mM AA could also affect susceptibility to antifungal agents, *C. albicans* biofilms were exposed 0.1 mM AA as well as 1  $\mu$ g/mL fluconazole (FLC) (**Figure 4.8**). Similar to the procedure of R6G-efflux, AA and FLC was added prior to biofilm formation and incubated for 6 hours or added for 1 hour after biofilm formation. Similarly to the increased R6G efflux, an increased resistance

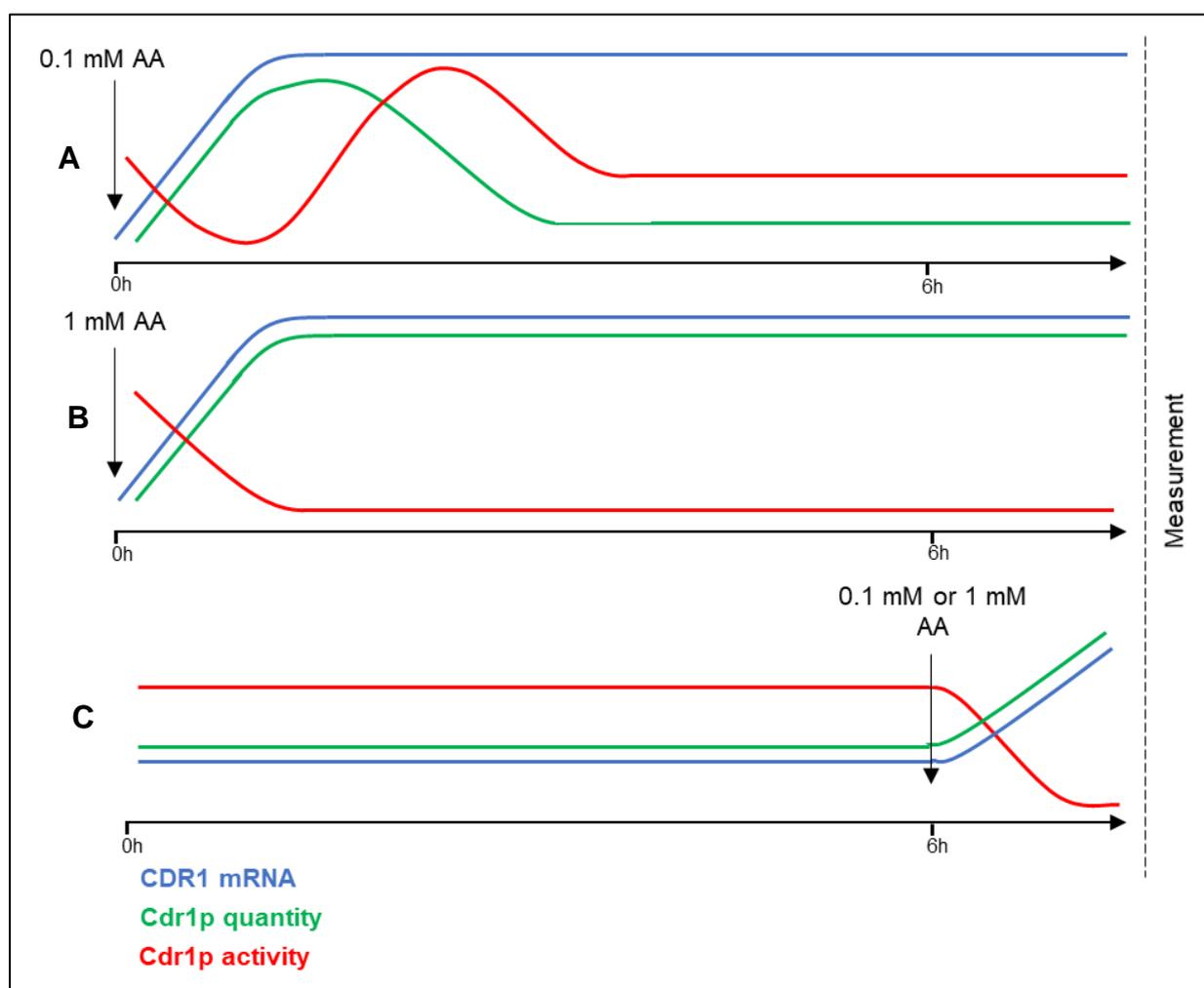


**Figure 4.8. Biomass and metabolic activity of *Candida albicans* wild type in the presence of 0.1 mM arachidonic acid (AA) and 1  $\mu$ g/mL fluconazole (FLC).** Percentage biofilm biomass (**A**) and metabolic activity (**B**) of *C. albicans* biofilms with AA and FLC added prior to biofilm formation and incubated for 6 hours compared to ethanol control. **C** and **D** – Percentage biofilm biomass and metabolic activity of biofilms treated with AA and FLC for 1 hour after

to FLC was observed for *C. albicans* biofilms grown in the presence of FLC and 0.1 mM AA compared to FLC alone for 6h (**Figure 4.8A** and **4.8B**), indicating increased

activity of Cdr1p under these conditions, and correlating with the increased expression of *CDR1*.

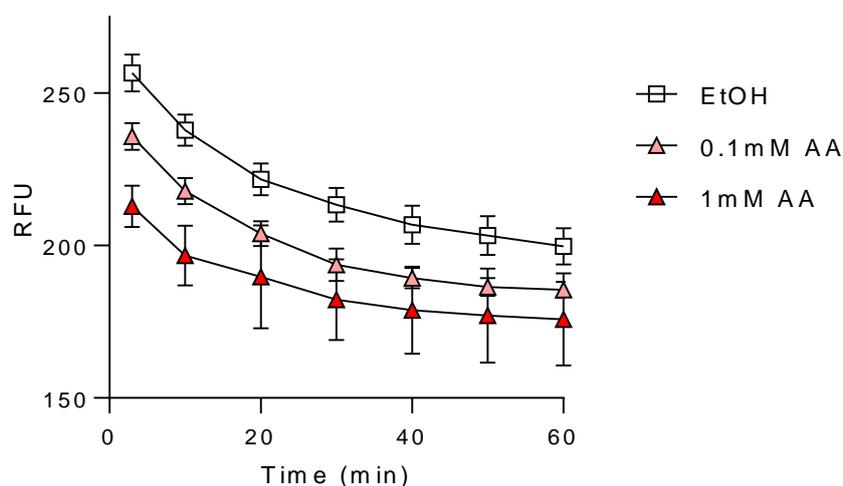
Interestingly, although short term exposure of biofilms to FLC (alone or in the presence of 0.1 mM AA) is not enough to cause a change in biomass or metabolic activity (**Figure 4.8C** and **4.8D**), due to its fungistatic nature, exposure of biofilms to 0.1 mM AA did reduce R6G efflux (**Figure 4.7**), indicating that exposure of *C. albicans* biofilms to 0.1 mM AA for 1 h may initially disrupt efflux of compounds such as R6G and FLC. We can thus hypothesise that the following may happen during exposure to 0.1 mM AA. Firstly, *CDR1* expression and Cdr1p translation is induced. However, the function of Cdr1p is diminished. As time progresses and AA is metabolised, the inhibition of Cdr1p-function is lifted and an increase in efflux of xenobiotic compounds such as R6G or FLC is seen (**Figure 4.9A**). As AA may not be at high enough concentration to elicit further effects, the induction of Cdr1p translation is reduced, however, not to control levels (higher R6G-efflux). An alternative possibility is that Cdr1p is not stable and may be degraded. Notably, the half-life Cdr1p is approximately 90 minutes (Manoharlal *et al.*, 2008), while the half-life of its mRNA is approximately 60 min (increased to >180 min in resistant isolates). After 6 hours *CDR1* expression is still increased, indicating that the stimulus for induction of *CDR1* expression is still present at the time of measurement of mRNA (6 hours), although Cdr1p concentration is no longer elevated. If a high concentration (1 mM) AA is added prior to biofilm formation (**Figure 4.9B**), an increase in Cdr1p is still observable after 6 hours (**Figure 4.6**). In addition, the inhibitory effect of AA is still apparent (**Figure 4.7**). This indicates that enough AA is still present to elicit increased translational and inhibitory effects on Cdr1p. The lower concentration of 0.1 mM AA was adequate to elicit the same inhibitory effect on Cdr1p function when *C. albicans* was only exposed to it for 1 hour (**Figure 4.9C**) (not enough time for metabolism of AA and accompanied reduction in concentration). The addition of AA prior to biofilm formation may not be physiologically relevant, as AA may be continuously released upon stimulation of immune cells. Therefore, adequate amounts of AA may be present during biofilm growth to elicit the inhibitory effects on Cdr1p. Therefore, we proceeded to evaluate the inhibitory effect of AA on Cdr1p.



