In vitro Arachidonic Acid Metabolism by Polymicrobial Biofilms of *Candida albicans* and *Pseudomonas aeruginosa*

by

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DECLARATIONS

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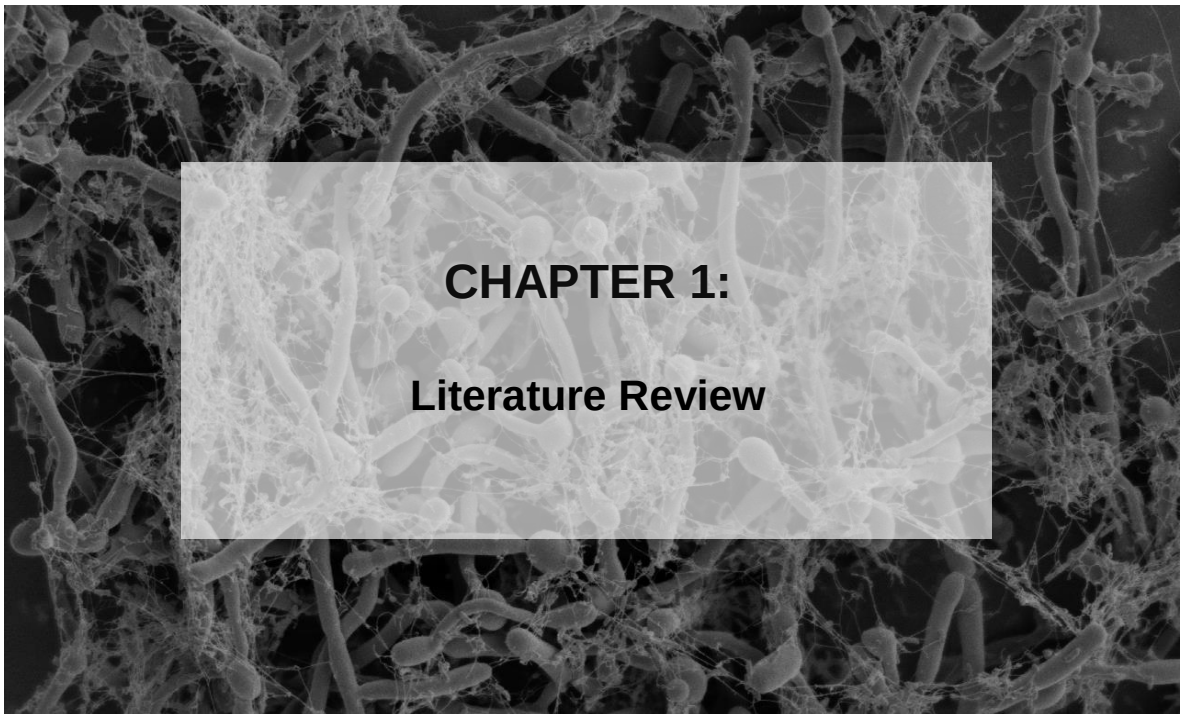


CHAPTER 1:

Literature Review

1.1. Motivation

Candida albicans is a dimorphic fungal opportunistic pathogen with the ability to form biofilms (Ramage *et al*., 2001). It has been implicated as the most prevalent fungal bloodstream fungal pathogen causing large problems in the nosocomial setting, in immunocompromised hosts as well as in cystic fibrosis infection (Rinzan, 2009). Treatment of *C. albicans* infection is highly problematic due to high resistance to antifungal agents (Dumitru *et al*., 2004; Kuhn *et al*., 2002; Ramage *et al*., 2001). *Candida albicans* is often not found alone, but causes disease in concert with other microorganisms such as bacteria (Diaz *et al*., 2014; Lindsay & Hogan, 2014). *Pseudomonas aeruginosa* is one of the frequent co-isolates during *C. albicans* infection. *Pseudomonas aeruginosa* is a major cause of morbidity and mortality in infection and it possesses several virulence factors, including the ability to form biofilms (Bragonzi *et al*., 2009). This pathogen is also highly resistant to antibiotic treatment (Drenkard, 2003). Several facets of interaction have been described between these two microbes, including physical interaction, where *P. aeruginosa* has been shown to kill hyphal filaments, but not yeast cells of *C. albicans* (Brand *et al*., 2008). Indirect interactions include the production of quorum sensing molecules, where *P. aeruginosa* secreted 3-oxo-dodecanoyl-homoserine lactone inhibits the yeast to hyphal switch of *C. albicans* (McAlester *et al*., 2008). *Pseudomonas aeruginosa* also forms phenazines, including pyocyanin that reduces the viability of *C. albicans* (Gibson *et al*., 2009; Kerr *et al*., 1999). *Candida albicans* quorum sensing molecule farnesol has also been shown to affect *P. aeruginosa* virulence and biofilm formation (Cugini *et al*., 2007; McAlester *et al*., 2008). Other factors, including iron sequestration, ethanol production, and extracellular DNA has been shown to affect the dynamic of the interaction between *C. albicans* and *P. aeruginosa* (Chen *et al*., 2014; Purschke *et al*., 2012; Sapaar *et al*., 2014; Trejo-Hernandez *et al*., 2014). During infection, both of these pathogens elicit the release of arachidonic acid (AA) from host cells (Agard *et al*., 2013; Castro *et al*., 1994). This AA can be used by the host to produce immunomodulatory eicosanoids such as prostaglandins, leukotrienes, thromboxanes and lipoxins (Dennis & Norris, 2015). These eicosanoids regulate the balance of inflammation during infection, and can either cause inflammation or resolution of the inflammatory response. In addition, *C. albicans* has been shown to produce immunomodulatory lipids from exogenous AA, including 3-

hydroxeicosatetraenoic acid (3-HETE) as well as prostaglandin E_2 (Deva *et al*., 2000; Erb-Downward & Noverr, 2007). *Pseudomonas aeruginosa* can also metabolize exogenous AA, producing prostaglandins (Lamacka & Sajbidor, 1995). In addition, a secretable 15-lipoxygenase is produced by *P. aeruginosa* that is able to convert AA to 15-HETE (Vance *et al*., 2004). These eicosanoids may play adverse roles in the dynamic of pathogen-host interaction, as well as pathogen-pathogen interaction. It is thus important to determine the production and role of eicosanoids by *C. albicans* and *P. aeruginosa* during combined incubation, as this could give valuable insight into the role of eicosanoids during polymicrobial infection by these microorganisms.

1.2. Introduction

Recently it has become increasingly evident that microorganisms, from bacteria to fungi, are not just found as free floating cells, but exist as surface associated, structured and cooperative consortia, called biofilms, that colonize and attach to biotic or abiotic surfaces (Burmølle *et al.*, 2006; Douglas, 2003; Harriott & Noverr, 2011; Hentzer *et al.*, 2003). In addition, these communities of microorganisms are embedded in an extracellular matrix of self-produced polymeric material. In these interactive organizations of microorganisms, secreted factors and physical proximity enable metabolic interactions (Diaz *et al.*, 2014). This often involves interkingdom interactions necessary for ecological balance and the survival of certain species (Rinzan, 2009).

Pseudomonas aeruginosa is a Gram-negative, aerobic rod, colonizing a remarkable assortment of niches, including aquatic environments, terrestrial environments and eukaryotic organisms (Pier, 1985; Tan *et al.*, 1999). It is an opportunistic pathogen, frequently isolated from healthy humans as part of the human microbiota and is commonly found in mixed infections with *C. albicans* (Kaleli *et al.*, 2006). *Candida albicans* is a major cause of opportunistic infections ranging from superficial to fatal systemic infections with the potential to infect and colonize almost every part of the human body, including skin and mucosa, as well as deep tissue and organs (Sandven, 2000). It is also found as part of the normal microbiota of the skin, gastrointestinal tract and female genital tract (Morales & Hogan, 2010). Fungal infections have become increasingly troublesome in the past decades, especially in immunocompromised patients and in the hospital setting, with *C. albicans* being the most frequently isolated fungal pathogen and the most commonly isolated bloodstream pathogen (Rinzan,

2009). Selective pressure of nutrient limitation and competition between bacteria and fungi regulate the colonization of potential pathogenic microorganisms such as *C. albicans* and *P. aeruginosa*, with a disruption in this equilibrium resulting in infection by opportunistic pathogens (Calderone & Fonzi, 2001).

These two medically significant microorganisms have tendencies to form polymicrobial biofilms and as such play extensive roles in nosocomial infections, infection in immunocompromised individuals and especially in cystic fibrosis (CF) patients (Bianchi *et al.*, 2008; El-Azizi *et al.*, 2004; McAlester *et al.*, 2008). This review therefore aims to evaluate the complex cross-kingdom relationship of these two pathogens and the impressive interaction and communication between them as well as the collateral damage to hosts caught in the cross-fire. Additionally, special attention will be given to the known immunomodulatory lipids produced by both microorganisms and the role this may play during infection.

1.3. Pathogenesis of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa possesses numerous virulence factors including exotoxin A, proteases and lipases, released by a type II secretion system (Xcp regulon), as well as exotoxins exoS, T, U and Y, secreted into host cells via an “injection needle” or type III secretion system (Hogardt *et al.*, 2004). Interestingly, it was found that *P. aeruginosa* possesses two type II secretory pathways, previously not seen in one organism (Ball *et al.*, 2002). Additionally, pyoverdine, rhamnolipids, lipopolysaccharide (LPS) and pili also form part of this formidable pathogen’s virulence arsenal (Gilligan, 1991; Méar *et al.*, 2013). A study by Bianchi *et al*. (2008) showed that *P. aeruginosa* impairs the engulfment of apoptotic cells through the action of yet another virulence factor, the phenazine, pyocyanin (PYO). Interestingly, it has been revealed that multiple drug resistant strains of *P. aeruginosa* show decreased production of PYO, and thus have a reduction in virulence, causing these strains to only cause subclinical colonization (Fuse *et al.*, 2013). As previously mentioned, *P. aeruginosa* forms biofilms, and a universal model for the formation of *P. aeruginosa* biofilm formation was suggested (O’Toole *et al.*, 2000). According to this model, *P. aeruginosa* cells move by means of flagella to an adequate surface and movement along this surface is accomplished through type IV-pili. Subsequent growth leads to aggregation of cells and subsequent microcolony formation. During maturation, large mushroom-shaped

structures are formed. Klausen *et al*. (2003) proposed an alternate model, with evidence indicating that flagella do not play a role in the attachment of *P. aeruginosa* cells. In addition, microcolony formation is the result of clonal growth, and type IV-pili results in the spreading of cells over a surface. The formation of *P. aeruginosa* biofilms are, however, highly dependent on the carbon source, as benzoate, citrate or casamino acids cause the formation of flat biofilms. Additionally, the circumstances during growth, for example, a flow through system versus stationary growth, might elicit large morphological changes.

In addition to the previously mentioned factors, the resistance of *P. aeruginosa* to antimicrobial agents is key to its pathogenic capabilities. Various mechanisms for antibiotic resistance in *P. aeruginosa* biofilms have been proposed. Figure 1 illustrates several processes of antimicrobial resistance reviewed by Drenkard (2003). These include the reduced transport of antimicrobial agents in the biofilm due to an extracellular matrix and accompanied nutrient and oxygen limitation of cells deeply embedded in the biofilm, causing a decrease in metabolic activity of the cells. Antibiotic resistant persisters embedded in the biofilm structure, stress responses of the cells, efflux pumps and quorum sensing among cells may all contribute to the increased resistance observed in bacterial biofilms (Drenkard, 2003). Evidence also suggests that a protein, PvrR, regulates susceptibility and resistance phenotypes of *P. aeruginosa* (Benamara *et al.*, 2011; Drenkard & Ausubel, 2002). The formation of biofilms is also speculated to impact the lipid composition of cells compared to planktonic growth. In this regard, Benamara *et al*. (2011) examined the effect of biofilm formation on inner membrane lipid composition in *P. aeruginosa* that indicated a reduced amount of uneven numbered phospholipids. In addition, an increase in long chain phosphatidylethanolamines was observed, suggesting an increase in bilayer lipid stability and a decrease in membrane fluidity. This could possibly influence the resistance of bacterial biofilm cells to certain antibiotics.

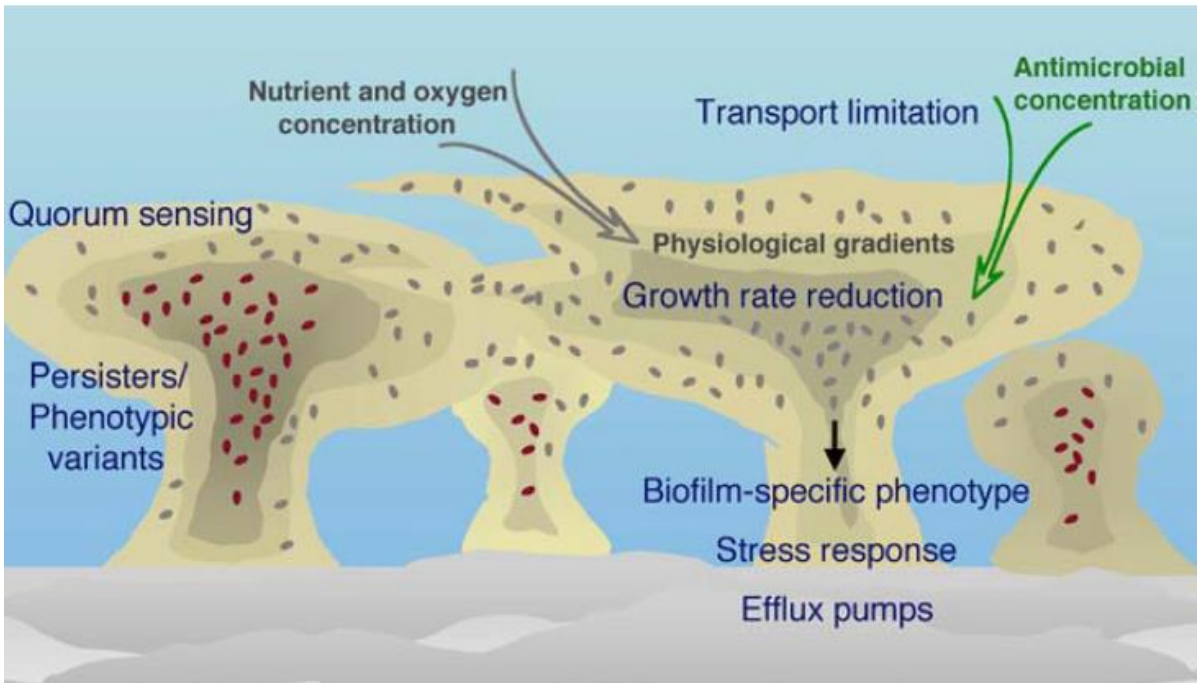


Figure 1. Illustration of factors that may, in concert, attribute to the antimicrobial resistance observed in *Pseudomonas aeruginosa* biofilms (Drenkard, 2003).

1.4. Pathogenesis of *Candida albicans*

Candida albicans is a dimorphic yeast, meaning that both yeast and hyphal morphology is shown, with a tendency to form drug resistant biofilms (Ramage *et al.*, 2001). The ability of this microorganism to switch between the planktonic single yeast cell and hyphal morphologies has a major influence on its virulence (Brand *et al.*, 2008; Gil-Bona *et al.*, 2015). In addition to this morphological plasticity, the aggressiveness of *C. albicans* colonization is due to a collection of virulence factors, including the aforementioned morphological plasticity and ability to form biofilms on tissue as well as medically implanted devices such as prosthetic heart valves and catheters (Andes *et al.*, 2004; Bruzual *et al.*, 2007; Pierce, 2005). Other virulence factors include adhesins (biomolecules that enable binding to host cells or host cell ligands), lipolytic and proteolytic enzymes and phenotypic switching (white to opaque switching) (Calderone & Fonzi, 2001). Interaction of *C. albicans* with the host is largely accomplished by contact with the *C. albicans* cell surface and subsequent biofilm formation (Gow & Hube, 2012). A study by Ramage *et al*. (2001) investigated the

formation of *C. albicans* biofilms through visualization of the biofilms at various stages of development, namely the adherence phase of the microorganism (0 to 2 hours), the formation of microcolonies (2 to 4 hours) during which the cells started budding, filamentation (pseudohyphae and true hyphae formation) in the biofilm (4 to 6 hours), monolayer development (6 to 8 hours) during which hyphae and budding yeast formed an intricate network, proliferation of the cells in the biofilm (8 to 24 hours), forming a multilayered structure, and maturation of the biofilm (24 to 48 hours). Through SEM and confocal scanning laser microscopy (CSLM) a dense network of hyphae and yeast cells in a matrix of exopolymeric material was visualized in the matured biofilm. The visualization of the biofilm also showed the three dimensional structure of the biofilm with a heterogeneous structure and spatial composition. Water channels between microcolonies aid in optimum nutrient transfer and waste disposal of the cells in the biofilm.