**Figure 4.9.** Schematic of *CDR1* mRNA (blue), Cdr1p (green) and its function (red) over time with exposure to arachidonic acid (AA). **A** – 0.1 mM AA added prior to biofilm formation. **B** – 1 mM AA added prior to biofilm formation. **C** – 0.1 mM or 1 mM AA added after biofilm formation for 1 hour. Measurements were taken after 6 hours of biofilm formation (A and B), or 6-hour biofilms were exposed to AA for 1 hour.

The reason for induction of *CDR1* expression, as well as the mechanism whereby AA inhibits Cdr1p function, is unknown. The absence of effect of 0.1 mM on metabolic activity after one-hour of exposure suggests that this is not due to a growth defect. Evaluation of conjugated linoleic acid's effect on *C. albicans* indicate that PUFAs may impair function of membrane proteins through mislocalisation in membrane microdomains (Shareck *et al.*, 2011). Prior to the R6G-efflux assay, R6G in the supernatant was measured over one hour after addition of the R6G. This would give an indication of the speed that R6G diffuses into cells. Notably, increased R6G-influx

was observed when biofilms were treated with AA prior to biofilm formation, suggesting increased membrane permeability (**Figure 10**). Consequently, it was suspected that changes in membrane permeability would affect membrane organisation. This effect of AA was dose dependent. Biofilms treated after 6 hours were not included as AA is added after R6G influx.



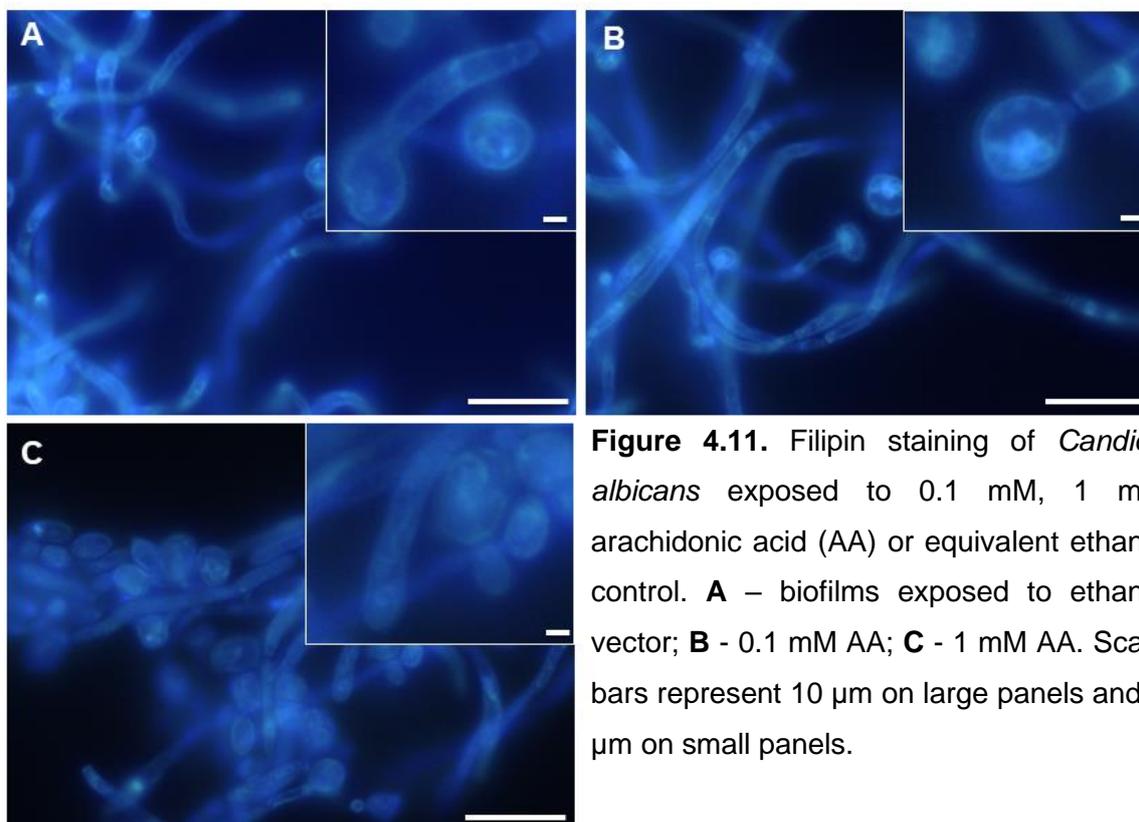
**Figure 4.10. Rhodamine 6G-influx of *Candida albicans* in the presence of ethanol, 0.1 mM or 1 mM arachidonic acid (AA) added prior to biofilm formation.** Y-axis represents relative fluorescent units (excitation/emission: 530/590 nm). Control – biofilms exposed to ethanol vector. In addition, 0.1 mM and 1 mM AA was added prior to biofilm formation and incubated for 6 hours.

#### 4.4.7. Possible mislocalisation of Cdr1p in the presence of arachidonic acid

Membrane microdomains, sometimes referred to as lipid rafts, are domains within the plasma membrane rich in sterols and sphingolipids (Mollinedo, 2012). These domains are characterised as detergent-resistant islands in the plasma membrane rich in proteins that are involved in processes including lipid metabolism, cell wall biogenesis, electron transport and ATP-synthesis (Insenser *et al.*, 2006; Mollinedo, 2012). Furthermore, Cdr1p localises to these lipid rafts and perturbations of these lipid raft structures affect the position and functionality of this protein (Pasrija *et al.*, 2005). The reduced functionality of Cdr1p due to exposure to AA may indicate the mislocalisation of Cdr1p and potential disruption of lipid raft formation. To evaluate this, filipin staining of biofilms exposed to AA was done.

#### 4.3.7.1. Filipin-staining of *C. albicans* exposed to arachidonic acid

Filipin is frequently used to visualise lipid raft formation due to its binding to ergosterol which can be visualised through fluorescence (Ghannoum and Rice, 1999; Martin and Konopka, 2004). Biofilms of *C. albicans* were prepared in the presence and absence of AA and the sterols stained with filipin. Localised fluorescence of filipin to the periphery of cells (plasma membranes), junctions between hyphae as well as intracellular organelle-membranes can be seen in biofilms in the absence of AA (**Figure 4.11A**) as reported previously (Martin and Konopka, 2004). The addition of 0.1 mM did not observably affect the localised fluorescence of filipin (**Figure 4.11B**). However, dispersed fluorescence inside *C. albicans* cells exposed to 1 mM AA was seen with less localisation of filipin staining to the cell periphery (**Figure 4.11C**). This may indicate the malformation of lipid rafts of *C. albicans* exposed to high concentrations of AA. This correlates well with the decreased R6G-efflux observed when 1 mM AA was added prior to biofilm formation. To confirm the phenomenon of mislocalisation due to AA, the plasma membrane H<sup>+</sup>-ATPase, Pma1p (Monk *et al.*, 1991) was C-terminally fused with a yeast optimised green fluorescent protein (GFP) (Granger, 2018).