The study also indicated the increased resistance against antimicrobial agents (Ramage *et al.*, 2001). The effect of fluconazole and amphotericin B was tested on *Candida* biofilms and planktonic *C. albicans* cells. It was found that cells in the biofilm had a 250 fold increase in resistance against fluconazole. Interestingly, Dumitru *et al.* (2004) argued that this increased resistance against azoles and polyenes might be due to the hypoxic conditions found in biofilms, with anaerobically grown *C. albicans* also showing resistance against certain antifungal agents. The increased resistance of *Candida* biofilms compared to planktonic counterparts have been accepted due to a multitude of evidence with routine antifungal therapies becoming obsolete in the treatment of fungal biofilms. Interestingly, a study by Kuhn *et al.* (2002) evaluated a range of antifungal agents against *Candida* and found that sub-inhibitory concentrations of certain antifungals elicited alterations in biofilm formation. Additionally, the group indicated significant repression of biofilm formation by lipid formulations of amphotericin B and echinocandins.

1.5. Interaction between *Pseudomonas aeruginosa* and *Candida albicans in vitro*

1.5.1. Physical/Direct interaction

Realization of the importance of polymicrobial biofilms lead to a range of studies being conducted to evaluate the interaction of clinically relevant microorganisms such as *P. aeruginosa* and *C. albicans*. The antagonistic interaction of *C. albicans* and *P. aeruginosa* was examined by Brand *et al.* (2008) and it was found that *P. aeruginosa* cells kill *C. albicans* hyphal cells, but do not kill *C. albicans* yeast cells. The data obtained in the study showed the colonization and lysis of *C. albicans* hyphae by *P. aeruginosa*. The deadly effect on *C. albicans* is thought to be due to PYO, which alters the cell wall of *C. albicans* (Kerr *et al.*, 1999). Further research into this interaction provided evidence that there is a difference in *P. aeruginosa*-mediated *C. albicans* killing among different morphotypes of *C. albicans* (Rinzan, 2009). Increased susceptibility to the killing effect of *P. aeruginosa* was seen with filamentous cells compared to planktonic counterparts, similar to the study by Brand *et al*. (2008), as well as a reversion of germ tube formation in the presence of *P. aeruginosa*. Figure 2 illustrates the increased cell death of *C. albicans* in the presence of *P. aeruginosa* in a polymicrobial biofilm.

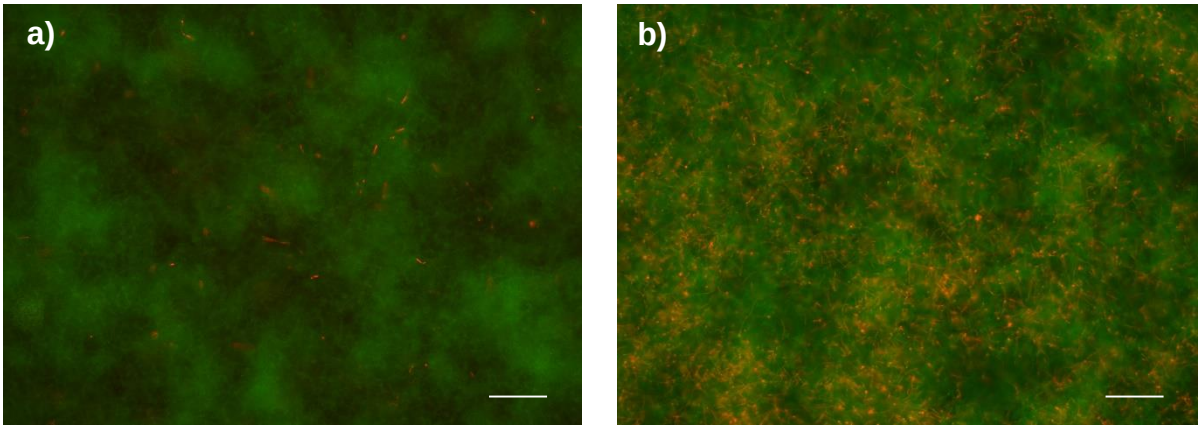


Figure 2. Visualization of 24 h biofilms of a) *Candida albicans* and b) *C. albicans* co-incubated with *Pseudomonas aeruginosa* showing increased cell death of *C. albicans* in the presence of *P. aeruginosa* (Fourie *et al*., unpublished observation). Live cells are stained green, whereas dead cells are stained red. Scale bars represent 100 µm.

Further analysis of this interaction indicated that attachment of *P. aeruginosa* to *C. albicans* and killing of *C. albicans* is mediated by lectin-carbohydrate interaction, type IV-pili and mannans. The authors also speculated on the possible involvement of O-linked mannans in the survival of *C. albicans* yeast cells during combined incubation, as was proposed previously (Brand *et al*., 2008; Rinzan, 2009). Mannosylation has also been identified as playing a critical role in the interaction of *C. albicans* with *Streptococcus gordonii* in the formation of polymicrobial biofilms, as well as playing a large role in stabilizing the *C. albicans* cell wall (Dutton *et al.*, 2014). In Figure 3, scanning electron micrographs of a dual species biofilm with *C. albicans* and *P. aeruginosa* is seen showing extensive colonization of *C. albicans* cells by *P. aeruginosa*.

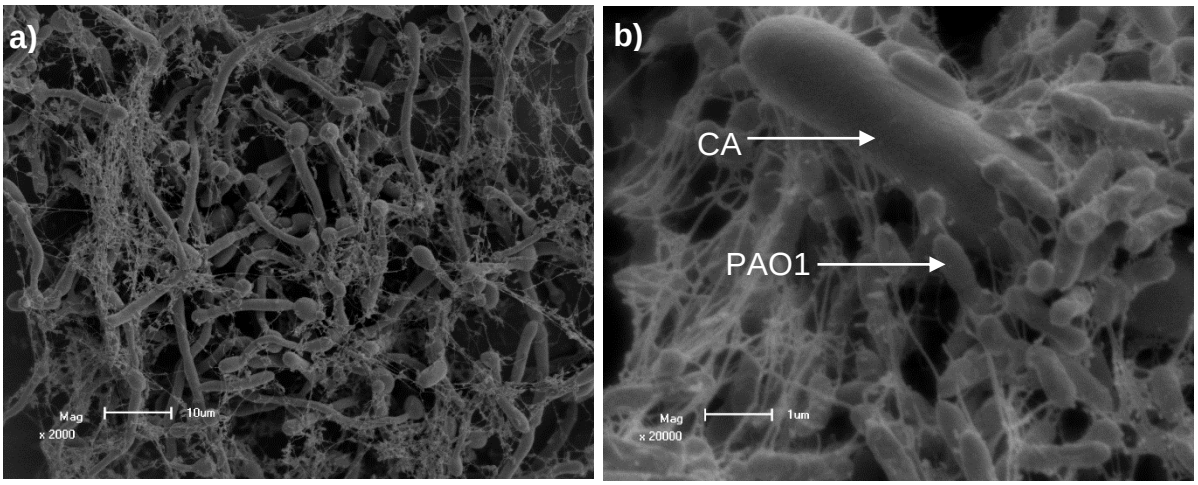


Figure 3. Scanning electron micrographs of *Candida albicans* (CA) colonized by *Pseudomonas aeruginosa* PAO1 (PAO1) showing adhesion to *C. albicans* hyphae (Fourie *et al*. unpublished observation). Scale bars represent (a) 10 µm and (b) 1 µm.

1.5.2. Indirect interaction

1.5.2.1 Role of Pseudomonas aeruginosa *quorum sensing molecules during* in vitro *interaction*

The interaction of *C. albicans* and *P. aeruginosa* is mediated by a range of quorum sensing molecules (QSMs) which are produced by both *P. aeruginosa* and *C. albicans* (Cugini *et al.*, 2007). The bulk of Gram-negative bacterial quorum sensing (QS)

systems utilize *N*-acyl homoserine lactones (AHL) signalling molecules. These molecules bind and activate their respective transcriptional activator (R protein) to induce expression of target genes (Figure 4) (de Kievit & Iglewski, 2000). When adequate population density is reached by bacterial cells, AHL concentrations are high enough to induce transcriptional changes. With low cell densities, the concentration of AHL is too low to elicit this effect.

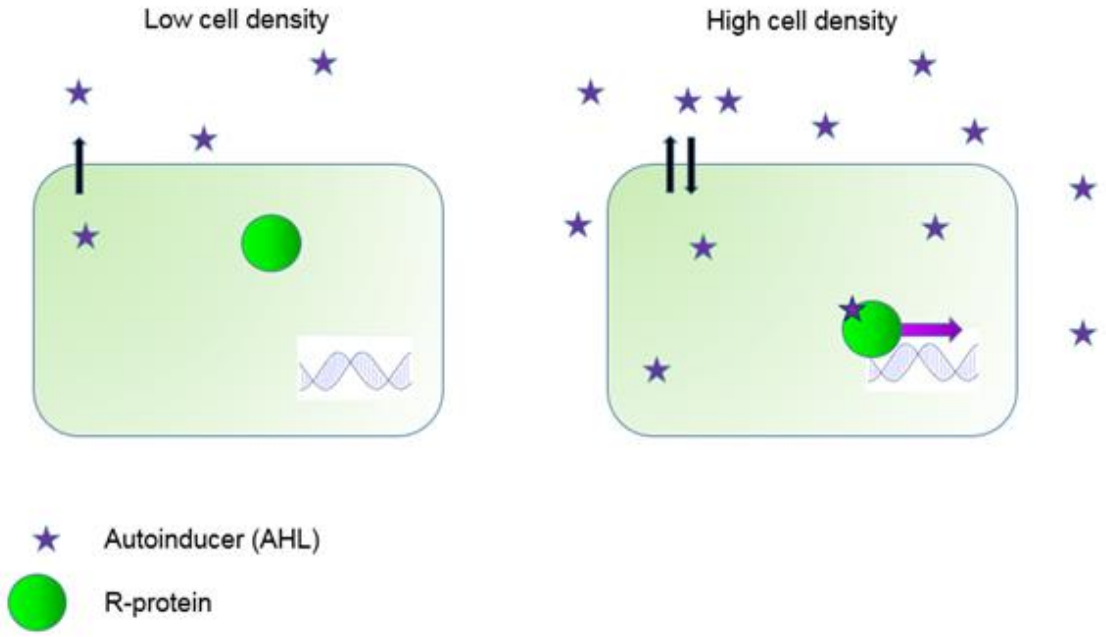


Figure 4. Illustration of quorum sensing by bacteria through N-acyl-homoserine lactone (AHL). When cell density is low, the concentration of AHL is too low to elicit an effect. However, during high cell density, these AHL elicit a transcriptional response in bacterial cells.

These QSMs have been shown to regulate up to 10 % of the genome of *P. aeruginosa* depending on culture conditions (Hentzer *et al.*, 2003; Wagner *et al.*, 2003). Two AHL-dependant QS systems were identified in *P. aeruginosa*, namely the *las* and *rhl* systems (de Kievit & Iglewski, 2000). 3-oxododecanoyl-L-homoserine lactone (3-oxo-HSL) is an autoinducer with its production directed by LasI autoinducer synthase (*las* QS system). The production of another autoinducer, butanoylhomoserine lactone, is similarly regulated by RhlI autoinducer synthase (*rhl* QS system). These bind and activate their respective transcriptional activators LasR and RhlR (Passador *et al.*, 1993; Pearson *et al.*, 1995). The structures of these autoinducers are shown in Figure 5.

a) b)

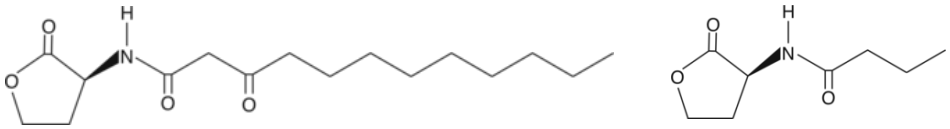


Figure 5. Structures of a) 3-oxododecanoyl-L-homoserine lactone and b) butanoylhomoserine lactone.

The QS molecule, 3-oxo-HSL, was studied for its effect on cell adherence in polymicrobial biofilms of *P. aeruginosa* and *C. albicans* (Ovchinnikova *et al.*, 2012). The study showed that mutant *P. aeruginosa* strains lacking the *lasI* gene for the LasI autoinducer synthase was unable to adhere to *C. albicans* hyphae, while a *P. aeruginosa* strain without the mutation was able to adhere to *C. albicans* cells. The study suggested that 3-oxo-HSL is needed for the adherence of *P. aeruginosa* cells to *C. albicans* hyphae, because 3-oxo-HSL is needed for the production of surface adherence proteins on *P. aeruginosa* cells. A study by McAlester *et al.* (2008) showed that if cell free supernatant (containing 3-oxo-HSL) from high 3-oxo-HSL producing *P. aeruginosa* strains is added to *C. albicans* cultures, the yeast to hyphal switch is inhibited in the *C. albicans* culture. *Pseudomonas aeruginosa* strains that produced low amounts of 3-oxo-HSL did not inhibit the yeast to hyphal switch when the supernatants of their cultures were added to *C. albicans* cultures, which suggests that 3-oxo-HSL affects yeast morphology in a dose dependant manner. To ensure that the 3-oxo-HSL was the cause of the inhibition of morphological switch, pure 3-oxo-HSL was added to a *C. albicans* culture with the same results obtained. The reaction of *C. albicans* towards 3-oxo-HSL may lead to the dispersal of *C. albicans* cells in the presence of *P. aeruginosa* (Morales & Hogan, 2010; Ovchinnikova *et al.*, 2012). These studies thus show that AHLs are not only important for bacterial communication, but are responsible for considerable interaction with other microorganisms such as *C. albicans*.

In addition to AHLs, a QS signal, 2-heptyl-3-hydroxyl-4-quinolone or *Pseudomonas* quinolone signal (PQS), is released in the late exponential phase of growth (Lépine *et al.*, 2003; Pesci *et al.*, 1999). The production of PQS is induced by the LasI/R system and inhibited by the RhlI/R system illustrated in Figure 6 (De Sordi & Mühlschlegel,

2009). Strikingly, PQS was shown to have both a damaging effect on *P. aeruginosa* through a pro-oxidative effect, as well as an anti-inflammatory effect (Haussler & Becker, 2008). This dual action is speculated to be similar to that of vitamin C. The authors speculate that this contradictory effect drives survival of the fittest through selection of phenotypic variants able to survive in stressful conditions and moulding populations sufficiently adapted. In addition to PQS, its immediate precursor, 2-heptyl-4-quinolone (HHQ), has been shown to repress *C. albicans* biofilm formation (Reen *et al.*, 2011).

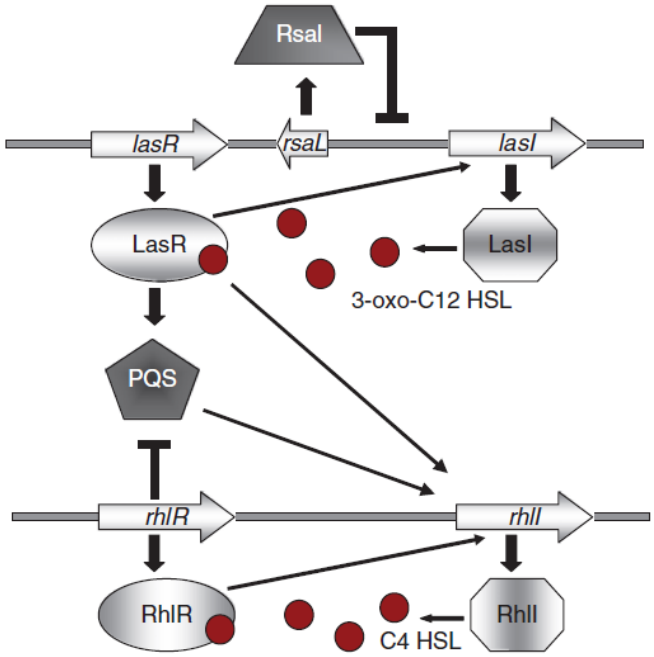
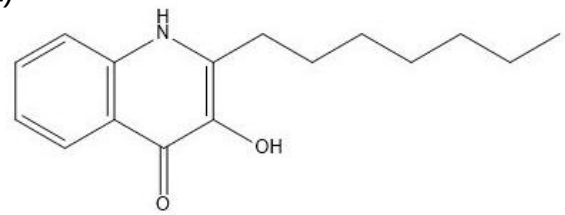


Figure 6. Schematic representation of regulation of *Pseudomonas* quinolone signal (PQS) through *lasI* and *rhlI* (De Sordi & Mühlschlegel, 2009).