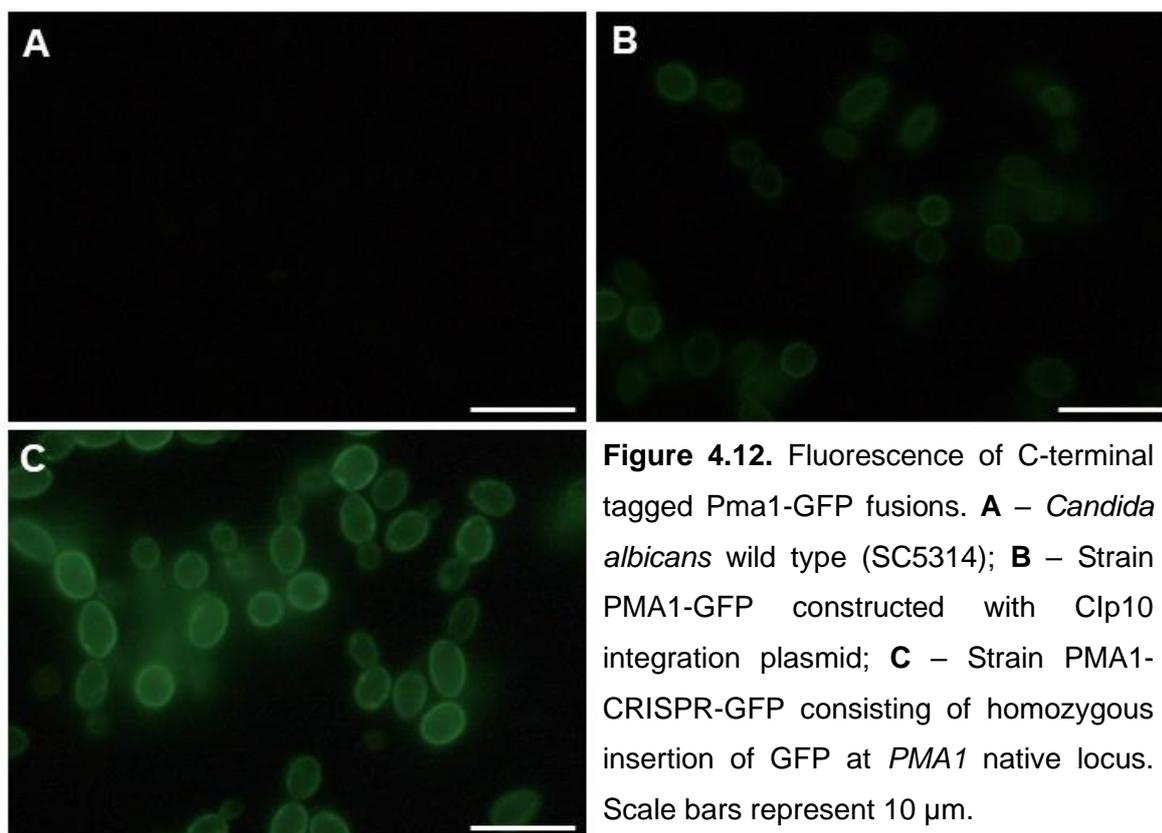


**Figure 4.11.** Filipin staining of *Candida albicans* exposed to 0.1 mM, 1 mM arachidonic acid (AA) or equivalent ethanol control. **A** – biofilms exposed to ethanol vector; **B** - 0.1 mM AA; **C** - 1 mM AA. Scale bars represent 10  $\mu$ m on large panels and 1  $\mu$ m on small panels.

#### 4.4.7.2. Construction of Pma1p-GFP fusions to evaluate lipid raft formation

Pma1p is frequently used as a marker for lipid rafts (Insenser *et al.*, 2006; Martin and Konopka, 2004) as it is highly expressed and localised to lipid rafts. Furthermore, malformation of lipid rafts causes delocalisation of Pma1p from the plasma membrane to the cytosol and intracellular compartments such as vacuoles (Suchodolski and Krasowska, 2019). To construct a C-terminal fusion of GFP to Pma1p, the Clp10 integration plasmid (Murad *et al.*, 2000) was used (see Section 4.3.13.1). This would allow for ectopic expression of a single copy of GFP-tagged Pma1p expressed from the *RP10* locus in *C. albicans*. Construction of PMA1-GFP was successful, however, when fluorescence was evaluated, low fluorescence was observed (**Figure 4.12B**). This may be as a result of defective initiation of transcription, due to incorrect cloning of the promoter region, or incorrect folding of the GFP-tagged Pma1p protein. To correct this, GFP was homozygously integrated at the native *PMA1* locus using CRISPR-Cas9 (Nguyen *et al.*, 2017) with a 13 amino acid linker (Janke *et al.*, 2004) to facilitate folding of GFP.

Integration of GFP at the native locus of *PMA1* was successful with bright green fluorescence visualised (**Figure 4.12C**). However, a growth defect and inability to form biofilms was seen with PMA1-CRISPR-GFP indicating an off-target modification during strain construction. Therefore, this strain was inadequate for use in determining the effect of AA on *C. albicans* biofilms. Due to this, another method was attempted to determine if the reduced functionality of Cdr1p is due to mislocalisation.

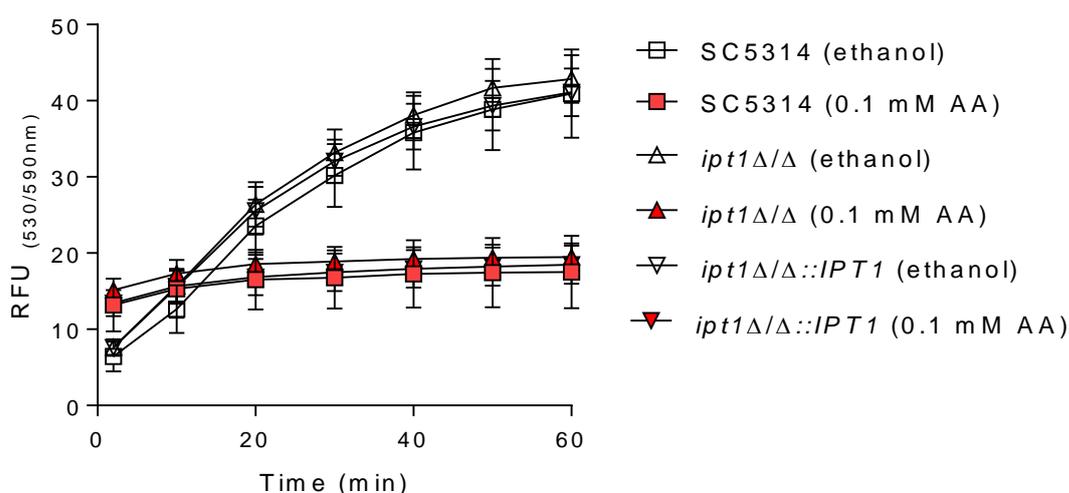


**Figure 4.12.** Fluorescence of C-terminal tagged Pma1-GFP fusions. **A** – *Candida albicans* wild type (SC5314); **B** – Strain PMA1-GFP constructed with Clp10 integration plasmid; **C** – Strain PMA1-CRISPR-GFP consisting of homozygous insertion of GFP at *PMA1* native locus. Scale bars represent 10  $\mu\text{m}$ .

#### 4.4.7.3. The use of *ipt1* $\Delta/\Delta$ in evaluating mislocalisation of *Cdr1p*

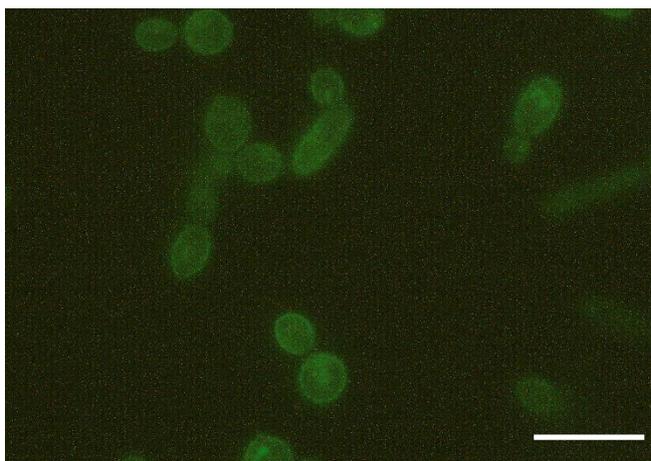
The loss of *IPT1* has been linked to mislocalisation of *Cdr1p* as *Ipt1p* is required for proper formation of lipid rafts (Prasad *et al.*, 2005). The constructed *ipt1* $\Delta/\Delta$  strain did not show significant alterations in the presence of AA in terms of biomass and metabolic activity compared to wild type biofilms (**Figure 4.3**). This was also seen with *ipt1* $\Delta/\Delta$ *cdr1* $\Delta/\Delta$  compared to *cdr1* $\Delta/\Delta$ . To determine if the decrease in functionality of ABC efflux pumps may be due to mislocalisation, we determined efflux of R6G of *ipt1* $\Delta/\Delta$  (**Figure 4.13**). If the effect of AA on *Cdr1p* was primarily due to mislocalisation, it would be expected that *ipt1* $\Delta/\Delta$  would present a similar profile as those biofilms treated with AA. However, this was not observed, with *ipt1* $\Delta/\Delta$  showing a decrease in R6G efflux in the presence of AA, similar to SC5314. Therefore, it can be speculated that the effect of AA on *Cdr1p* may not primarily be due to mislocalisation. However, deletion of *IPT1* is known to cause decreased R6G efflux through impairment of *Cdr1p*-function (Pasrija *et al.*, 2005). In the present study, *ipt1* $\Delta/\Delta$  efflux activity was not impaired compared to wild type *C. albicans*. However, the R6G assay used in this study was done on early phase biofilms, whereas planktonic cells were used by Pasrija

and co-workers (2005). This is of importance as the main product of activity of Ipt1p ( $M(IP)_2C$ ) is nearly undetectable in early (6h) and late phase (48h) biofilms compared to high amounts detectable in planktonic cells (Lattif *et al.*, 2011). Therefore, the effect of *IPT1* deletion on R6G export may be less pronounced in biofilms compared to planktonic cells. Due to this, the use of *ipt1Δ/Δ* may not provide enough information on the localisation of Cdr1p in biofilms cells.



**Figure 4.13.** Rhodamine 6G-efflux of *Candida albicans* in the presence of ethanol or 0.1 mM of arachidonic acid (AA). Y-axis represents relative fluorescent units (excitation/emission: 530/590 nm) and X-axis represents time in minutes. This was done for *C. albicans* wild-type (SC5314), the homozygous deletion of *IPT1* (*ipt1Δ/Δ*) and the add-back of *IPT1* (*ipt1Δ/Δ::IPT1*).

In a last attempt to determine if localisation of Cdr1p is affected, the strain AsCa1 (Cr1p-GFP; Szczepaniak *et al.*, 2015) was exposed to 1 mM AA (**Figure 4.14**). Due to the low fluorescence of AsCa1 in the control condition (without AA) in **Figure 4.6**, we were unable to compare the localisation of AsCa1 between the ethanol control and treatment with 1 mM AA. However, localised fluorescence to the cell periphery can be seen in AsCa1 treated with AA, which may indicate that correct localisation of Cdr1p may still take place. A *C. albicans* strain that constitutively expresses Cdr1p fused to GFP may provide valuable insight and may be attempted in future research.



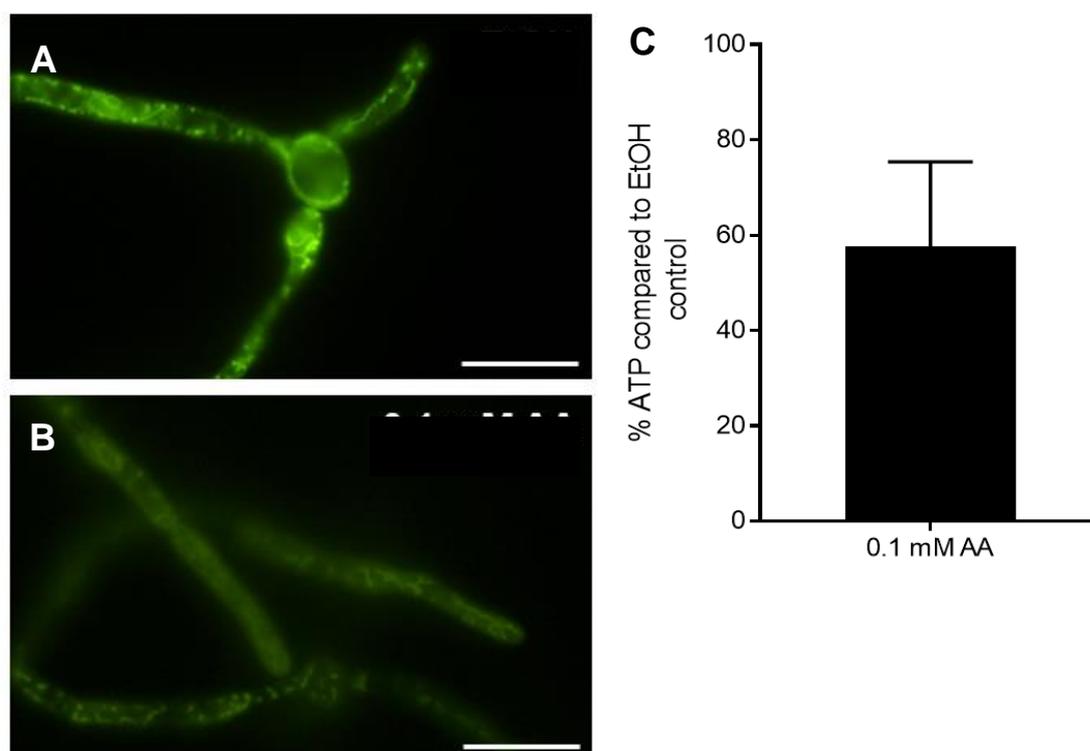
**Figure 4.14.** Fluorescence of *Candida albicans* AsCa1 in the presence of 1 mM arachidonic acid indicating visible localisation of fluorescence at cell periphery and intracellular organelles. Scale bar indicates 10  $\mu$ m.

#### 4.4.8. Arachidonic acid induces mitochondrial dysfunction

Changes in sterol levels and sphingolipid levels can affect Cdr1p functionality and alter antifungal resistance (Pasrija *et al.*, 2005; Vu *et al.*, 2019). In addition, alterations in phospholipids also affect Cdr1p function (Kundu *et al.*, 2019). Notably, unlike alterations in sterols and sphingolipids, alterations in phospholipids do not affect localisation of Cdr1p. Interestingly, alterations in phospholipids can result in mitochondrial dysfunction (Baker *et al.*, 2016; Böttinger *et al.*, 2012; Kundu *et al.*, 2019). As AA can be incorporated into yeast-phospholipids and increase the unsaturation index of phospholipids (Ells *et al.*, 2009; Kock and Ratledge, 1993), this may subsequently affect mitochondrial activity. Previous studies have reported a link between mitochondrial activity and Cdr1p function (Thomas *et al.*, 2013). Thus, as alterations in phospholipids can affect mitochondrial oxidative phosphorylation and ATP production (Kundu *et al.*, 2019), this may be a mechanism by which AA affects the activity of Cdr1p.

To evaluate the functionality of the mitochondria in the presence of AA, the DiOC<sub>6</sub>(3) stain was used (**Figure 4.15**). DiOC<sub>6</sub>(3) labels intracellular organelles such as the mitochondria and endoplasmic reticulum, however, at low concentrations it can be used to determine mitochondrial depolarisation (Korchak *et al.*, 1982; Yun and Lee, 2016). Control biofilms, treated with ethanol, exhibited strong localised fluorescence of tubular structures inside the cell indicating healthy mitochondria (**Figure 4.15A**). However, when treated with 0.1 mM AA, a decrease in localised fluorescence is seen,

indicating possible mitochondrial depolarisation (**Figure 4.15B**). Interestingly, smaller spots of fluorescence are seen, potentially indicating enhanced fission of the mitochondria. These fluorescent micrographs indicate a potential defect in mitochondrial function in the presence of AA that could decrease the functionality of Cdr1p (Calderone *et al.*, 2015).