Pseudomonas quinolone signal (Figure 7a) induces the formation of several virulence factors, including phenazine compounds like 1-hydroxyphanzine (1-HP), phenazine-1-carboxamine (PCN), 5-methylphenazine-1-carboxylic acid (5-MPCA), PYO and phenazine-1-carboxylic acid (PCA) (Phelan *et al.*, 2014). It also modulates swarming motility of *P. aeruginosa* (Déziel *et al.*, 2004; Ha *et al.*, 2011).

a)



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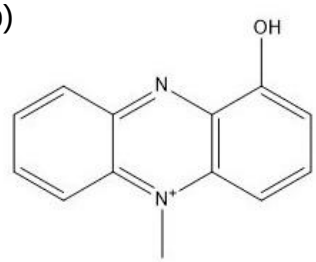


Figure 7. Structures of a) Pseudomonas quinolone signal and b) pyocyanin.

Pyocyanin or methyl-1-hydroxyphenazine (Figure 7b), is a chloroform soluble compound with a blue colour (Cox, 1986). It has been shown to be a QS molecule produced in the early stationary phase (Hernandez *et al.*, 2004; Price-Whelan *et al.*, 2007) and a study by Dietrich *et al.* (2006) suggested that PYO is a terminal signalling molecule that controls its own cycling. It plays a major role in maintaining NADH/NAD^+ ratio stability in *P. aeruginosa* cells when they encounter oxygen limiting conditions due to the limited fermentation capability of *Pseudomonas* (Dietrich *et al.*, 2006). Pyocyanin can then act as an alternate terminal electron acceptor and decrease the NADH/NAD^+ ratio in the stationary phase of growth. It can then later be reoxidized by oxygen when it becomes available and this could be a mechanism for the production of reactive oxygen species (ROS). In addition, PYO, PCA, 1-HP and PCN play extensive roles in the interaction between *Pseudomonas* species and eukaryotes, including fungal microorganisms (Kaleli *et al.*, 2006; Phelan *et al.*, 2014). Pyocyanin has also been shown to have an antimicrobial activity against a wide range of cells including a bactericidal effect against a wide variety of bacterial species with Gram-positive species being more susceptible than Gram-negative species (Hassan & Fridovich, 1980). Interestingly, *Pseudomonas* species seem to be resistant to this bactericidal effect (Baron & Rowe, 1981), but it is toxic to eukaryotic cells (O'Malley *et al.*, 2003). The mechanism of this effect is thought to be the ability of this compound to undergo non-enzymatic redox cycling intracellularly, resulting in the generation of ROS. This effect of PYO was later confirmed using A549 respiratory cells (Gloyne *et*

al., 2011). Another effect of PYO is the reduction in cyclic adenosine monophosphate (cAMP) (Kerr *et al.*, 1999). This enables PYO to inhibit the shift from yeast to hyphal morphology in *C. albicans*, as the yeast-mycelium transition is promoted by increased levels of intracellular cAMP (Kerr *et al*., 1999). A recent study indicated that the phenazines phenazine-1-ol, PCA and PCN has a synergistic effect with three antifungals: fluconazole, itraconazole and clotrimazole against *Candida* species (Kumar *et al.*, 2014). This then suggests that the presence of phenazine producing organisms such as *Pseudomonas* can drastically alter the treatment of simultaneous fungal infection.

Gibson *et al.* (2009) observed a red pigment with the co-incubation of *P. aeruginosa* and *C. albicans* produced during close proximity of the yeast and bacterial cells. This pigment was localized to fungal cells. The authors speculate that *C. albicans* enzymes participate in the formation of this product intracellularly. The precursor of this red pigment was identified as 5-MPCA through the use of *P. aeruginosa* strains with disruptions in the phenazine biosynthesis pathway. The presence of the red pigmented compound was linked to significant repression of *C. albicans* viability.

1.5.2.2. *The role of* Candida albicans *quorum sensing molecules during* in vitro *interaction*

Candida albicans also produces QSMs (Hornby *et al.*, 2001). The QSM, farnesol (Figure 8a), was shown to inhibit germ tube formation and also caused a morphological shift from predominantly mycelial state to predominantly yeast morphology, indicating the same effect as 3-oxo-HSL. The effect on the morphology of *C. albicans* is thought to be due to inhibition of the *Ras1*-controlled pathway involved in hyphal growth (Morales & Hogan, 2010). Recently, this QSM was identified to attract macrophages in hosts (Hargarten *et al*., 2015). The authors speculate that engulfment and movement of these immune cells then aid in dissemination as macrophages are killed by *C. albicans* after engulfment. Farnesol induces the generation of ROS which could play a role in the competition of *C. albicans* with bacteria. Resistance of *C. albicans* to oxidative stress has also been linked, in part, to farnesol (Westwater *et al.*, 2005). Interestingly, farnesol caused ROS generation through affecting the electron transport chain in *Saccharomyces cerevisiae* (Machida *et al.*, 1998).

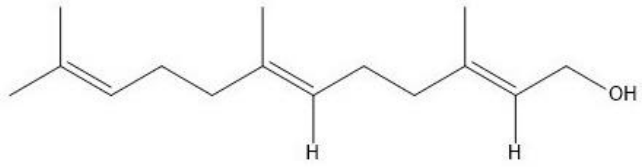


Figure 8: Structure of farnesol.

A study by Cugini *et al.* (2007) indicated that farnesol inhibits *P. aeruginosa* PQS and a subsequent virulence factor, PYO, whose production is controlled by PQS, in a dose dependant manner. Interestingly, there was no effect on the overall growth of *P. aeruginosa*. Additionally, when *P. aeruginosa* was co-cultured with *C. albicans,* reduction in PQS and PYO produced by *P. aeruginosa* was also observed, suggesting that high enough concentrations of farnesol is produced by *C. albicans* to exert an effect on *P. aeruginosa*. Later, the same research group found that *C. albicans* and its secreted factors increase PQS and butanoylhomoserine lactone in *lasR* defective mutants of *P. aeruginosa*, with a downstream increase in phenazine production (Cugini *et al.*, 2010). The authors speculate that oxidative stress may trigger downstream quorum sensing pathways.

In addition to decreasing PQS and PYO production, farnesol inhibits swarming motility in *P. aeruginosa* (McAlester *et al*., 2008). Rhamnolipids, a class of glycolipids, play a role in swarming motility and has been implicated as playing part in the development of ventilator associated pneumonia (VAP) (Köhler *et al.*, 2010). Due to the fact that rhamnolipid production is partly regulated by PQS, the mechanism of decreased swarming motility may be due to the reduction in PQS production by farnesol. Together with proteomic analysis by Jones-Dozier, (2008), a possible decrease in virulence of *P. aeruginosa* is evident when exposed to farnesol, due to alterations in protein expression of *P. aeruginosa* subjected to this compound.

1.5.2.3. Other factors influencing in vitro interaction

1.5.2.3.1. Iron availability

In a study by Purschke *et al.* (2012), *C. albicans* exhibited a lower metabolic activity in mixed biofilms with *P. aeruginosa* when compared to monomicrobial biofilms. Secretome analysis of the proteins of the mono- versus polymicrobial biofilms revealed an overall increase of secreted proteins of polymicrobial biofilms of *C. albicans* and *P. aeruginosa* compared to the monomicrobial counterparts. This increase was largely found to be due to increased secreted proteins by *P. aeruginosa*. Interestingly, a large proportion of the increased protein production was attributed to a siderophore, pyoverdine, specific to *Pseudomonas*. This increase in pyoverdine was thought to be due to the increased iron utilization by the two species in the mixed biofilm. This was confirmed by the addition of iron, which abolished the production of pyoverdine. The authors speculated that sequestration of the available iron by pyoverdine results in decreased availability to *C. albicans*, although *C. albicans* is able to utilize iron bound to certain other microbial siderophores. However, recent evidence suggests that this phenomenon may not be of importance during *in vivo* interaction (Lopez-Medina *et al.*, 2015). In this study, *C. albicans* secreted factors significantly reduced pyoverdine and another siderophore, pyochelin, expression by *P. aeruginosa* during gastrointestinal colonization in a murine model. This decrease of expression by *P. aeruginosa* was linked to diminished virulence of *P. aeruginosa*. The authors suspect the heterogeneity of the biofilms or difference in surface may cause the differential results when comparing *in vivo* and *in vitro* studies.

The importance of siderophores in interkingdom microbial interactions has further been evaluated using *P. aeruginosa* and *Aspergillus fumigatus* in terms of phenazine production by *P. aeruginosa* (Phelan *et al.*, 2014). This study indicated that phenazine production by *P. aeruginosa* is linked to siderophore production by *P. aeruginosa* as well as *Aspergillus fumigatus*, with an increase in triacetylfusarinine, a siderophore, production by *A. fumigatus* in the presence of phenazines. This interaction is however greatly dependant on the phenazine produced.

Recently, Trejo-Hernandez *et al*. (2014) also evaluated polymicrobial biofilms of *C. albicans* and *P. aeruginosa* through proteomic analysis. The authors found that

hypoxia influences the ability of *P. aeruginosa* to inhibit *C. albicans* filamentation *in vitro* compared to aerobic conditions. This was attributed to decreased AHL production by *P. aeruginosa* in the presence of *C. albicans*. Previously, it was shown that hypoxic conditions promote filamentation in *C. albicans* and reduces farnesol production (Dumitru *et al.*, 2004). Additionally, the authors also speculated that competition for iron may also be greater during hypoxia (Synnott *et al.*, 2010; Trejo-Hernandez *et al.*, 2014). Therefore, the interaction of *P. aeruginosa* with *C. albicans*, the concentration of oxygen and iron competition influence the production of AHLs (Trejo-Hernández *et al*., 2014). The authors also found that proteins known to play roles in iron uptake in *P. aeruginosa* were upregulated in mixed biofilms, confirming previous observations. Additionally, iron supplementation increased the growth of *P. aeruginosa* in mono- and polymicrobial biofilms, with this effect not seen with *C. albicans*. This increase in growth of the bacterium may exacerbate the destruction of the fungal population. Lamont *et al.* (2002) indicated that pyoverdine may act as a signalling molecule to regulate other virulence factors including exotoxin A and pyoverdine itself. Due to this possible increase in virulence by pyoverdine, the increased production of this siderophore in polymicrobial biofilms may increase the virulence of *P. aeruginosa* during co-incubation (Trejo-Hernández *et al*., 2014). Additionally, PQS as well as products of the PQS system, including rhamnolipids and PYO, were upregulated. A significant increase in *P. aeruginosa* mutability frequency was seen with a large number of antibiotic resistant mutant phenotypes arising over time. The authors speculate that the decreased catalase activity observed in polymicrobial biofilms may result in increased oxidative stress, concomitantly increasing mutability. In the case of *C. albicans*, the same trend was seen, with hypermutability arising with a high frequency of antimicrobial resistant phenotypes, possibly attributed to the increased oxidative stress caused by PYO. Additionally, *C. albicans* iron dependant processes, including aerobic respiration, were downregulated. Glycolytic enzyme activity in *C. albicans* was also altered, possibly leading to other pathways for energy utilization. To confirm the increased virulence of *C. albicans* and *P. aeruginosa* in polymicrobial biofilms *in vitro*, the authors utilized a rat infection model. *Candida albicans* was shown to promote pathogenicity of *P. aeruginosa*. Therefore, the ability of these pathogens to compete for iron may alter population dynamics and influence the nature of the interaction.

1.5.2.3.2. Bacterial cell wall components

In addition to various secreted factors produced by *P. aeruginosa* in polymicrobial interaction (Holcombe *et al.*, 2010), bacterial lipopolysaccharide (LPS) from various bacterial species has been shown to have adverse modulatory effects on *Candida* biofilms (Bandara *et al.*, 2010). The same group later confirmed these results by evaluating the effect of *P. aeruginosa* LPS on *C. albicans* hyphae formation and gene expression in biofilms, with an inhibition of hyphae formation seen in *C. albicans* (Bandara *et al.*, 2013). The study suggested a decrease of *C. albicans* biofilm metabolic activity, including glycolysis, and growth with the addition of high (100 µg/mL) concentrations of *P. aeruginosa* LPS. In addition to this, peptidoglycan was shown to trigger filamentation in *C. albicans* (Xu *et al.*, 2008).

1.5.2.3.3. Ethanol

Chen *et al.* (2014) evaluated the effect of *C. albicans*-produced ethanol on *P. aeruginosa* and found that ethanol stimulated adhesion and biofilm formation of *P. aeruginosa*. In addition, swarming motility by *P. aeruginosa* decreased and a stimulation of phenazine derivitization and production of 5-MPCA by *P. aeruginosa* in the presence of ethanol was observed. The authors speculate that there is a positive feedback loop where *C. albicans* ethanol production increases *P. aeruginosa* 5-MPCA production and biofilm formation. In turn, 5-MPCA stimulates ethanol production in *C. albicans* (Morales *et al.*, 2013). In addition, ethanol acts as an immunosuppressant during lung infection, possibly affecting the ability of the host to clear infection (Goral *et al.*, 2008).

1.5.2.3.4. Extracellular DNA

A recent study also identified extracellular DNA as a large factor in biofilm formation by *C. albicans* (Sapaar *et al.*, 2014). Low amounts of extracellular DNA (1.0 µg/mL) was shown to promote biofilm formation and increase biofilm stability, whereas higher concentrations (10 µg/mL) hampered the formation of biofilms by *C. albicans* as well as the stability of the biofilms. The study also indicated that the source of the extracellular DNA, whether it is from *C. albicans*, or from bacterial sources such as *P. aeruginosa*, does not matter. This increase in biofilm formation by *C. albicans* due to

extracellular DNA may increase the virulence of the fungus. In turn, due to previous research indicating that prior *C. albicans* colonization may increase susceptibility and severity of bacterial infections, this may also increase severity of bacterial infection by *P. aeruginosa* (Hamet *et al.*, 2012). Evidence also suggests that the concentration of extracellular DNA can reach 4 mg/mL in CF patient sputum samples, raising the question if this facet of interaction might have clinical relevance (Sapaar *et al.*, 2014). A summary of several facets of interaction between *C. albicans* and *P. aeruginosa* can be seen in Figure 9.

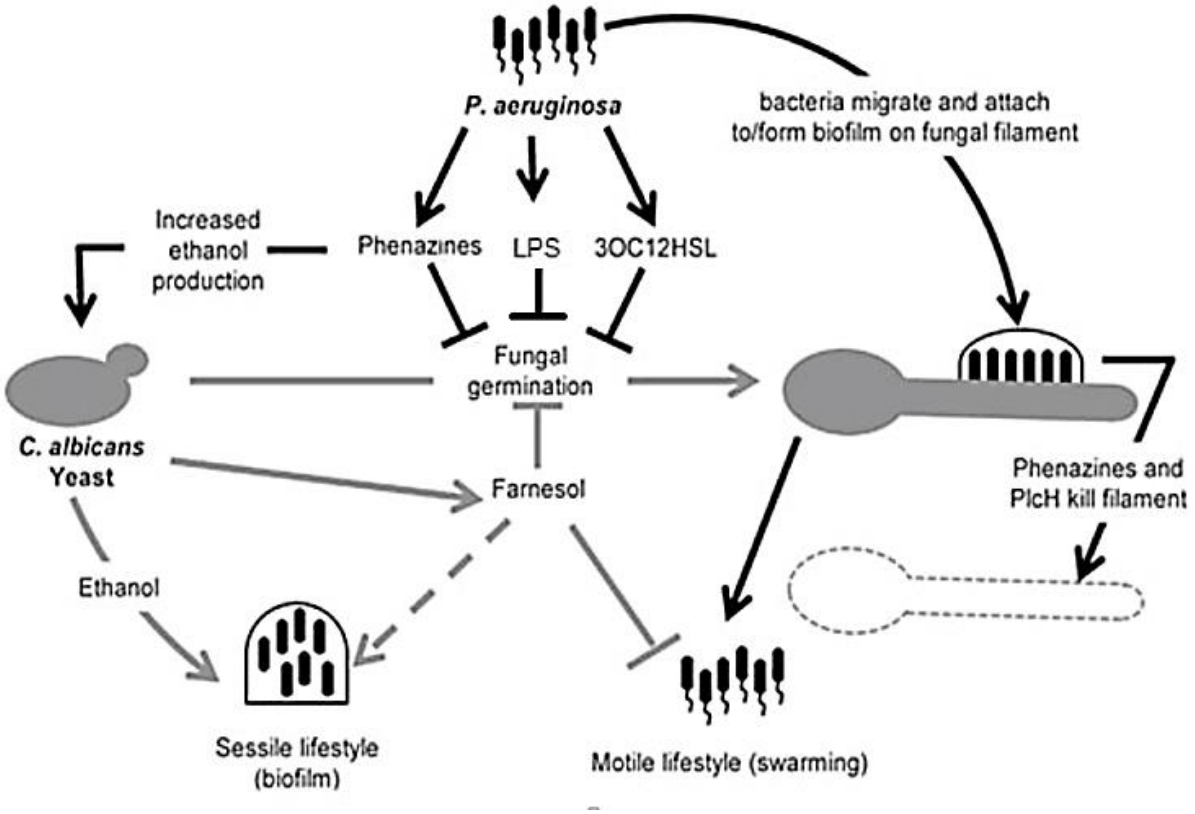


Figure 9. Illustration of competition between *Candida albicans* and *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* attaches to *C. albicans* hyphae and kills hyphal cells through secreted hydrolytic enzymes such as hemolytic phospholipase C (PlcH) and phenazines such as pyocyanin and 5-methylphenazine-1-carboxylic acid. 3-Oxo-homoserine lactone (3OC12HL) produced by *P. aeruginosa* and phenazines inhibit filamentation by *C. albicans*, similar to farnesol, produced by *C. albicans*. *Pseudomonas aeruginosa* lipopolysaccharide (LPS) inhibits *C. albicans* filamentation. Ethanol production is increased by the fungus, inhibiting the motility of *P. aeruginosa* (adapted from Lindsay & Hogan, 2014).

1.6. Interaction between *Pseudomonas aeruginosa* and *Candida albicans in vivo*

A high number of *C. albicans* nosocomial infections are polymicrobial (over 25 %) with *P. aeruginosa* a frequent co-isolate in blood stream infections and pneumonia (Lindsay and Hogan, 2014). Kerr (1994) was the first to describe the anticandidal activity of *P. aeruginosa in vivo*. The study evaluated lung infection of three surgery patients postoperatively with inhibition of *C. albicans* growth seen after *P. aeruginosa* colonization. This inhibition was confirmed with the regrowth of *C. albicans* seen after eradication of *P. aeruginosa*, even with fluconazole treatment. Additional *in vitro* susceptibility experiments confirmed the suppression of *Candida* growth by *P. aeruginosa* (Kerr, 1994). Gupta *et al.* (2005) evaluated 300 burn patients over two years and found the repression of *Candida* spp. in the presence of *P. aeruginosa*. Several studies also indicate that prior colonization of *Candida* may promote the susceptibility of the host to *P. aeruginosa* infection (Hamet *et al.*, 2012; Roux *et al.*, 2009; Xu *et al.*, 2014). Nseir *et al*. (2007) reported that antifungal treatment during *Candida* spp. tracheobronchial colonization may be associated with reduced risk for *P. aeruginosa* colonization. The case is strengthened by Azoulay *et al.* (2006), who reported a possible link between *Candida* colonization of the respiratory tract and an increased risk for *Pseudomonas* VAP. Roux *et al.* (2013) reported that *C. albicans* infection in a rat model was associated with increased interferon ɣ (INFɣ) (associated with the T_h1 response) and interleukin-17 (IL-17) (T_h17 response) in addition to decreased IL-2. The authors speculate decreased alveolar macrophage phagocytosis of bacteria after prior *C. albicans* colonization due to IFNɣ.

Remarkably, contradictory results to the notion that *P. aeruginosa* infection is more aggressive after prior *C. albicans* colonization, was provided by Ader *et al.* (2011). In a murine model, *C. albicans* short term colonization prior to *P. aeruginosa* colonization caused a reduction in *P. aeruginosa* bacterial load compared to the absence of *C. albicans* colonization. Additionally a reduction in *P. aeruginosa* induced lung injury was observed with the prior colonization of *C. albicans*. Interestingly this effect was reversed with treatment by the antifungal caspofungin during *C. albicans* colonization. *Candida albicans* initiates alveolar innate immunity in a murine model, protecting the host against subsequent *P. aeruginosa* infection (Mear *et al.*, 2014). The authors showed that prior *C. albicans* infection induces IL-17 and IL-22 secretion through

innate lymphoid cell recruitment. The cytokines produced, induce the production of antimicrobial peptides as well as the mobilization of phagocytic cells. In a murine gut model, *C. albicans* secreted factors inhibited expression of siderophores as well as cytotoxic molecules by *P. aeruginosa*, reducing the virulence of the bacteria (Lopez-Medina *et al.*, 2015). Due to this, increased survival of the host was observed during co-incubation of *P. aeruginosa* with *C. albicans*. Interestingly, Neely *et al*. (1986) demonstrated increased mortality in a murine model when *C. albicans* infection was preceded by *P. aeruginosa*. This reciprocal effect may also be due to alterations in innate immune response, as Faure *et al*. (2014) reported that the *P. aeruginosa* type III secretion system induced IL-18 secretion, causing substantial neutrophil recruitment and host cell damage, and decreased IL-17 secretion in a mouse model, possibly leading to the reduced clearance of pathogens.

1.6.1. Cystic fibrosis

The co-infection of *P. aeruginosa* and *C. albicans* has been well documented in cystic fibrosis (CF) patients and thus is relevant to this discussion. Cystic fibrosis is one of the most commonly encountered autosomal recessive disorders, with the occurrence varying in race (Andersen, 1938). The disease is caused by a genetic disorder where a mutation exists in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Delhaes *et al.*, 2012).

Cystic fibrosis is a disease with two pathophysiological properties, namely pancreatic insufficiency with malnutrition and airway infections due to extremely viscous secretions (Andersen, 1938). The viscous secretions lead to blockage of pancreatic ducts and autodigestion of the pancreas due to the inability to secrete digestive enzymes. The increased viscosity of the lung secretions is thought to be due to the increased sodium absorption of the respiratory epithelium and the defective regulation of chloride ion secretion (Gilligan, 1991). This is thought to be the reason why CF patients have comparatively dehydrated surface liquid which leads to defective mucociliary clearance. The thick bronchial mucus traps viral particles, fungal spores and bacteria and provides a suitable environment for the fungal spores and bacteria to grow, causing infection (Delhaes *et al*., 2012). Ninety percent of deaths in CF are due to pulmonary dysfunction and in effect, chronic airway infection (Gilligan, 1991).

The infection is characterized by fever, weight loss, increased cough, a change in the appearance of sputum and the progressive deterioration of the function of the lungs. The pulmonary dysfunction occurs in repeated episodes between relatively 'healthy' times. With each exacerbating cycle the lung function declines, ultimately leading to pulmonary failure and death. Increased oxidative stress is observed in CF patients (Brown & Kelly, 1994). Patients with CF exhibit increased lipid and protein peroxidation with susceptibility to oxidative damage, however this lipid and protein peroxidation varied between patients. A contributory factor may also be malabsorption of nutrients facilitating protection against oxidative stress.

A study by Güngör *et al.* (2013) evaluated the most prevalent fungal colonisations in Turkish CF patients. The most prevalent fungal microorganisms isolated from these CF patients, were shown to be *C. albicans* at 62.5 % (30 patients) with the most frequently isolated filamentous fungus being *Aspergillus fumigatus* (10.4 %). Fungal-bacterial co-colonization in the CF patients was shown to be 100 % in *A. fumigates* infections and 98.2 % in *C. albicans* infections. The most frequent bacterial co-colonizer of CF patients with *C. albicans* infections was found to be *Staphylococcus aureus* (53.57 %), with *P. aeruginosa* at 48.21 %, *Staphylococcus maltophilia* at 16.07 % and *Haemophilus influenza* at 5.97 %. Other similar studies have also implicated *S. aureus* and *P. aeruginosa* as the most prevalent bacterial species isolated (Valenza *et al.*, 2008; Williamson *et al.*, 2011). Several studies addressed the correlation between *C. albicans* and *P. aeruginosa* in CF infection, with a recent study suggesting a significant correlation (Conrad & Bailey, 2015). Discrepancies may arise due to method of analysis and the population analysed. Although there has been a debate over the presence of *P. aeruginosa* as an indication of severe decline in lung function, recent evidence cannot conclusively identify this (Paganin *et al.*, 2015).

1.7. Production and role of oxylipins during infection

Lipids have crucial cellular significance, forming cellular membranes, as well as acting as cellular signals (Reviewed by Tehlivets *et al.*, 2007). The latter is of great importance in multicellular eukaryotic organisms such as mammals, as lipids act as immunomodulatory signals, for example, arachidonic acid (AA) and its various metabolites. Conserved mechanisms for synthesis of fatty acids, as well as elongation of longer fatty acids from short chain fatty acids exist (Tehlivets *et al*., 2007). The

mechanism for elongation consists of acetyl-CoA, which is carboxylated by CO_2 to malonyl-CoA. This is then used as a donor of two carbon atoms to elongate fatty acids in a stepwise manner through various reactions. These are mediated by fatty acid synthases as well as elongases. The process of fatty acid synthesis and elongation is however an energy consuming process. Cells are nevertheless able to take up various fatty acids for use as a carbon source to be modified by various enzymes to fulfil cellular requirements (Hou, 2008; Tehlivets *et al*., 2007).

Oxylipins, the oxygenated products after lipid peroxidation of unsaturated fatty acids, are widely distributed in mammalian and non-mammalian organisms including plants and even fungi (Brodhun & Feussner, 2011; Deva *et al.*, 2000). These molecules are highly bioactive, playing significant roles in cellular signalling. Although these molecules have been well studied in mammals and plants, their roles in fungi are not nearly as well characterized.

Arachidonic acid, or 5,8,11,14-eicosatetraenoic acid (Figure 11a), a major constituent of the mammalian host cell phospholipids, together with its wide range of metabolites (termed eicosanoids), have substantial roles as lipid signals (Chilton *et al.*, 1996). Arachidonic acid can be obtained by mammalian cells through diet, or the elongation of C-18 fatty acids such as linoleic acid. As the constituent of cellular membranes, it is predominantly found in the sn2 position of 1-ether-linked phospholipids with incorporation into inflammatory cells through CoA-dependant acyl transferases. The autotoxic response arising from excessive amounts of AA and metabolites is circumvented through modulation by acylation of AA. The amount of AA released during infection is attributed to phospholipase A_2 activity (Dennis & Norris, 2015). In disease, lipids may be a contributory factor. In testament to this, in CF an imbalance is seen in fatty acid levels as well as dysfunction in inflammatory regulation (Zaman *et al.*, 2010). Linoleic acid supplementation in CF increased AA and consequently the immunomodulatory molecules arising from its metabolism. Eicosanoids play a crucial role in the innate immune response (Rodríguez *et al.*, 2014). Signalling cascades, including recognition of pathogen-associated molecular patterns (PAMPs), modulate phospholipase activity, inducing the release of large quantities of AA.

The metabolism of AA to form lipid mediators involves various pathways in vertebrates, including the action of cyclooxygenases (COX), lipoxygenases (LOX) monooxygenases (CYP450) and non-enzymatic (NE) pathways (Figure 10).

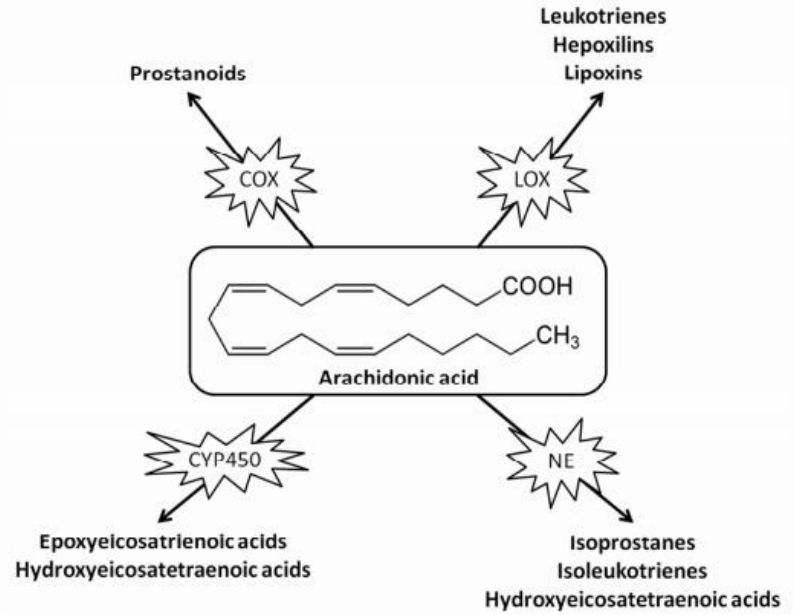


Figure 10. Pathways for eicosanoid synthesis from arachidonic acid (Ells, 2011)

Cyclooxygenases (COX), or prostaglandin endoperoxide synthases are enzymes catalysing the insertion of two oxygen atoms into AA (Marnett *et al*., 1999). In mammalian cells, two isoforms exist, namely, COX-1, which is constitutively expressed, and COX-2, which is inducible. The initial reaction of AA oxidation, mediated by COX, yields prostaglandin G_2 (PGG_2) (Rodriguez *et al*., 2014). This is accomplished by hydrogen abstraction at carbon 13 of AA, with subsequent oxygen insertion at C-9 and C-11. This is followed by the formation of a ring structure and oxygen insertion at C-15. Through peroxidase activity, PGG_2 is reduced to PGH_2. This is accomplished through reduction of C-15 hydroperoxide to a hydroxide. This product serves as a precursor for various other immunomodulatory compounds, including prostaglandins as well as thromboxanes. For example, further action by prostaglandin E synthase converts PGH_2 to PGE_2.

Lipoxygenases (LOX) are a large group of dioxygenases that catalyse oxygen insertion into polyunsaturated fatty acids (PUFAs) in animals, plants as well as microorganisms (Kuhn & O'Donnell, 2006). The reaction of oxygenation consists of various steps, starting with hydrogen abstraction, followed by radical rearrangement

and the insertion of oxygen. In stressful cellular reactive potential circumstances, such as hypoxia, LOX preferentially catalyse hydroperoxide activity with resultant radical production. Due to the detrimental radicals produced by LOX hydroperoxidation, suicide inactivation in animal cells has been proposed to limit damage after cycles of activity. In addition to oxygenation and hydroperoxidation, LOXs also catalyse the synthesis of leukotrienes, lipoxins and hepoxilins through the combination of various enzymatic activities (Dennis & Norris, 2015; Kuhn & O'Donnel, 1996).

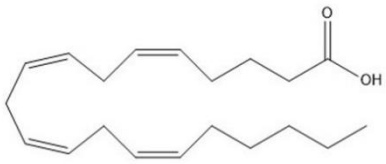
LOXs are classified by the carbon atom number at which oxygen is inserted into the carbon backbone of fatty acids, for example 5-LOX and 15-LOX insert oxygen at number 5 and 15 carbon atoms, respectively (Kuhn & O'Donnel, 2006). Various isoforms of LOX exist with different stereospecificity and activities, for example 12/15-LOX catalyses oxygenation and hydroperoxidation of PUFAs at the 12 or 15 carbon position. Various products of LOX have also been implicated in anti-inflammatory responses in neutrophils. Many LOX products, including hydroperoxyeicosatetraenoic acids and hydroxyeicosatetraenoic acids are intermediate products leading to the formation of lipoxins and leukotrienes (Dennis & Norris, 2015). The interaction of COX- and LOX-derived lipid mediators as well as the combination of these two pathways leads to the modulation of the inflammatory response (Dennis & Norris, 2015). The non-steroidal anti-inflammatory drug (NSAID), acetylsalicylic acid (ASA), was shown to acetylate COX isozymes leading to the formation of 15(*R*)-HETE, which acts as substrate for LOX for the formation of lipoxins (Serhan, 2002). These lipoxins are potent anti-inflammatory molecules, inhibiting neutrophil recruitment and leukotriene formation. In addition to these pathways, cytochrome P450s are also responsible for the formation of epoxyeicosatetraenoic acids from AA, with concurrent modification to diHETEs, playing differential effects on the host.