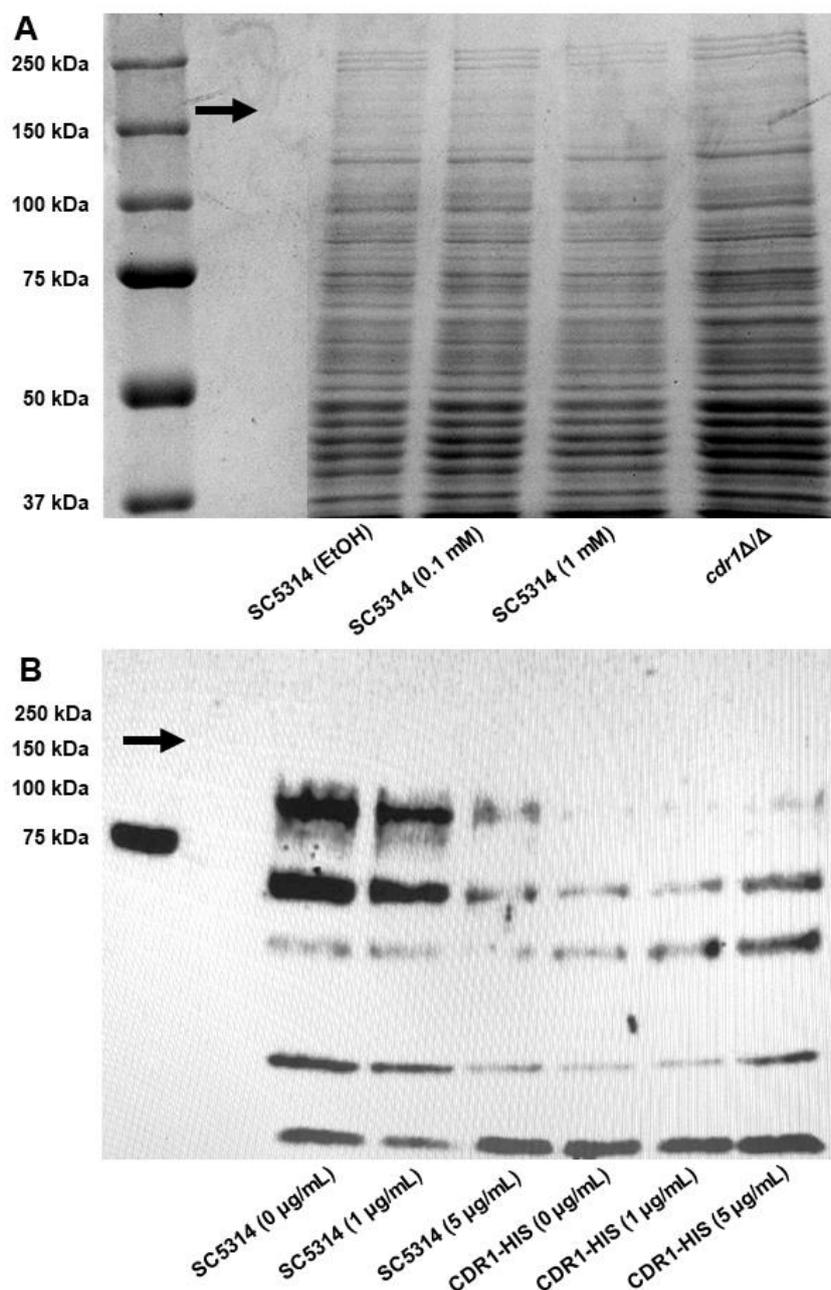
**Figure 4.15. Effect of 0.1 mM arachidonic acid (AA) on *Candida albicans* mitochondrial function.** **A** - DiOC<sub>6</sub>(3) staining of mitochondria of *C. albicans* biofilms exposed to ethanol (0.076%), or 0.1 mM AA (**B**). Scale bars indicate 10  $\mu$ m. **C** – ATP production by *C. albicans* biofilms exposed to 0.1 mM AA.

To evaluate if ATP synthesis is affected by the addition of AA, ATP production was measured through an ATP-bioluminescent assay. Confirming results seen with the DiOC<sub>6</sub>(3) staining, ATP production was decreased in biofilms treated with 0.1 mM AA (**Figure 4.15C**). The reduction in functionality of Cdr1p may then be, in part, due to mitochondrial membrane depolarisation and decrease in ATP production.

Cdr1p is not only dependent on ATP for function but is subject to post-translational modification through phosphorylation of the N-terminal extension (Tsao *et al.*, 2016). Due to the decreased functionality of mitochondria and reduced ATP generation

imposed by AA, phosphorylation of Cdr1p may also be affected, due to the dependence of phosphorylation on high energy phosphate-donating molecules. To determine if we could identify Cdr1p on an SDS-PAGE and subject it to LC-MS/MS to determine phosphorylation status of the protein (Tsao *et al.*, 2016), *C. albicans* biofilms were prepared in the presence of AA. Proteins were extracted and separated via SDS-PAGE using a 7.5% polyacrylamide gel. In addition, *cdr1Δ/Δ* was included to potentially identify Cdr1p in wild type samples due to its absence in *cdr1Δ/Δ*. However, Cdr1p could not be identified through this method (**Figure 4.16A**). This may be due to the low expression of Cdr1p, or due to the presence of additional proteins of similar size (Cdr1p is ~170 kDa), such as Cdr2p (168 kDa) (The Uniprot Consortium).

To combat this, we attempted to insert a polyhistidine sequence at the C-terminal of Cdr1p. This would then aid in identification of Cdr1p through the use of anti-His HRP-conjugated antibody. Cdr1p migrates as a single broad band or a doublet in Western blots (Coste *et al.*, 2006; Tsao *et al.*, 2009; 2016). The double band is due to the presence of both phosphorylated and non-phosphorylated Cdr1p. Therefore, the phosphorylation status of Cdr1p may be visible on a Western blot. The insertion of a polyhistidine sequence was done via the CRISPR-Cas9 system through introduction of modified dDNA. Two CRISPR-sites were used to accomplish this. The first CRISPR-site is located 562bp upstream of the stop codon of *CDR1*. Introduction of the an in-frame polyhistidine sequence at the 3'-end of *CDR1* was successful as confirmed with sequencing with primers CDR1-HIS-S-1F and CDR1-HIS-S-1R. However, a band on the Western blot corresponding to the expected size of Cdr1p (170kDa) was not observed, even in the presence of fluconazole, a known inducer of *CDR1* expression (Hernández *et al.*, 1998; Vu *et al.*, 2019). Sequencing with primers CDR1-HIS-1F and CDR1-HIS-1R revealed a deletion of a portion of *CDR1* at the CRISPR-site used to introduce a DSB (data not shown). This deletion may have resulted in incorrect formation of Cdr1p. To alleviate this, another CRISPR-site was used to create a DSB 177bp downstream of the *CDR1* coding sequence. In-frame integration of the polyhistidine sequence was confirmed with sequencing, however, the band corresponding to Cdr1p was still not visible on a Western blot using an anti-his antibody (**Figure 4.16B**).