The effects of eicosanoids on host cells are highly dependent on the type of target tissue and the physiological state of these tissues (Dennis & Norris, 2015). Considerable research is being done to determine the eicosanoids that play a role in host protection against pathogens during infection as they can enhance the clearance of pathogens. For this review, the focus will fall on lipid mediators in terms of *C. albicans* and *P. aeruginosa* infection.

1.7.1. Role of mammalian oxylipins during Pseudomonas aeruginosa *infection*

Several studies have addressed the effect of various invading pathogens on the production of PGE_2 to gain a better understanding of the immunological aspects of infection. This gaining of knowledge is of great interest in treating bacterial infections. Prostaglandin E_2 (PGE_2, Figure 11d), an AA metabolite, plays a crucial role in infection. The recent identification of PGE_2 produced *in vitro* by *Saccharomyces cerevisiae* in fermentation products adds to the importance of this lipid mediator in human health (Chikhalya, 2013). Immune cells are the main source of PGE_2 in mammals, although this compound is also produced by various other cell types (Agard *et al.*, 2013; Kalinski, 2012). It elicits a response through activation of four receptors in mammalian cells, designated EP1 to EP4, with the effect dependant on the receptor activated. The effects of PGE_2 vary from anti- to proinflammatory effects. The infection of various bacterial species is discussed by Agard *et al*. (2013) in terms of PGE_2. *Pseudomonas aeruginosa* pulmonary infection is associated with an overproduction of PGE_2 by the host and concurrent decrease in phagocytosis by alveolar macrophages (Agard *et al.*, 2013; Ballinger *et al.*, 2006). This increase in PGE_2 is due to the large amount of AA released during *P. aeruginosa* infection, mediated by ExoU, an intracellular phospholipase (Agard *et al.*, 2013; König *et al.*, 1996; Sadikot *et al.*, 2007; Saliba *et al.*, 2005). This potent virulence factor plays a crucial role in initial infection and infiltration of *P. aeruginosa* through causing significant release of AA through phospholipase activity. The absence of ExoU in *P. aeruginosa* was linked to diminished severity of infection and PGE_2 production. The importance of PGE_2 during *P. aeruginosa* infection was seen when a COX-2 inhibitor was employed, resulting in a decrease in severity of infection by this pathogen. Several other virulence factors also elicit changes in PGE_2 levels. The QSM 3-oxo-homoserine lactone produced by *P. aeruginosa* was shown to induce COX-2 and therefore PGE_2 production in human lung fibroblasts (Smith *et al.*, 2002). Similarly, *P. aeruginosa* PYO and LPS increased the release of PGE_2 and IL-6 in urothelial cells in a concentration dependant manner (McDermott *et al.*, 2013).

a)



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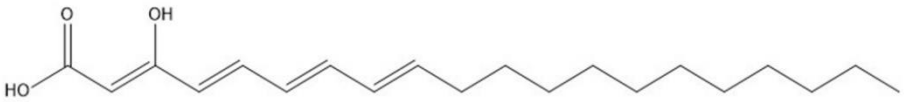

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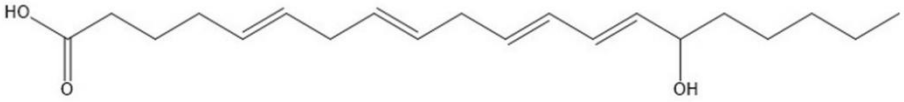

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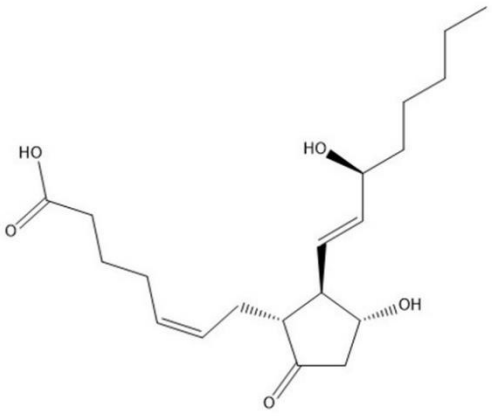

Figure 11: Structures of a) arachidonic acid (AA); b) 3-hydroxyeicosatetraenoic acid (3-HETE); c) 15-HETE and d) Prostaglandin E_2

1.7.2. Role of Pseudomonas aeruginosa *oxylipins during infection*

A number of microorganisms including bacteria are able to form eicosanoids from fatty acid precursors (Lamacka & Sajbidor, 1995). Although the presence of LOX in plants and animals has long been known, their presence in lower eukaryotes and prokaryotes has only recently been established with *P. aeruginosa* one of the few bacteria with typical LOX genes. *Pseudomonas aeruginosa* has been found to possess a secretable 15-LOX, homologous to mammalian LOX, producing 15-hydroxyeicosatetraenoic acid

(15-HETE, Figure 11c) which is similar to host 15-HETE and elicits anti-inflammatory effects on the host, through acting as a substrate for lipoxin formation (Serhan, 2002; Vance *et al.*, 2004). It is tempting to speculate that the action of *P. aeruginosa* 15-LOX during infection is able to produce intermediate eicosanoids for the formation of lipoxins or other eicosanoids by host cells. The formation of these lipoxins may alter the severity of infection through inhibiting neutrophil recruitment and generation of leukotrienes (Serhan, 2002). However, the role of this *P. aeruginosa* 15-LOX in infection has not been addressed. In addition, the production of prostaglandins and prostaglandin-analogue compounds have been identified in *P. aeruginosa* (Lamacka & Sajbidor, 1995), however, the effect of these compounds during infection has also not been addressed.

Pseudomonas aeruginosa is able to utilize other fatty acids to produce a range of products including dihydroxy unsaturated fatty acids such as 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) (Hou, 2008). This compound has been shown to have antimicrobial activity towards *Bacillus subtilis* and *C. albicans*. A study by Giamarellos-Bourboulis *et al.* (1998) suggested that gamma-linolenic acid (GLA) in concert with AA exhibit a bactericidal effect on *P. aeruginosa* strains. Additionally, the exposure of 19 *P. aeruginosa* strains to both these PUFAs caused a development of resistance against various aminoglycosides and β-lactams. This phenomenon is thought to be mediated by the action of peroxides (Giamarellos-Bourboulis *et al.*, 1998).

1.7.3. Role of mammalian oxylipins during Candida albicans *infection*

The alteration of immune response is not unique to bacteria, but plays a significant role as a virulence factor during *C. albicans* infection. The presence of *C. albicans* in respiratory tract secretions of VAP has been speculated to be linked to worse clinical outcomes (Delisle *et al.*, 2011). Arachidonic acid, found in significant quantities in mammalian cells, is not found in *C. albicans*, but this pathogen is able to utilize external AA, liberated from host cells by phospholipase A_2 (Brash, 2001; Castro *et al.*, 1994; Filler *et al.*, 1994; Parti *et al.*, 2010). *Candida albicans* possesses enzymes with phospholipase A_2 activity, also possibly contributing to the release of AA during tissue invasion (Niewerth & Korting, 2001; Theiss *et al.*, 2006). In addition, *C. albicans* can induce significant production of PGE_2 by mammalian cells (Deva *et al.*, 2001; Filler *et*

al., 1994). *Candida albicans* mannans, forming part of the *C. albicans* cell wall, can directly induce PGE_2 production by mammalian cells (Smeekens *et al.*, 2010). The induction of the release of AA and the eicosanoid metabolites from alveolar macrophages has been shown to be partly regulated by mannose and β-glucan receptors interacting with fungal cell wall components and have also been shown to inhibit phagocytosis during *C. albicans* infection (Castro *et al*., 1994). Eradication of infection by the immune system is highly dependent on the balance of T_h1 and T_h2 responses (Romani, 2000). The T_h1 response is associated with the removal of pathogens through phagocytosis, in contrast to the hampering of this effect during the T_h2 response. Prostaglandin E_2 is able to modulate this balance and frequently promotes colonization of pathogens such as *C. albicans*, as well as causing tissue eosinophilia. Prostaglandin E_2 production in response to *C. albicans* invasion also induces the protective T_h17 response in mammalian systems (Smeekens *et al*., 2010).

1.7.4. Role of Candida albicans *oxylipins during infection*

Arachidonic acid can be used as a carbon source by *C. albicans* at low concentrations (maximum at 3 μM) with higher concentrations (20 μM) showing an inhibiting effect on growth (Deva *et al.*, 2000). The authors also identified a 3-hydroxy fatty acid produced by *C. albicans* from AA namely 3,18-dihydroxy-5,8,11,14-eicosatetraenoic acid (3,18-diHETE), that is associated with hyphal forms and may play a role in adhesion during infection (Deva *et al.*, 2000; Ells *et al.*, 2012). Additionally, the authors showed that most AA is metabolized by non-mitochondrial pathways, concurrent with the speculation that fatty acids are metabolized by peroxisomal β-oxidation in yeasts.

Aspirin-sensitive 3(*R*)-hydroxyoxylipins (3-HETE, Figure 11b), produced by *C. albicans* was later speculated to be linked to *C. albicans* morphogenesis (Deva *et al.*, 2001). These 3-HETEs can also serve as substrate for COX-2 in mammalian cells to form various 3-hydroxyeicosanoids due to the similarity to AA (Ciccoli *et al.*, 2005). Further analysis of one of these metabolites, 3-OH-PGE_2, indicated a similar or even more robust effect on mammalian cells eliciting a pro-inflammatory response. This could indicate that the ability of *C. albicans* to form 3-HETE from exogenous AA during infection could lead to the production of 3-hydroxyeicosanoids with significant immunomodulatory capabilities.

The anti-inflammatory lipid, resolvin E_1, which *Candida albicans* is able to produce from eicosapentaneioc acid, plays a role in the interspecies signalling via lipids (Haas-Stapleton *et al.*, 2007). This compound was shown to protect *C. albicans* against host immunity at low doses, however, at higher concentration, this protective effect is lost. An important aspect of the determination of *C. albicans* virulence was the identification of a PGE_2 cross reactive compound produced by *C. albicans* from exogenous AA (Noverr *et al.*, 2001). This compound was later identified as PGE_2, identical to the host product (Erb-Downward & Noverr, 2007). This was especially surprising as *C. albicans* does not possess homologues to COX present in mammalian cells responsible for the formation of PGE_2. The involvement of various enzymes or homologous enzymes was speculated in the production of PGE_2. A multicopper oxidase homolog (Fet3p), fatty acid desaturase homolog (Ole2p) and cytochrome P450s has been shown to be involved in the production of PGE_2 by *C. albicans* (Ells *et al.*, 2011; Erb-Downward & Noverr, 2007; Krause *et al.*, 2015). Biofilm formation in *C. albicans* as well as germ tube formation has been shown to be enhanced by the addition of synthetic PGE_2. Various COX-inhibitors drastically affect the formation of biofilms and germ tubes (Alem & Douglas, 2004). Acetylsalicylic acid had the most drastic effect on biofilm formation. Interestingly, the simultaneous addition of PGE_2 abolished the inhibitory effect of ASA on *C. albicans* biofilm formation. In a later study by the same researchers, a comparison of PGE_2 production by *C. albicans* planktonic cells and biofilms showed a significant increase in PGE_2 production of *C. albicans* biofilms (Alem & Douglas, 2005). In addition, various COX-inhibitors, namely ASA, diclofenac and etodolac, significantly decreased the production of PGE_2 by *C. albicans* biofilms. These findings suggest a possible link between prostaglandin production and biofilm formation. Deva *et al*. (2001) reported significant decrease in yeast to hyphal transition by *C. albicans* with the addition of ASA. This morphological effect was attributed to the possible suppression of 3(*R*)-hydroxyoxylipins.

Through the aforementioned importance of lipids in infection, it is not surprising that lipids are under the spotlight for treating infections. In a recent study the simultaneous addition of the AA and fluconazole or terbinafine indicated an increase in PGE_2 production by *C. albicans* and *Candida* non-*albicans* biofilms (Nath Mishra *et al.*, 2014). Several studies have also indicated the increased susceptibility of *C. albicans* to antifungals in the presence of polyunsaturated fatty acids (Ells *et al.*, 2009; Nath

Mishra *et al.*, 2014; Thibane *et al.*, 2012). This was attributed to possible changes in membrane fluidity and/or increased oxidative stress.

Considering the information regarding the direct and indirect interaction of *C. albicans* and *P. aeruginosa*, as well as the information regarding the role of eicosanoids during single species infection, several questions can be asked relating to polymicrobial infection with *C. albicans* and *P. aeruginosa*. Figure 12 indicates the interaction of *C. albicans* and *P. aeruginosa* with host cells with the focus on eicosanoid production. In the figure, arrows indicate the production or increase of an eicosanoid, whereas a question mark indicates an unknown effect or interaction.

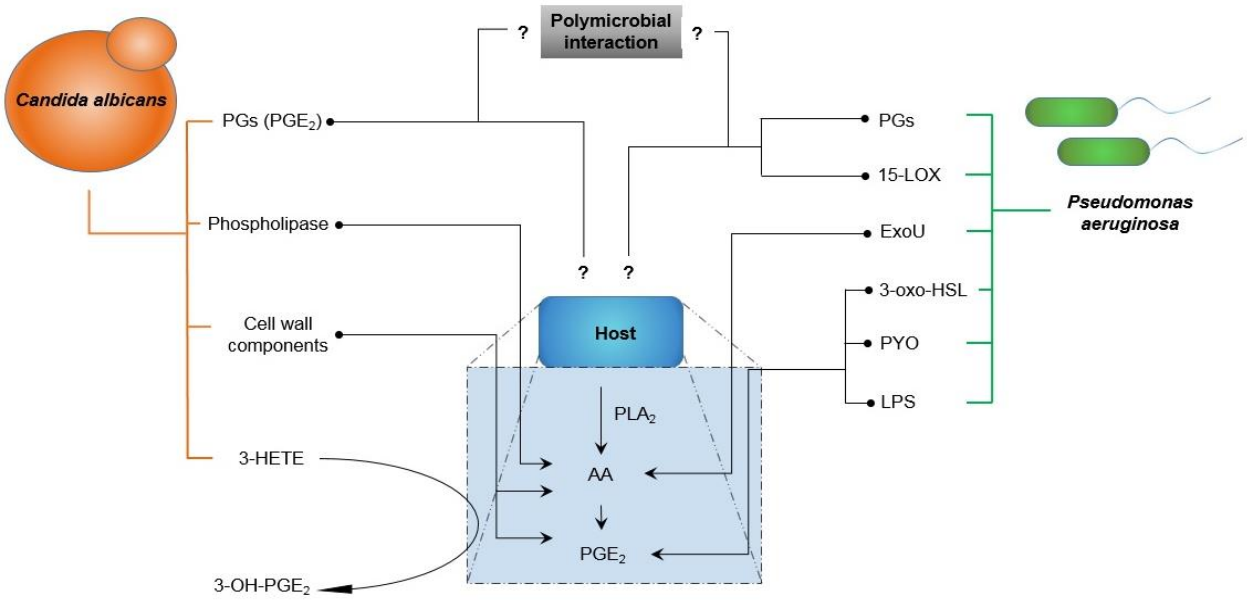

Figure 12: Interaction of *Candida albicans* and *Pseudomonas aeruginosa* with host cells highlighting the production of eicosanoids. Arrows indicate production/increase. Question marks indicate uncharacterized interactions (AA – Arachidonic acid; PLA_2 – Phospholipase A_2; PGs – Prostaglandins; PGE_2 – Prostaglandin E_2; ExoU – Exotoxin U; PYO – pyocyanin; LPS – lipopolysaccharide; 3-HETE – 3-hydroxyeicosatetraenoic acid; 3-oxo-HLS - 3-oxododecanoyl-L-homoserine lactone; 15-LOX – 15-Lipoxygenase).

1.7.5. Role of oxylipins in polymicrobial infection

Although information is available regarding the eicosanoids produced, and their effect during monomicrobial infection, very little is known regarding the effect of microbially-produced eicosanoids on co-infecting pathogens, as well as on the host. In a study by Peters & Noverr (2013), the role of eicosanoids during polymicrobial infection in another microbial interaction became evident. In this study, a murine model was used to elucidate the effect of polymicrobial interactions using *C. albicans* and *S. aureus*. Through their work, it was evident that polymicrobial infections with these organisms resulted in a significant increase in morbidity and mortality during murine peritoneal infection, with this effect not seen with monomicrobial infection. In addition, disease progression and microbial load in infected mice was significantly higher, compared to monomicrobial infections. The authors also detected a significant increase in pro-inflammatory chemokines released, as well as recruitment of polymorphonucleocytes. To determine the effect of the pro-inflammatory response on infection dynamics, indomethacin was used. Indomethacin, a non-selective COX inhibitor, similar to ASA, caused a significant reduction in morbidity and mortality in polymicrobial infection, as well as a reduction in cytokine release. Indomethacin also caused a significant reduction in microbial load during co-infection. This effect was not seen with monomicrobial infection. *In vitro* experiments showed that this effect was not due to inhibition of growth of *C. albicans* and *S. aureus*. The authors also observed a significant increase in PGE_2 release by the host cells in response to polymicrobial infection with indomethacin also causing a significant reduction in PGE_2. The authors suggested that PGE_2 may play a role in the non-protective pro-inflammatory response during polymicrobial infection, as PGE_2 induces the release of several cytokines. To further determine the effect of PGE_2 in co-infection, mice were co-infected with *C. albicans* and *S. aureus* and treated with indomethacin as well as PGE_2. Under this circumstance, high morbidity and mortality was observed in the mice, although they were treated with indomethacin. In the absence of infection, with only the administration of indomethacin and PGE_2, no mortality was observed. Further, the administration of PGE_2 caused a significant increase in microbial burden during co-infection. During this study, the production of eicosanoids, such as PGE_2, by *C. albicans* itself was not addressed. Interestingly, Krause *et al*. (2015) indicated that *C. albicans* PGE_2 production significantly enhances *S. aureus* biofilm growth *in vitro*. The

authors also found that co-incubation of *C. albicans* with *S. aureus* did not elicit an increase in PGE_2 production by *C. albicans*.

1.8. Conclusions

In addition to the ample evidence supporting the interaction of *C. albicans* and *P. aeruginosa*, not only with their host, but also with each other, it is evident that the interaction is multifaceted. Various virulence factors such as morphogenesis, hypermutabililty and secreted factors (including lipid mediators) affect and damage hosts to facilitate rapid and aggressive colonization and infection. Any disequilibrium in host defences, such as in CF, immune disorders, and breaching of host barriers, is rapidly exploited by these opportunistic pathogens.

On their own, *C. albicans* and *P. aeruginosa* pose a rather frightening risk to compromised hosts. Recent studies however illuminate that the interaction of these microorganisms is antagonistic with substantial damage caused to the host during the chemical war they play part in. Host immunity also plays a large role in damage to its own tissue due to radical generation by the innate immune response and various lipid mediators. This is even more evident in the work by Peters & Noverr (2013). It is also evident that various secreted factors of *C. albicans* (including farnesol and various hydrolytic enzymes) and *P. aeruginosa* (including AHL's, PQS, PYO and various peptides) form a large amount of radicals and can elicit oxidative damage to each other, as well as the host.

Although the effect of lipid mediators have been studied between single pathogens and hosts, a gap in knowledge still has to be filled describing the effect of lipid mediators in polymicrobial infection of *C. albicans* and *P. aeruginosa*. Polymicrobial infection by *C. albicans* and *S. aureus*, however, highlight the importance of lipid mediators, such as PGE_2, in infection by multiple microbes. Due to the fact that *C. albicans* and *P. aeruginosa* are both able to produce significant amounts of prostaglandins and other eicosanoids with exogenous AA, this could affect the dynamics of this co-infection as well as host survival during infection. This warrants investigation to fully understand the interaction of *C. albicans* and *P. aeruginosa* in terms of eicosanoids, as well as the effect of these eicosanoids in the host during co-infection.

1.9. References

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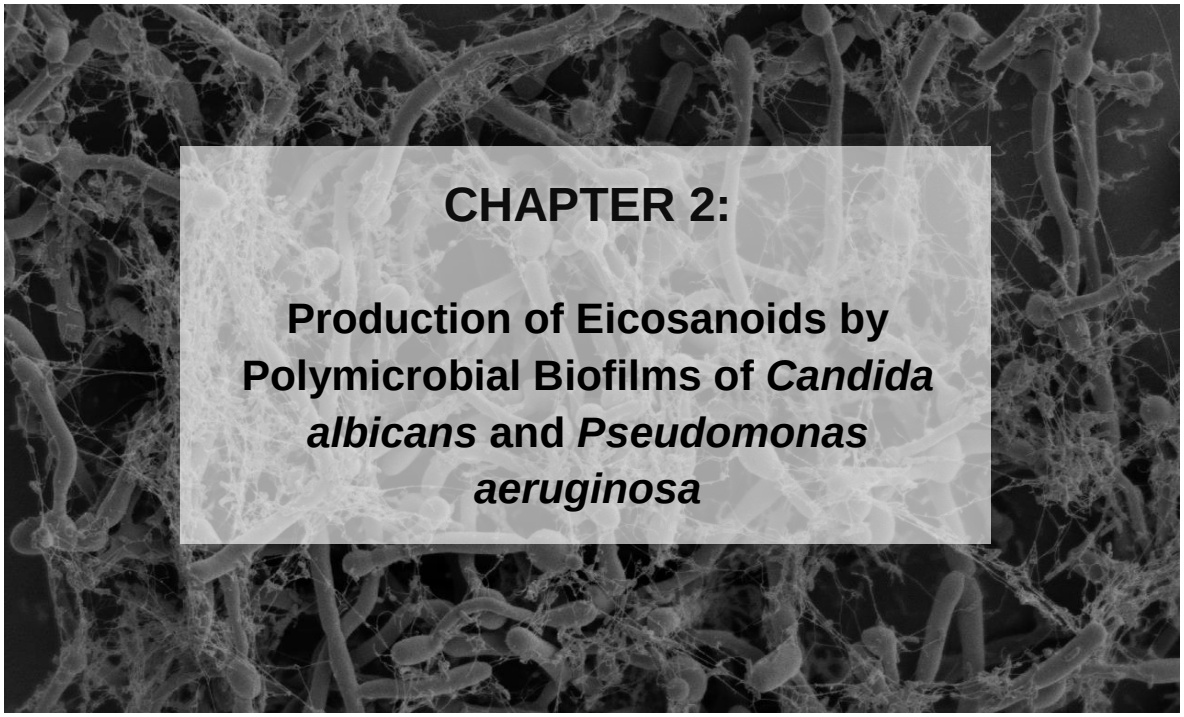

CHAPTER 2:

Production of Eicosanoids by Polymicrobial Biofilms of *Candida albicans* and *Pseudomonas aeruginosa*

2.1. Abstract

Pseudomonas aeruginosa and *Candida albicans* are opportunistic pathogens causing a range infections in a nosocomial setting as well as in immunocompromised individuals. Previous research indicates significant interaction between these two microorganisms, as well as between them and hosts during infection. Arachidonic acid metabolites, termed eicosanoids, play an important role in infection and both these pathogens are capable of producing eicosanoids from host derived arachidonic acid. Therefore, this study evaluated the production of eicosanoids by polymicrobial biofilms of *P. aeruginosa* and *C. albicans* with the focus on prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and 15-hydroxyeicosatetraenoic acid (15-HETE) *in vitro*. Production of PGE_2 by *C. albicans* was confirmed, together with $PGF_{2\alpha}$ and 15-HETE through the use of enzyme-linked immunosorbent assay (ELISA). In addition, *P. aeruginosa* biofilms were identified to produce PGE_2, as well as $PGF_{2\alpha}$ and 15-HETE. Through ELISA and LC-MS/MS the identification and quantification of PGE_2 production *in vitro* was assessed with the use of PGE_2 isomers. This study highlights potentially problematic identification of enzymatically produced eicosanoids, due to the formation of autoxidation products. Significantly, an increase in production of all three eicosanoids by polymicrobial biofilms was found in comparison to monomicrobial counterparts. This may contribute to the increased virulence of these polymicrobial biofilms and difficulty in clearance of polymicrobial infection by the host.

2.2. Introduction

Candida albicans is an opportunistic pathogen frequently isolated from healthy individuals as part of the normal commensal microbiota (Rinzan, 2009). During compromised immunity (such as during antibiotic use, immunodeficiency disorders and individuals with cystic fibrosis), *C. albicans* causes superficial to systemic infections. The ability of *C. albicans* to cause invasive candidiasis is due to its morphological plasticity (Gil-Bona *et al*., 2015). Yeast, pseudohyphae and true hyphae are frequently found at infection sites. In addition to this, *C. albicans* is able form biofilms on biotic (i.e. epithelial surfaces) as well as abiotic surfaces (i.e. catheters and other indwelling devices) (Andes *et al*., 2004; Bruzual *et al*., 2007; Pierce, 2005; Ramage *et al*., 2001a). These structures, consisting of cells embedded in an extracellular matrix, are highly resistant to antifungal treatment. *Candida albicans* colonization of the host and subsequent biofilm formation is characterized by invasion of tissues by hyphae (Gil-Bona *et al*., 2015).

This opportunistic pathogen is rarely found alone and is the subject of study for interdomain interactions with bacteria, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Diaz *et al*., 2014; Lindsay & Hogan, 2014). The latter is a Gram-negative bacterium and opportunistic pathogen, frequently infecting immunocompromised individuals (Pier, 1985; Tan *et al*., 1999). *Pseudomonas aeruginosa* also possesses the ability to form antibiotic resistant biofilms (Bragonzi *et al*., 2009; Drenkard, 2003). A large amount of research is available describing the antagonistic interaction between *C. albicans* and *P. aeruginosa in vitro* (Hogan & Kolter, 2002; Lindsay & Hogan, 2014; Méar *et al*., 2013; Rinzan, 2009; Shirtliff *et al*., 2009; Xu *et al*., 2014). During close proximity, such as during the formation of polymicrobial biofilms, *P. aeruginosa* has been shown to kill *C. albicans* hyphae through the involvement of various virulence factors, including the production of a phenazine pigment, pyocyanin (Brand *et al*., 2008; Kerr *et al*., 1999). The interaction of these two opportunistic pathogens is expanded by non-physical interactions promoting further antagonism between these microorganisms with the dynamic shifted towards inhibition of fungal growth. These interactions are mediated by quorum sensing molecules, produced by both species (Cugini *et al*., 2007). These include 3-oxo-homoserine lactone, a quorum sensing molecule produced by the bacterium,

which inhibits the yeast to hyphal switch by *C. albicans* with possible involvement in dissemination in the host (Hornby *et al*., 2001; McAlester *et al*., 2008; Morales & Hogan, 2010). Farnesol, a quorum sensing molecule produced by *C. albicans*, reduces *Pseudomonas* quinolone signal (PQS) with downstream reduction in phenazine production (Cugini *et al*., 2007). The latter causes a reduction in *C. albicans* viability. *Candida albicans* ethanol production has also been shown to promote biofilm formation and to inhibit motility of *P. aeruginosa* (Chen *et al*., 2014). Other factors, including iron sequestration, extracellular DNA as well as peptidoglycan and lipopolysaccharide (LPS) affect the interaction of *C. albicans* and *P. aeruginosa* (Bandara *et al*., 2013; Purschke *et al*., 2012; Sapaar *et al*., 2014; Trejo-Hernandez *et al*., 2014; Xu *et al*., 2008).

During infection, *C. albicans* and *P. aeruginosa* elicit the release of arachidonic acid (AA) from host cells (Castro *et al*., 1994; Saliba *et al*., 2005). Arachidonic acid is the substrate for the production of various eicosanoids, with crucial involvement in the innate and adaptive immunity of hosts (Dennis & Norris, 2015). During *C. albicans* infection, AA liberation is significantly induced by cell wall components as well as phospholipases produced by *C. albicans* (Castro *et al*., 1994; Smeekens *et al*., 2010). This leads to the increased production of eicosanoids, the most important being prostaglandin E_2 (PGE_2), by the host. Prostaglandin E_2 can affect the cytokine responses of helper T cells, including inhibition of T_h1 and promotion T_h2 responses in the host, ultimately affecting the ability of the host to clear *C. albicans* infection (Romani, 2000; Smeekens *et al*., 2010). Interestingly, *C. albicans* is also able to produce a significant amount of a PGE_2 cross-reactive compound, later identified as authentic PGE_2 using LC-MS/MS, from exogenous AA (Erb-Downward & Noverr, 2007; Noverr *et al*., 2001). Inhibition of PGE_2 production by *C. albicans* was shown to reduce germ tube formation and biofilm development in *C. albicans* (Alem & Douglas, 2004). In addition, PGE_2 potentially plays a crucial role in interkingdom interactions between *C. albicans* and bacteria, as PGE_2 significantly increased *S. aureus* biofilm growth (Krause *et al*., 2015).

The ability to produce eicosanoids is not unique to *C. albicans*. *Pseudomonas aeruginosa* also has the ability to produce prostaglandins and prostaglandin-like molecules with potential effects on host cells during infection (Lamacka & Sajbidor,

1995). *Pseudomonas aeruginosa* possesses a secretable 15-lipoxygenase capable of converting AA to 15-hydroxyeicosatetraenoic acid (15-HETE), which has a dramatic anti-inflammatory effect on the host (Serhan, 2002; Vance *et al.*, 2004). In addition, *P. aeruginosa* elicits the release of large amounts of AA through exotoxin U (ExoU) and the subsequent production of PGE_2 by the host (Saliba *et al*., 2005). Pyocyanin, 3-oxo-homoserine lactone and LPS also induce the production of PGE_2 by the host (McDermott *et al*., 2013; Smith *et al*., 2002).

Although the effect of *C. albicans*-produced PGE_2 on *S. aureus* has been studied, the role of microbially produced eicosanoids has not been elucidated in the interaction between *C. albicans* and *P. aeruginosa*. Therefore, the aim of this study is to determine how AA is metabolized by *C. albicans* and *P. aeruginosa* polymicrobial biofilms compared to the monomicrobial counterparts *in vitro.* This facet of interaction may give valuable insight into the possible interaction between *C. albicans* and *P. aeruginosa* during co-infection.

2.3. Materials and methods

2.3.1. Strains used

Candida albicans strain CBS 8758 (SC5314) was used in the following studies and was maintained on yeast malt extract (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 also used. This strain was provided by Professor Hancock from the Department of Microbiology and Immunology at the University of British Columbia and revived according to American Type Culture Collection (ATCC) method from lyophilized strain ampoules, and maintained on nutrient agar (NA) (1 g/L malt extract, 2 g/L yeast extract, 5 g/L peptone, 8 g/L sodium chloride and 20 g/L agar) at 37 °C.

2.3.2. Formation of mono- and polymicrobial biofilms

2.3.2.1. Monomicrobial biofilm formation by Candida albicans

Candida albicans 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 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 then counted with a hemocytometer and diluted to 1 x 10^6 cells/mL in 20 mL filter sterilized (0.22 µm 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, 500 µM of AA (Sigma-Aldrich, USA) (Stock of 1 g in 25 mL of absolute ethanol reaching a concentration of 131.4 mM) was added to each petri dish containing medium plus cells (Ells *et al*., 2011). Petri dishes were incubated for 48 h at 37 °C to allow biofilm formation (Ramage *et al*., 2001b). The final ethanol concentration in the biofilms before incubation was 0.38 %. Appropriate controls were included, consisting of a cell free control (RPMI-1640 without AA) as well as a cell free control consisting of RPMI-1640 and 500 µM AA. These controls were repeated for monomicrobial biofilms of *P. aeruginosa* as well as polymicrobial biofilms.

2.3.2.2. Monomicrobial biofilm formation by Pseudomonas aeruginosa

Pseudomonas aeruginosa was grown on NA plates for 24 h at 37 °C and was 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). Cells were diluted to an optical density (OD_{600}) of approximately 0.05 in 20 mL filter sterilized RPMI-1640 medium and dispensed into 90 mm polystyrene petri dishes. In addition to the cells, 500 µM of AA was added to each petri dish containing medium plus cells. Petri dishes were incubated for 48 h at 37 °C to allow biofilm formation. The final ethanol concentration in the biofilms before incubation was 0.38 %. Appropriate controls were included.