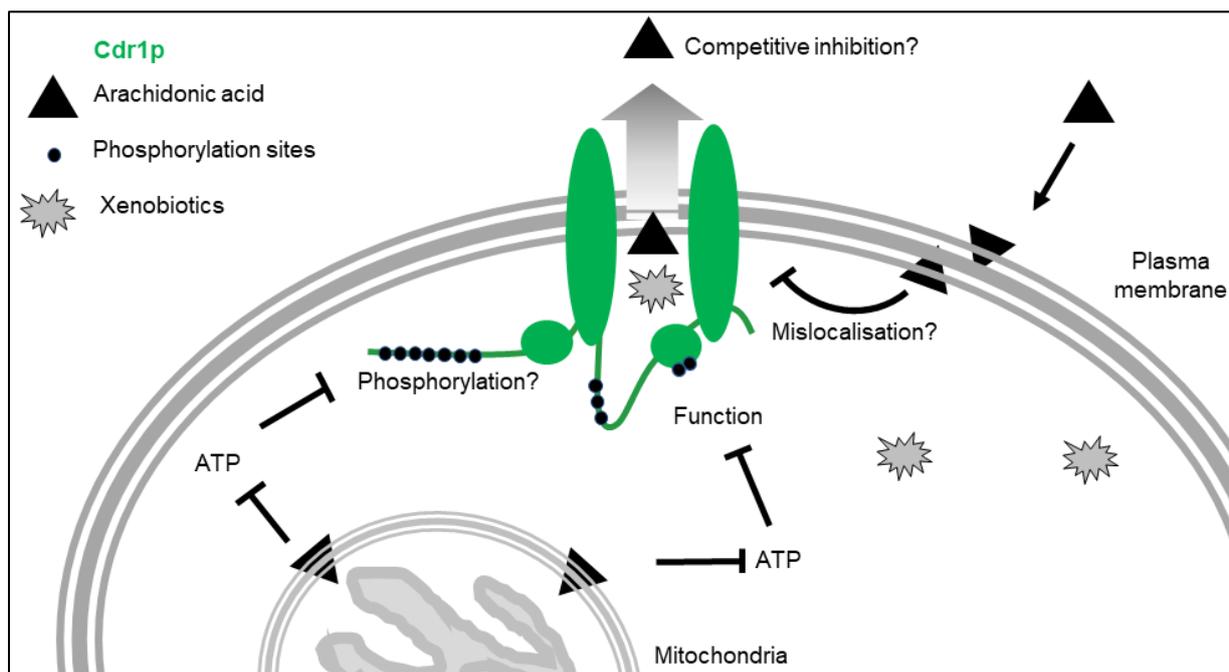
**Figure 4.16. Identification of Cdr1p.** **A** – SDS-PAGE of *Candida albicans* wild type (SC5314) and *cdr1Δ/Δ* in the presence of 0.1 mM or 1 mM arachidonic acid or ethanol control (EtOH). **B** – Western blot of *C. albicans* SC5314 or Cdr1p with polyhistidine insertion (CDR1-HIS) exposed to increasing concentrations of fluconazole. Bands indicate the presence of a polyhistidine sequence (SuperSignal® West HisProbe™ Kit). The arrows indicate the expected region where Cdr1p would migrate in an SDS-PAGE corresponding to ~170kDa.

To determine if this was due to an inappropriate protein extraction procedure, multiple methods for protein extraction were attempted (discussed in *Section 4.3.18.1*). However, this did not resolve the inability to identify Cdr1p. This inability to identify Cdr1p may be due to masking of the polyhistidine epitope on the Western blot, or errors during translation of Cdr1p with the polyhistidine sequence. Future studies would benefit from the use of an anti-Cdr1p antibody such as those used by Szczepaniak co-workers (2015) and Tsao and co-workers (2016) where the unmodified Cdr1p can be

detected. Therefore, the phosphorylation status of Cdr1p of *C. albicans* in the presence of AA is yet to be determined.

#### **4.4.9. The effect on Cdr1p functionality may be due to a combination of factors**

In this chapter, the reduction of Cdr1p functionality was discussed. Literature regarding factors that would influence the activity of Cdr1p was assessed. This section aims to summarise the information regarding the effect of AA on the function of Cdr1p. Firstly, it was speculated that mislocalisation of Cdr1p may be the reason for its decrease in functionality. This was based on increased diffusion of R6G into cells treated with AA as well as staining of ergosterol in lipid rafts using filipin. Furthermore, Shareck and co-workers (2011) observed mislocalisation of another membrane-bound protein due to CLA. In addition to mislocalisation, the reason for reduced function of Cdr1p can be due to other reasons. We therefore assessed other methods whereby AA could inhibit Cdr1p function. We found that mitochondrial function is reduced with exposure to AA for one hour. This effect may also be due to incorporation of AA into membrane phospholipids, such as in the mitochondrial membrane. As Cdr1p is dependent on ATP for function, this may indicate that a decrease in ATP may be responsible for the decreased functioning of Cdr1p. Lastly, Cdr1p requires phosphorylation to function, also dependent on high energy phosphate molecules. However, this needs confirmation. Lastly, lipophilic compounds have been reported to competitively inhibit the efflux of xenobiotic compounds by Cdr1p (Galkina *et al.*, 2020). This would explain that AA needs to be present in a high amount to elicit long term inhibitory effects on Cdr1p. In addition, the increased sensitivity of *cdr1Δ/Δ* to AA may indicate that it is responsible for extrusion of AA from the cell and that this may be at the expense of export of xenobiotic compounds such as R6G, and possibly antifungal agents. Therefore, at this time, we could not definitively confirm the reason for the reduction in Cdr1p function due to AA, but we suggest that this may be due to a combination of occurrences visualised in **Figure 4.17**.



**Figure 4.17. Schematic of proposed mechanisms whereby arachidonic acid (AA) inhibits Cdr1p function.** Arachidonic acid can insert into the plasma membrane and may affect localisation of Cdr1p. Insertion into the membrane of the mitochondria causes dysfunction and subsequent decrease in ATP production, possibly affecting function of Cdr1p due to its energy requirements, as well as phosphorylation. Lastly, AA may compete with xenobiotic compounds for efflux by Cdr1p.

#### 4.5. CONCLUSIONS

Transcriptomic analysis of *C. albicans* exposed to AA revealed the differential expression of genes associated with membrane organisation (**Supplementary Table S2.2**). Genes associated with membrane organisation can affect virulence as well as susceptibility to antifungal agents. *CDR1*, *IPT1* and *RTA3*, all associated with membrane organisation and susceptibility to antifungal agents, are induced upon exposure to AA. In addition, these genes were induced in response to *P. aeruginosa*. To evaluate the role of these genes further, homozygous deletion mutants were successfully constructed. Whereas *CDR1* and *RTA3* did not significantly affect survival in the presence of *P. aeruginosa*, an increase in *ipt1Δ/Δ* CFUs was observed in polymicrobial biofilms. This may be due to an increase in yeast-form cells as well as reduced killing by *P. aeruginosa*, implicating *IPT1*, and sphingolipids, as potentially playing a role in the interaction with *P. aeruginosa*.

*Caenorhabditis elegans* was used to evaluate the effect of *cdr1Δ/Δ*, *ipt1Δ/Δ* and *rta3Δ/Δ* on virulence. Whereas deletion of *RTA3* had no significant effect on virulence, deletion of *IPT1* caused a minor but significant increase in virulence, potentially through reduced immune induction and inability of the host to clear infection. Interestingly, deletion of *CDR1* decreased the virulence of *C. albicans* in the *C. elegans* infection model, warranting further investigation.

Among *cdr1Δ/Δ*, *ipt1Δ/Δ* and *rta3Δ/Δ*, only *cdr1Δ/Δ* exhibited a decrease in biofilm biomass in the presence of 0.1 mM AA. Increased lipid peroxidation and oxidative stress suggest that AA accumulates in *cdr1Δ/Δ*, implicating Cdr1p in the export of AA out of the cell. Evidence presented in this chapter indicates that AA not only increases transcription of *CDR1*, but increases the formation of Cdr1p in a dose dependent manner. Evidence was presented that this may be due to various factors, or a combination thereof. The results in this chapter provides evidence that PUFAs, such as AA, can reduce the function of membrane-bound proteins important in *C. albicans*'s antifungal resistance. Further research is required to determine if this effect extends to other PUFAs and to other pathogenic yeast species.