2.3.2.3. Polymicrobial biofilm formation by Candida albicans *and* Pseudomonas aeruginosa

The formation of polymicrobial biofilms follows the combination of monomicrobial biofilm formation of both *C. albicans* and *P. aeruginosa*. Briefly, *C. albicans* and *P. aeruginosa* were grown as described above, and diluted to 20 mL RPMI-1640 medium. The medium thus contains 1x10^6 cells/mL *C. albicans* cells, as well as *P. aeruginosa* cells (approximately 0.05 OD_{600}). In addition to the cells in the medium, 500 µM of AA

was added to each petri dish. Petri dishes were incubated for 48 h at 37 °C to allow biofilm formation. The final ethanol concentration in the biofilms before incubation was 0.38 %. Appropriate controls were included.

A visual representation of the experimental procedure that was followed to induce biofilm formation of mono- and polymicrobial biofilms is shown in Figure 1.

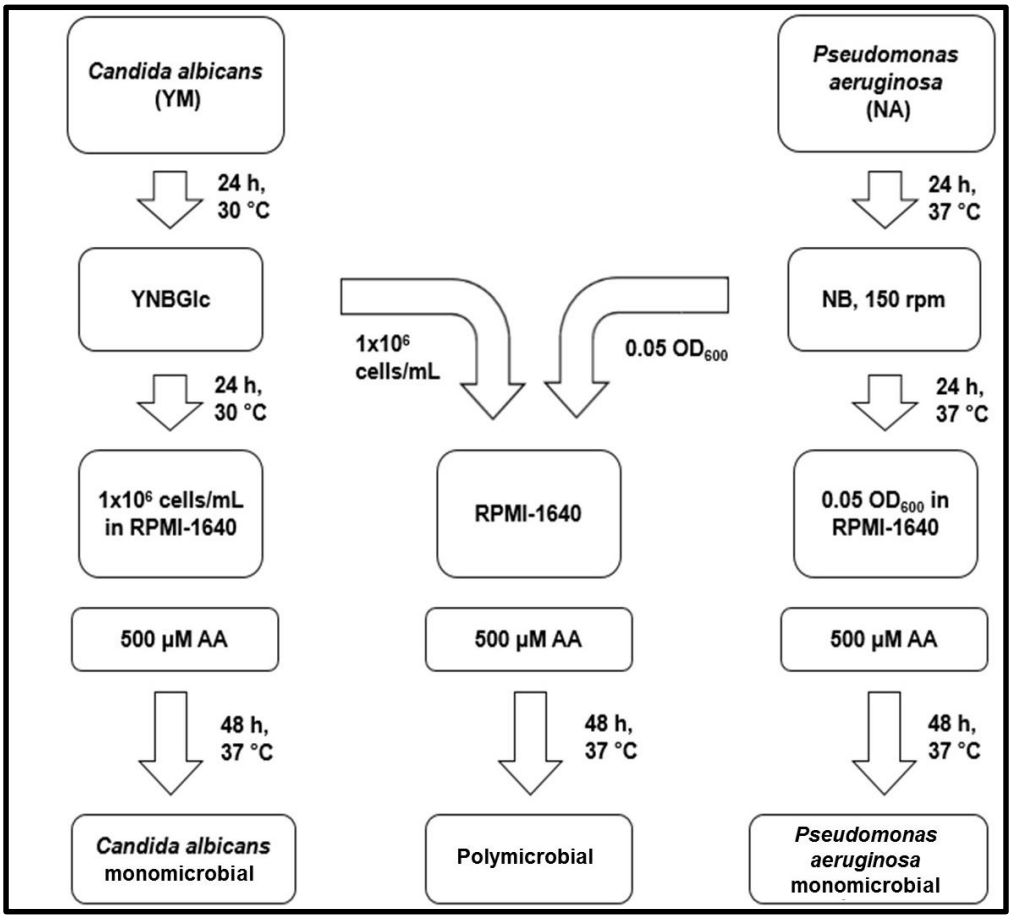

Figure 1. A schematic representation of the experimental procedure that was followed to induce biofilm formation by strains representing *Candida albicans* and *Pseudomonas aeruginosa*.

2.3.3. Determination of morphology of mono- and polymicrobial biofilms using scanning electron microscopy (SEM)

Biofilms were prepared as described above in flat bottom 6 well culture plates (Corning Incorporated, USA) in 3 mL medium. After incubation the supernatants were removed

and 5 mm rectangular sections of the bottom of the wells were cut out and washed with PBS and fixed overnight with the primary fixative, 3 % (v/v) gluterdialdehyde (Merck, Germany) in phosphate buffer (pH 7.0) (Hawser & Douglas, 1994). The cells were then washed with phosphate buffer and then fixed with the secondary fixative, 1 % (v/v) osmium tetroxide (Merck, Germany) for 1 h at room temperature. The biofilms were then sequentially dehydrated with 50 %, 70 % and 95 % ethanol for 15 min each and twice for 1 h with 100 % ethanol. After critical point drying (Samdri-795 Critical point dryer, Tousimis, USA), the biofilms were sputter coated with gold using a SEM coating system (Bio-Rad Microscience division, UK) and examined using a Shimadzu SSX-550 Superscan scanning electron microscope.

2.3.4. Determination of metabolic activity of mono- and polymicrobial biofilms

Biofilms were prepared as described above for a 96-well plate (Corning Incorporated, Costar®, USA) with the volume of medium adjusted to 100 µL. The plate was incubated for 48 h 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. The XTT assay was performed according to Kuhn *et al.* (2003). Briefly, 50 µL 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. Following incubation, the optical density of each well was measured at 492 nm on a Labotec Spectramax M2 microplate reader (Molecular devices, USA) and data was obtained. Appropriate controls were included, consisting of a cell free control (RPMI-1640 without AA) as well as a cell free control consisting of RPMI-1640 and 500 µM AA. This experiment was performed in triplicate.

2.3.5. Determination of biomass of mono- and polymicrobial biofilms

Biofilms were prepared as described above. After incubation, the individual biofilms were scraped off and washed twice with sterile PBS, where after the resuspended cells were filtered through pre-weighed filters (0.22 µm nitrocellulose filter, Merck Millipore, Ireland). The filters were dried at 37 °C overnight and the dry biomass of the

mono- and polymicrobial biofilms determined (Thibane *et al*., 2010). This experiment was performed in triplicate.

2.3.6. Arachidonic acid localization in mono- and polymicrobial biofilms using TOF-SIMS

Biofilms were prepared as described above in flat bottom 6 well culture plates (Corning Incorporated, USA) in 3 mL medium. After incubation the supernatants were removed and 5 mm rectangular sections of the bottom of the wells were cut out and washed with PBS, fixed, dehydrated and critical point dried as described above.

Analysis of samples was performed with the use of an ionTOF^5 (IONTOF, Germany) TOF-SIMS using a pulse beam of 30 kV Bi^+ (100 µs, 1 pA, 10 kHz) with an analysis area of 500x500 µm (Lockyer & Vickerman, 2004). In order to obtain the chemical composition of underlying biofilm structures, the surface of samples were etched using an Argon cluster gun (0.9 nA, 2.5 kV). Sputter intervals lasted 10 seconds on an area of 1000x1000 µm. Sputter and analysis was repeated for between 40-100 scans depending on biofilm thickness. Data was analysed using system software (SurfaceLab 6).

2.3.7. Quantification of eicosanoid production by mono- and polymicrobial biofilms

Biofilms were prepared in 90 mm petri dishes as described before. Supernatant and cells were collected in 50 mL Falcon tubes (Corning Incorporated, Mexico) and cells were removed with centrifugation (5750 *g* @ 4 °C for 5 minutes, Eppendorf, Germany). After centrifugation the supernatants were filtered (0.2 µm nitrocellulose filter). Extraction of eicosanoids were performed according to a modified protocol proposed by Cayman chemicals for PGE_2 purification for enzyme linked immunosorbent assay (ELISA). Briefly, supernatants were acidified by to a pH of approximately 4 with the addition of 1 M formic acid (Merck, Germany). Solid phase extraction (SPE) classic C18 cartridges (Waters, Ireland) were prepared with 5 mL methanol (Merck, Germany), followed by 5 mL deionized water. Samples (10 mL) were applied to cartridges and subsequently washed with 5 mL deionized water to remove impurities. Eicosanoids were then eluted from the SPE cartridges with 5 mL ethyl acetate

containing 1 % methanol and collected in pre-washed poly top glass vials (Lasec, South Africa). The eluent was dried under a stream of N_2 and stored at -80 °C until use.

Samples were dissolved in eicosanoid affinity (EIA) buffer provided by the manufacturers and samples were assayed for PGE_2, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and 15-HETE using ELISA (Cayman Chemicals, USA) according to manufacturer's specifications. Samples were assayed in two dilutions in duplicate. This experiment was done in triplicate. Data was analysed according to manufacturer's specifications.

2.3.8. Determination of PGE_2 enzyme linked immunosorbent assay (ELISA) cross-reactivity with known PGE_2 isomers

Prostaglandin E_2 isomers, namely, 8-*iso*-PGE_2, 15-keto-PGE_2, 5-*trans*-PGE_2, 11β-PGE_2, 15(*R*)-PGE_2 and *ent*-PGE_2 were dissolved in EIA buffer and assayed at 4 concentrations with the highest concentration (1000 ng/mL) sequentially diluted by a factor of 10 (Personal communication with Cayman Chemical technical assistance).

During PGE_2 ELISA, a PGE_2 derivative (bicyclo-PGE_2) competes with a PGE_2 bound tracer to bind to polyclonal antibody on the ELISA plate wells. The amount of PGE_2 in a sample is then calculated according to the percentage of bound PGE_2 tracer to give a % bound over maximum binding capacity (% B/B_o). To determine the cross reactivity of PGE_2 isomers, the concentration of the PGE_2-cross-reactive compound at which 50 % of PGE_2 tracer is misplaced (50 % B/B_o), is expressed according to the 50 % B/B_o of the supplied PGE_2 standard (Personal communication with Cayman Chemical technical assistance).

2.3.9. Identification of eicosanoids produced by mono- and polymicrobial biofilms by LC-MS/MS

2.3.9.1. Extracellular eicosanoid extraction

Biofilms were prepared and supernatants were removed from biofilms, centrifuged (5750 *g* @ 4 °C for 5 minutes) and filtered as described previously. Lipids were extracted using a modified protocol proposed by Cayman chemicals for PGE_2

purification for enzyme linked immunosorbent assay (ELISA) as described above. The eluent was dried under a stream of N_2 and stored at -80 °C until use.

2.3.9.2. Intracellular eicosanoid extraction

Biofilms were prepared as described with the exception that these biofilms were incubated for 24 h. This was done as previously described (Ells *et al*., 2013) as little to no PGE_2 was detected intracellularly by these authors after 48 h. After incubation, a modified protocol proposed by Ells *et al*. (2013) was used to extract eicosanoids. Biofilms were scraped off and washed three times with PBS. The biomass of three biofilms were pooled to obtain sufficient biomass. Washed biofilms were vortexed twice for 1 min to disperse biofilms and centrifuged (5 min @ 5750 *g*, Eppendorf) to collect cells. Cells were resuspended in 1 mM ethylenediaminetetraacetic acid (EDTA) with lyticase (625 U/mL, Sigma-Aldrich, USA) and gassed with N_2. This was incubated for 60 min at 30 °C to create spheroplasts. After incubation, 1 N citric acid (160 µL) and 20 µL (10 %) butylated hydroxytoluene (BHT, Sigma-Aldrich) was added as antioxidant to combat degradation of PGE_2. Samples were sonicated according to a protocol described for *P. aeruginosa* biofilms with modification (Chaturvedi & Kumar, 2012). Briefly, cell suspensions were sonicated at 60 % power (Bandalin Sonopuls, Germany) for 30 seconds and incubated on ice for 30 seconds. This was repeated for 10 cycles. Microscopic analysis revealed that sonication was inadequate for breaking of *C. albicans* cells, therefore, *C. albicans* cells were further broken by the use of French press cell disruptor at 34 psi. After breaking the cells, eicosanoids were extracted with 2 mL hexane: ethyl acetate (1:1) (Ells *et al*., 2013). After addition of hexane:ethyl acetate, samples were vortexed for 1 minute and centrifuged (at 1878 *g* for 10 minutes, 4 °C) to remove cellular debris after which the organic phase was removed. This was done three times and the organic phase was pooled and evaporated under stream of N_2 in pre-washed glass vials. Samples were stored at -80 °C until use.

In addition to samples, AA, PGE_2 isomers (8-*iso*-PGE_2, 15-keto-PGE_2, 5-*trans*-PGE_2, 11β-PGE_2, 15(*R*)-PGE_2, *ent*-PGE_2) and HETEs (5-HETE, 8-HETE, 9-HETE, 15-HETE, 19-HETE and 20-HETE) (Cayman Chemical Company, USA) were extracted with a method identical to the protocol described above. In addition, a deuterated PGE_2

isomer (PGE_{2d4}) (Sigma-Aldrich) (5 ng) was added to each sample prior to extraction to evaluate efficiency of extraction and to identify retention time of authentic PGE_2, as the retention time of the deuterated PGE_2 is identical to PGE_2 (Brose *et al*., 2011).

2.3.9.3. Detection of eicosanoids by LC-MS/MS

Samples were analysed using a 4000 QTRAP hybrid triple quadrupole ion trap mass spectrometer (AB Sciex) and Shimadzu UFLC stack with LC-20AB binary pump and SIL-20A HT autosampler as front end. All data acquisition and processing was performed using Analyst 1.5 (AB SCIEX) software.

To analyse the sample a targeted multiple reaction monitoring (MRM) workflow was followed on the instrument. During an MRM scan the instrument is used in triple quadrupole mode where every ionised analyte (the precursor) eluting off the column is fragmented in the collision cell to produce fragment masses. A set of masses, the precursor mass and one fragment mass constitutes a transition. The instrument alternates between different transitions in an MRM transition list during an analysis cycle, each cycle typically lasting less than a second. If a transition is detected, the instrument's response is registered and this ion intensity value is plotted as a chromatogram. All compound and source dependant parameters were optimised using compound optimization in Analyst 1.5.2.

The peak area on the chromatogram generated from the first and most sensitive transition was used as the quantifier while the other transitions (if available) were used as qualifiers. The qualifier serves as an additional level of confirmation for the presence of the analyte, the retention time for these transitions needs to be the same.

2.3.9.4. Analyses of prostaglandin E_2 and prostaglandin E_2 isomers

Two separation methods were employed for PGE_2 analysis. For detection of PGE_2 in samples, the method proposed by Ells *et al*. (2013) was used. In this method, 20 µL of each sample was separated on a C18 (XDB-C18, 1.8 µm, 4.6x50 mm, Agilent) column at a flow rate of 300 µL/min using 10 mM ammonium acetate (mobile phase A) and methanol/10 mM ammonium acetate (mobile phase B). The column was equilibrated and loaded at 5 % B, increasing to 10 % B over 4 minutes, 95 % B for 5 minutes, followed by re-equilibration at 5 % for a total runtime of 15 minutes. Eluting

analytes were ionised in negative electrospray mode with a 4500 V ion spray voltage and 500 °C heater temperature to evaporate excess solvent, 50 psi nebuliser gas, 50 psi heater gas and 25 psi curtain gas.

For separation of PGE_2 isomers, a method described by Brose *et al*. (2011) was used. Each sample was separated on a C18 (Luna 3 µm C18 (2), 150x3 mm, Phenomenex) column at a flow rate of 200 uL/min using 0.1 % formic acid (mobile phase A) and acetonitrile with 0.1 % formic acid (mobile phase B). The column was equilibrated and loaded at 20 % B, increasing to 42.5 % B over 50 minutes, 95 % B for 10 minutes, followed by re-equilibration at 20 % B for a total run time of 70 minutes.

The targeted analysis for the extracted PGE_2 and the PGE_2 isomers described above used 5 MRM transitions: 351.1 > 315.2; 351.1 > 271.2; 351.1 > 333.3; 351.1 > 189.0; 351.1 > 235.1. For targeted analysis for PGE_{2d4} the following transitions were used: 355.17 > 275.2; 355.17 > 337.2; 355.17 > 319.2; 355.17 > 193.0; 355.17 > 239.1.