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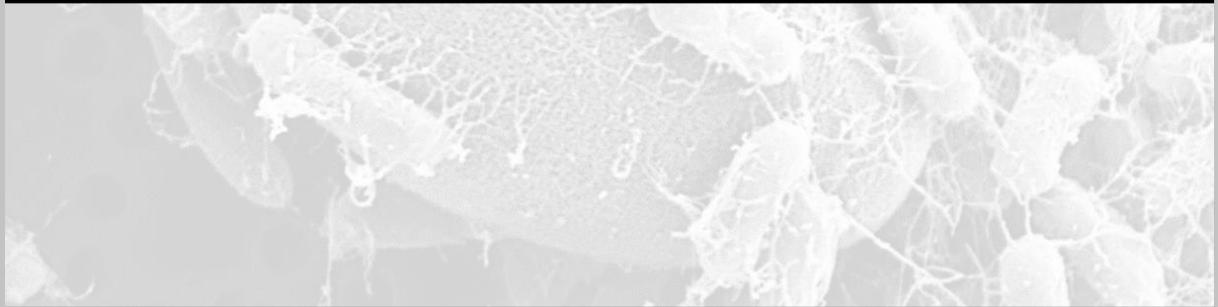
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***CHAPTER 5***

**General discussion and conclusions**



## 5.1. GENERAL DISCUSSION AND CONCLUSIONS

The interaction between *Candida albicans* and *Pseudomonas aeruginosa* can be regarded as a model for polymicrobial interactions with clinical significance. During infection by these opportunistic pathogens, arachidonic acid (AA) is released (Deva *et al.*, 2000; Saliba *et al.*, 2005). This polyunsaturated fatty acid is not only crucial for normal function in mammalian systems (Tallima and El Ridi, 2018), but is also proposed to act as an endogenous antimicrobial molecule (Das, 2018). However, the effect of AA on infective agents is frequently overlooked during *in vitro* studies. An increase in antifungal sensitivity and reduction in virulence-associated characteristics, such as morphogenesis, is seen in *C. albicans* in response to high concentrations of AA (Ells *et al.*, 2009; Shareck and Belhumeur, 2011). However, the mechanisms associated with these effects are unknown. To address this, transcriptomic profiles of *C. albicans* biofilms in response to a sub-inhibitory, and more physiologically relevant concentration of AA (0.1 mM), co-incubation with *P. aeruginosa* and a combination of these two factors, were compared.

### 5.1.1. The response of *C. albicans* towards *P. aeruginosa*

The response of *C. albicans* towards *P. aeruginosa* in polymicrobial biofilms was evaluated to potentially identify novel facets of interaction as well as to determine a baseline to compare the effect of AA on polymicrobial biofilms. Among the conditions evaluated, the response of *C. albicans* to *P. aeruginosa* in the absence of AA elicited the largest transcriptional response. The response of *C. albicans* towards *P. aeruginosa* was dominated by a response towards hypoxia and fermentative growth (Askew *et al.*, 2009). This may be due to the rapid depletion of oxygen by the aerobic growth of *P. aeruginosa*. Furthermore, phenazine production by *P. aeruginosa* contributes to this fermentative profile (Morales *et al.*, 2013). Importantly, genes associated with drug efflux, such as *CDR1* and *CDR2*, were induced during co-incubation with *P. aeruginosa*. Holcombe and co-workers (2010) also observed the induction of multidrug efflux pumps in response to the supernatant of *P. aeruginosa*, suggesting that the induction of these genes may be in response to toxic metabolites produced by *P. aeruginosa*. The role of induction of these genes in the antifungal resistance of *C. albicans* in polymicrobial biofilms were not addressed in the present study and requires further attention.

### 5.1.2. Morphogenesis of *C. albicans* in polymicrobial biofilms

Several factors imposed by co-incubation with *P. aeruginosa* can influence the morphogenesis of *C. albicans* and research has suggested that *C. albicans* morphogenesis is mediated by interplay between transcription factors in a complex network (reviewed in Fourie and Pohl, 2019; Huang, 2012; Hogan and Kolter, 2002; Shareck and Belhumeur, 2011). Chromatin remodelling by the Set3/Hos2 histone deacetylase complex (Set3C) is known to affect morphogenesis and may act as a transcriptional buffer in the case of multiple stimuli (Hnisz *et al.*, 2012). However, chromatin remodelling is also known to affect morphogenesis in *C. albicans*, acting as an additional layer of regulation above transcription factors. Overexpression of components of the Set3C is known to inhibit the yeast-to-hyphal switch in *C. albicans* and promotes dispersal of yeast cells (Hnisz *et al.*, 2010; Nobile *et al.*, 2014). We observed an increase in expression of *SET3*, encoding a component of the Set3C. Therefore, we asked if the Set3C may play a role in the protection of *C. albicans* against *P. aeruginosa* and induce the yeast's dispersal, as the bacterium is known to preferentially kill hyphal cells (Hogan and Kolter, 2002). As deletion of *SET3* may confer a hyperfilamentous phenotype under certain laboratory conditions and increase the proclivity of *P. aeruginosa* for killing hyphal cells, we expected increased killing of *set3Δ/Δ*. Furthermore, as the Set3C has little overlap with human histone deacetylases, it is an attractive target for intervention (Garnaud *et al.*, 2016). However, deletion of *SET3* did not affect morphogenesis of *C. albicans* in the presence of *P. aeruginosa*, nor did it increase the capacity of *P. aeruginosa* to kill *C. albicans* cells. Therefore, deletion of *SET3* had a limited influence on the interaction of *C. albicans* with *P. aeruginosa* *in vitro*. Interestingly, deletion of *SET3* was able to abrogate virulence in a *Caenorhabditis elegans* model. Additionally, altered virulence of *set3Δ/Δ*, even in the presence of *P. aeruginosa*, is seen. Although the *C. elegans* infection model used can provide valuable information regarding human pathogens due to similarity to human innate immunity, it lacks valuable information regarding the multiple factors imposed by a systemic infection (Marsh and May, 2012). Therefore, a systemic infection model may provide more resolution on the effect of *set3Δ/Δ* during co-infection with *P. aeruginosa*, as a more pronounced abrogation of virulence is seen during single species infection of a murine systemic model (Hnisz *et al.*, 2010).

### 5.1.3. The role of *WOR1* in polymicrobial biofilms with *P. aeruginosa*

In addition to its role in regulating the classical morphological transition between yeast and hyphal morphologies, the Set3C also plays a role in white-opaque switching (Hnisz *et al.*, 2009). The white-opaque regulator, *WOR1*, is the master switch of white-opaque switching, an epigenetic, heritable change to the opaque phenotype with altered phenotype, carbon utilisation and virulence (Lan *et al.*, 2002). *WOR1* is induced by various bacteria that *C. albicans* may encounter in the gastrointestinal tract and promotes competitive fitness with co-inhabiting bacteria in the gastrointestinal tract (Pande *et al.*, 2013). Interestingly, *WOR1* was also induced in a polymicrobial biofilm with *P. aeruginosa*. This induction of *WOR1* may have been due to the hypoxic environment of the polymicrobial biofilm, the presence of bacterial cell wall components, as well as the physiological temperature of 37°C. Notably, these environmental cues may also be present at infection sites where *C. albicans* and *P. aeruginosa* are both present, such as the cystic fibrosis lung (Montgomery *et al.*, 2017). The occurrence of *WOR1* induction encouraged us to determine if non-classical phenotypes of *C. albicans* may affect its interaction with *P. aeruginosa*. To determine this, *WOR1* was deleted and the polymicrobial biofilms with *P. aeruginosa* were characterised. Deletion of *WOR1* did not affect polymicrobial biofilm morphology or the survival of *C. albicans* in the presence of *P. aeruginosa*. Furthermore, although transcriptomic evaluation revealed an induction of *WOR1*, it did not indicate that the full opaque transcriptional profile was induced in response to *P. aeruginosa*, similar to the results of Fox and co-workers (2014) with *Klebsiella pneumoniae*, *Escherichia coli* and *Enterococcus faecalis*. However, a unique transcriptional response to *P. aeruginosa* was seen when compared to other bacterial species, indicating a species-specific transcriptional response to *P. aeruginosa*. Importantly, deletion of *WOR1* did not alter the virulence of *C. albicans*, even when combined with *P. aeruginosa*, in the *C. elegans* infection model. Therefore, in the strain of *C. albicans* used (SC5314), *WOR1* expression does not play a role in the interaction of *C. albicans* with *P. aeruginosa*, at least under the circumstances used in this study. Other reports have indicated that homozygosity at the mating type locus (MTL; *a/a* or  $\alpha/\alpha$ ), is required for white-opaque switching (Lockhart *et al.*, 2002). However, strain SC5314 (used in this study) is heterozygous at the MTL (*a*/ $\alpha$ ) (Ramirez-Zavala *et al.*, 2008). Of note is the results reported by Lockhart and co-workers (2002), where 220 independent *C.*

*albicans* isolates indicated that only 3.2% of isolates were homozygous at the MTL, with 96.8% heterozygous. Therefore, the use of SC5314 may represent the majority of *C. albicans* clinical isolates. However, a high frequency of (a/α) isolates were able to become homozygous at MTL, indicating that a significant amount of *C. albicans* clinical isolates may be able to undergo white-opaque switching. Therefore, the use of SC5314 as wild type has a limitation in this aspect as it may not represent (a/a) and (α/α) isolates. The use of a *WOR1*-overexpressing strain, or a strain with homozygosity at the MTL may be advantageous in future research.

#### 5.1.4. The effect of arachidonic acid on *C. albicans* monomicrobial biofilms

As expected, exposure of *C. albicans* to AA resulted in the differential expression of genes associated with membrane organisation and cell periphery. This observation is validated by numerous reports that indicated effects of PUFAs on membrane integrity and fluidity in mammalian cells, fungal cells and bacteria (Baker *et al.*, 2018; Brash, 2001; Pohl *et al.*, 2011; Shaikh and Edidin, 2008; Yoon *et al.*, 2018). Three genes involved in membrane organisation were chosen (*CDR1*, *IPT1* and *RTA3*) for further research due to their importance in antifungal susceptibility (Pasrija *et al.*, 2005; Prasad *et al.*, 1995, 2005; Srivastava *et al.*, 2017; Whaley *et al.*, 2016), and the observed increase in antifungal susceptibility of pathogenic yeast caused by PUFAs. When evaluating virulence, both *cdr1Δ/Δ* and *ipt1Δ/Δ* altered survival of *C. elegans*. However, these changes were minor, with *cdr1Δ/Δ* reducing virulence. Interestingly, the virulence of *ipt1Δ/Δ* was higher than the wild type, possibly through an inability to elicit immune induction and subsequent clearing of infection (Rouabhia *et al.*, 2011). Among *CDR1*, *IPT1* and *RTA3*, *C. albicans* indicated dependence on *CDR1* for survival in the presence of AA. We suspect that this pleiotropic transporter may be responsible for efflux of AA, as increased oxidative stress, potentially imposed by lipid peroxidation, is observed in the absence of *CDR1*. This may also explain the reduction in virulence of *cdr1Δ/Δ* in *C. elegans*. However, whereas AA is the main PUFA in phosphatidylinositol in mammalian phospholipids, eicosapentaenoic acid is the predominant PUFA in phospholipids in *C. elegans* (Lee *et al.*, 2008). If the reduction in virulence of *cdr1Δ/Δ* is due to increased lipid peroxidation, it may indicate that other PUFAs could have a similar effect.

As the main transporter involved in antifungal efflux, deletion of *CDR1* is known to significantly reduce efflux of xenobiotic compounds, such as antifungal agents (Sanglard *et al.*, 1996). Furthermore, antifungal resistant isolates exhibit an overexpression of *CDR1* (Chen *et al.*, 2010). We observed an increase in both *CDR1* mRNA, as well as Cdr1p in response to AA in a dose dependent manner. However, functionality of Cdr1p was severely diminished in the presence of AA. The increased expression of *CDR1*, but decreased function, prompted us to evaluate possible mechanisms associated with loss of function of Cdr1p. We speculate that it may be due to one of three effects of AA, or a combination thereof. Firstly, loss of functionality of Cdr1p may be due to mislocalisation of the protein in the membrane, however, this needs further confirmation. Secondly, the decrease in Cdr1p functionality may be due to mitochondrial dysfunction and inhibition of phosphorylation of Cdr1p, required for functionality of Cdr1p. However, further investigation is still required. Lastly, competitive inhibition of rhodamine 6G (used to determine functionality of Cdr1p) may occur, as AA may act as a substrate for Cdr1p. This study only addressed *CDR1*, however, its homolog, *CDR2*, is also induced upon exposure to AA. Therefore, other ABC transporters may also be affected by this PUFA. Notably, the mechanism by which AA induces *CDR1* expression was not investigated. However, increased expression of *FCR1*, encoding a negative regulator of *CDR1* (Shen *et al.*, 2007), was induced, indicating that it does not play a role in the induction of *CDR1* by AA. Furthermore, *TAC1*, encoding a transcriptional activator of *CDR1* (Coste *et al.*, 2004), was not induced at the time of measurement. However, other Tac1p regulated genes are differentially expressed, including *CDR2*, *PDR16* and *RTA3* (Liu *et al.*, 2007). Further research is required to determine if AA may have induced *TAC1* at an earlier time point, as this may explain the induction of Tac1p-regulated genes.

These proposed effects on Cdr1p, may form a general concept of the influence that PUFAs have on membrane-bound proteins in pathogenic yeast. Notably Shareck and co-workers (2011) also indicated the mislocalisation of another membrane-bound protein, Ras1p (involved in morphogenesis), due to conjugated linoleic acid. However, the dependence of protein function on mitochondrial function may only apply to ATP-driven or phosphorylated proteins. Further research is required to determine if the effect of AA on Cdr1p extends to other PUFAs, as well as other pathogenic yeast species, such as the intrinsically resistant *Candida auris*.

### 5.1.5. The effect of arachidonic acid on *C. albicans* polymicrobial biofilms

The transcriptomic profile of *C. albicans* in polymicrobial biofilms exposed to AA revealed a further reduction of hyphae formation, compared to polymicrobial biofilms without the fatty acid. Components of the Set3C are unaffected by the addition of AA. However, *NRG1*, encoding a component of the Tup1-Nrg1p repression complex (Braun *et al.*, 2001; Shareck *et al.*, 2011), is induced, similar to single species biofilms exposed to AA. Therefore, morphogenesis in *C. albicans* is reduced by both *P. aeruginosa*, as well as by AA as reported before. However, repression of filamentation by AA is mediated by Tup1p-Nrg1p repression, similar to what was observed for conjugated linoleic acid (Shareck *et al.*, 2011). Therefore, AA seems to further inhibit filamentation in the presence of *P. aeruginosa* through an additive mechanism. Yet, AA did not affect expression of *WOR1*.

Similar to the response of single species biofilms to AA, polymicrobial biofilms exhibited differential expression of genes whose products are associated with the periphery of the cell. However, *CDR1*, *CDR2*, *IPT1* and *RTA3* were not further induced with the addition of AA beyond their induction by the presence of *P. aeruginosa*. A threshold of induction may have been reached by co-incubation with *P. aeruginosa*. This may also be due to the rapid metabolism of AA by *P. aeruginosa*, abrogating its effect on *C. albicans*. Increased oxidative stress is also seen with the addition of AA to polymicrobial biofilms. This effect was not observed with single species biofilms exposed to AA. Notably, phenazine production by *P. aeruginosa* is known to induce oxidative stress (Morales *et al.*, 2010). Arachidonic acid has been reported to reduce swarming motility and increase biofilm formation by *P. aeruginosa* (Baker *et al.*, 2018), however, other factors, such as phenazine production, was not addressed. Further research is required to establish if phenazine production may be induced by the exposure to AA.

The present research then indicates that the most pronounced effect of AA, in terms of antifungal resistance-associated proteins, are on single species biofilms of *C. albicans*. In polymicrobial biofilms, AA seems to exacerbate the inhibition of morphogenesis by *C. albicans* by *P. aeruginosa*, potentially through an additive effect. Furthermore, an enhanced response towards oxidative stress is observed, prompting further investigation. This research then provides information regarding the

transcriptional changes of *C. albicans* in both single and polymicrobial biofilms in response to a component of the host during infection that is frequently overlooked. Furthermore, evidence is provided that the previously observed increased susceptibility of *C. albicans* to antifungal agents in the presence of AA is due, in part, to reduced function of Cdr1p, possibly mediated through various factors, including competitive inhibition, mislocalisation of Cdr1p and dysfunction of the mitochondria.

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