2.3.9.5. Analyses of hydroxyeicosatetraenoic acids (HETEs)

Twenty microliter of each sample was separated on a C18 (Discovery C18, 5 µm, 2.1x150 mm, Supelco) column at a flow rate of 300 uL/min using 0.1 % formic acid (mobile phase A) and acetonitrile with 0.1 % formic acid (mobile phase B) (Yue *et al*., 2007). The column was equilibrated and loaded at 60 % B, increasing to 80 % B over 30 minutes, 85 % B over the following 5 minutes, 100 % over 1 minute, followed by re-equilibration at 60 % B for a total run time of 45 minutes. The transitions used are listed in Table 1.

Table 1. Transitions used for targeted analysis of HETEs by LC-MS/MS.

Eicosanoid	Transitions
5-HETE	319.3 > 115.0
8-HETE	319.3 > 155.0
9-HETE	319.3 > 167.1 319.3 > 69.2
11-HETE	319.3 > 167.1
12-HETE	319.3 > 179.1
15-HETE	319.3 > 219.1
19-HETE	319.3 > 209.0
20-HETE	319.3 > 289.3 319.3 > 245.1

In addition, AA was identified using the following transitions: 303.1 > 259.3; 303.1 > 205.1.

2.3.10. Statistical analysis

To evaluate significant differences between mono- and polymicrobial biofilms, one-way analysis of variance (ANOVA) was used between data sets of mono- and polymicrobial biofilms ($P < 0.05$). In addition, this was followed by Tukey's multiple comparisons test with an alpha value of 0.05. Significant differences are indicated with ' * '.

2.4. Results and discussion

2.4.1. Morphology of mono- and polymicrobial biofilms of Candida albicans *and* Pseudomonas aeruginosa

Previous research indicates the antagonistic interaction between *C. albicans* and *P. aeruginosa* with bacterial cells killing *C. albicans* and causing a reduction in fungal biofilm growth (Brand *et al*., 2008). Figure 2 contains scanning electron micrographs of *C. albicans* and *P. aeruginosa* monomicrobial biofilms as well as a polymicrobial

biofilm in the presence of 500 µM AA. *Candida albicans* shows predominantly yeast morphology in the presence of AA as previously described (Figure 2a) (Shareck & Belhumeur, 2011). The *P. aeruginosa* monomicrobial biofilm (Figure 2b) exhibits a large amount of extracellular material. Interestingly, an increase in hyphal growth is observed during co-incubation of *C. albicans* with *P. aeruginosa* (Figure 2c). This is possibly due to bacterial components such as peptidoglycan as well as increased DNA in the biofilm matrix which has previously been shown to induce filamentation (Sapaar *et al*., 2014; Xu *et al*., 2008). In addition, this could be due to the increased metabolism of AA in the presence of *P. aeruginosa* diminishing the inhibition of filamentation of *C. albicans* by AA.

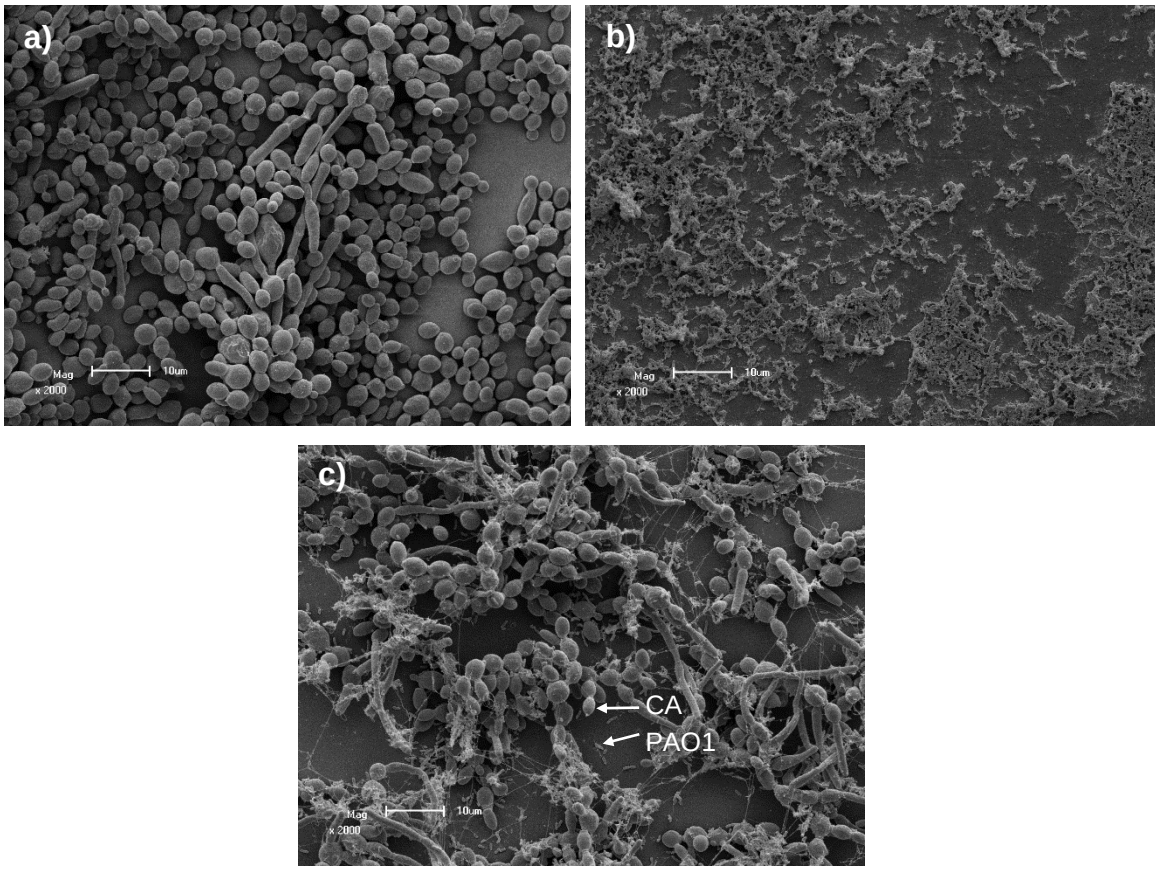

Figure 2. Scanning electron micrograph of mono- and polymicrobial biofilms grown in the presence of arachidonic acid (AA) for 48 h. Monomicrobial biofilm of *Candida albicans* is shown in (a); monomicrobial biofilm of *Pseudomonas aeruginosa* (b) and polymicrobial biofilm of *C. albicans* (CA) and *P. aeruginosa* (PAO1) (c). Arrows indicate respective cell types. Scale bars represent 10 µm.

2.4.2. Effect of co-incubation on biofilm metabolic activity and biomass

To discuss the production of eicosanoids by mono- and polymicrobial biofilms, it is first necessary to evaluate the effect of co-incubation on biofilm growth and metabolic activity. Metabolic activity of monomicrobial *C. albicans* biofilms and polymicrobial biofilms do not differ significantly, however a reduction in dry biomass is observed by polymicrobial biofilms compared to *C. albicans* monomicrobial biofilms (Figure 3a, b). The metabolic activity and dry biomass of *P. aeruginosa* monomicrobial biofilms is significantly lower. The fact that there was not a correspondingly low dry biomass value obtained for *P. aeruginosa* monomicrobial biofilm (Figure 3b), may reflect a large amount of extracellular material also contributing to the dry biomass. Therefore eicosanoid production was not normalised against dry biomass. In addition, although the use of XTT in the determination of fungal and bacterial biofilm metabolic activity has been proven to be useful, previous research indicates that additional electron carriers (in addition to menadione used in this study), such as phenazine methosulphate, may facilitate the assay for metabolic activity by *P. aeruginosa* biofilms and give higher values (Koban *et al*., 2012; Kuhn *et al*., 2003). It should also be noted that biomass values reflect the total (planktonic and biofilm) biomass, rather than only the cells embedded in the biofilm.

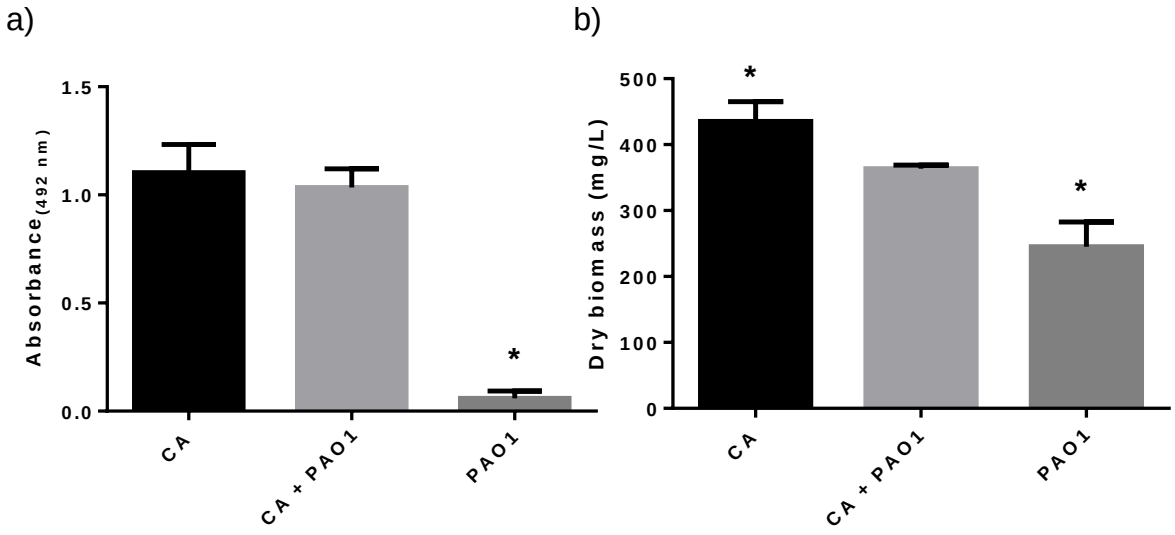

Figure 3. a) Metabolic activity of *Candida albicans* (CA) and *Pseudomonas aeruginosa* (PAO1) mono- and polymicrobial biofilms in the presence of 500 µM arachidonic acid (AA) measured by XTT assay; b) Dry biomass (mg/L) of *C. albicans* and *P. aeruginosa* mono- and polymicrobial biofilms in the presence of 500 µM AA. * Significantly different from polymicrobial biofilms ($P < 0.05$).

2.4.3. Localization of arachidonic acid in mono- and polymicrobial biofilms

To determine the distribution of AA and potential eicosanoid products in mono- and polymicrobial biofilms, a novel method with high efficacy in chemical analysis of biological samples, was employed (Belazi, 2009; Brulet *et al*., 2010; Tyler *et al*., 2006; Vaidyanathan *et al*., 2008). Biofilms were dehydrated and analysed using time of flight-secondary ion mass spectrometry (TOF-SIMS) to determine the chemical composition. To do this, samples are bombarded with a short pulse beam (Belazi, 2009). This pulse has high kinetic energy and causes the release of secondary ions from surface molecules. The secondary ions are accelerated and the time they take the reach the detector, as well as their charge, gives a mass-to-charge ratio (m/z). In TOF-SIMS, this is done in parallel for a large number of secondary ions to give an image of the chemical composition of a specific area on a biological sample. Through bombarding the sample spot with heavy argon clusters, a layer of the biological surface is removed and spectroscopy analysis is repeated to reveal the chemical composition of underlying layers. By consecutive bombarding and analysis, the chemical and molecular composition of a biological sample can be obtained. Through this method mono- and polymicrobial biofilms were analysed to detect AA and possible products (e.g. hydroxyeicosatetraenoic acids and prostaglandins). In addition, hydrogen, carbon and oxygen were mapped in biofilms to produce a colour image (Figure 4). No AA and corresponding fragments (m/z: 59; 80; 177; 205; 231; 259; 285; $[M-H]^-$:303.2330) were detected in mono- and polymicrobial biofilms. This could indicate that most or all of the AA is metabolized by cells during 48 h incubation with the subsequent release of metabolites into the supernatant. The lack of visualization could indicate that the AA may have been incorporated into cells to facilitate growth and increased biomass, as well as the possible production of eicosanoids, released into the medium during biofilm growth. The lack of AA and metabolites are however not due to inadequate sample preparation, as low amounts of AA and fragments could be detected in samples exposed to AA for 14 hours (data not shown).

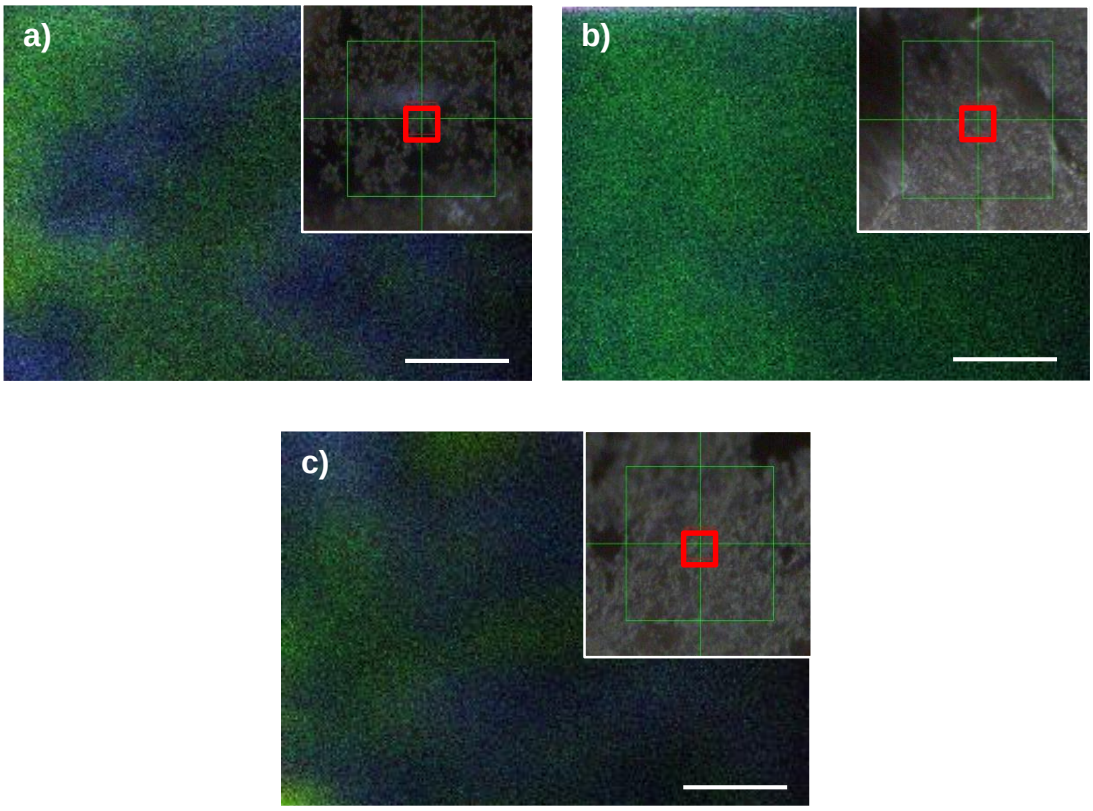

Figure 4. Time of flight secondary ion mass spectroscopy (TOF-SIMS) images of biofilms using overlay of hydrogen (H^-, red), carbon (C^-, green) and oxygen (O^-, blue) during negative ionization mode spectroscopy analysis. Small images show area that is analysed indicated by red box. *Candida albicans* monomicrobial biofilm (a); *Pseudomonas aeruginosa* monomicrobial biofilm (b); polymicrobial biofilm of *C. albicans* and *P. aeruginosa* (c). Scale bars represent 20 μm.

2.4.4. Comparison of mono- and polymicrobial biofilm prostaglandin E_2, $PGF_{2\alpha}$ and HETE production

Figure 5 represents data obtained with the use of ELISA for PGE_2, $PGF_{2\alpha}$ and 15-HETE (Figure 5a, b and c respectively). These eicosanoid profiles represent eicosanoid production by cells embedded in the biofilm, as well as planktonic cells. In Figure 5a, the production of PGE_2 by mono- and polymicrobial biofilms of *C. albicans* and *P. aeruginosa* is provided. Our results validate previous research indicating the production of PGE_2 by *C. albicans* biofilms in significant quantities, as well as the presence of $PGF_{2\alpha}$ and 15-HETE (Erb-Downward & Noverr, 2007; Noverr *et al*., 2001).

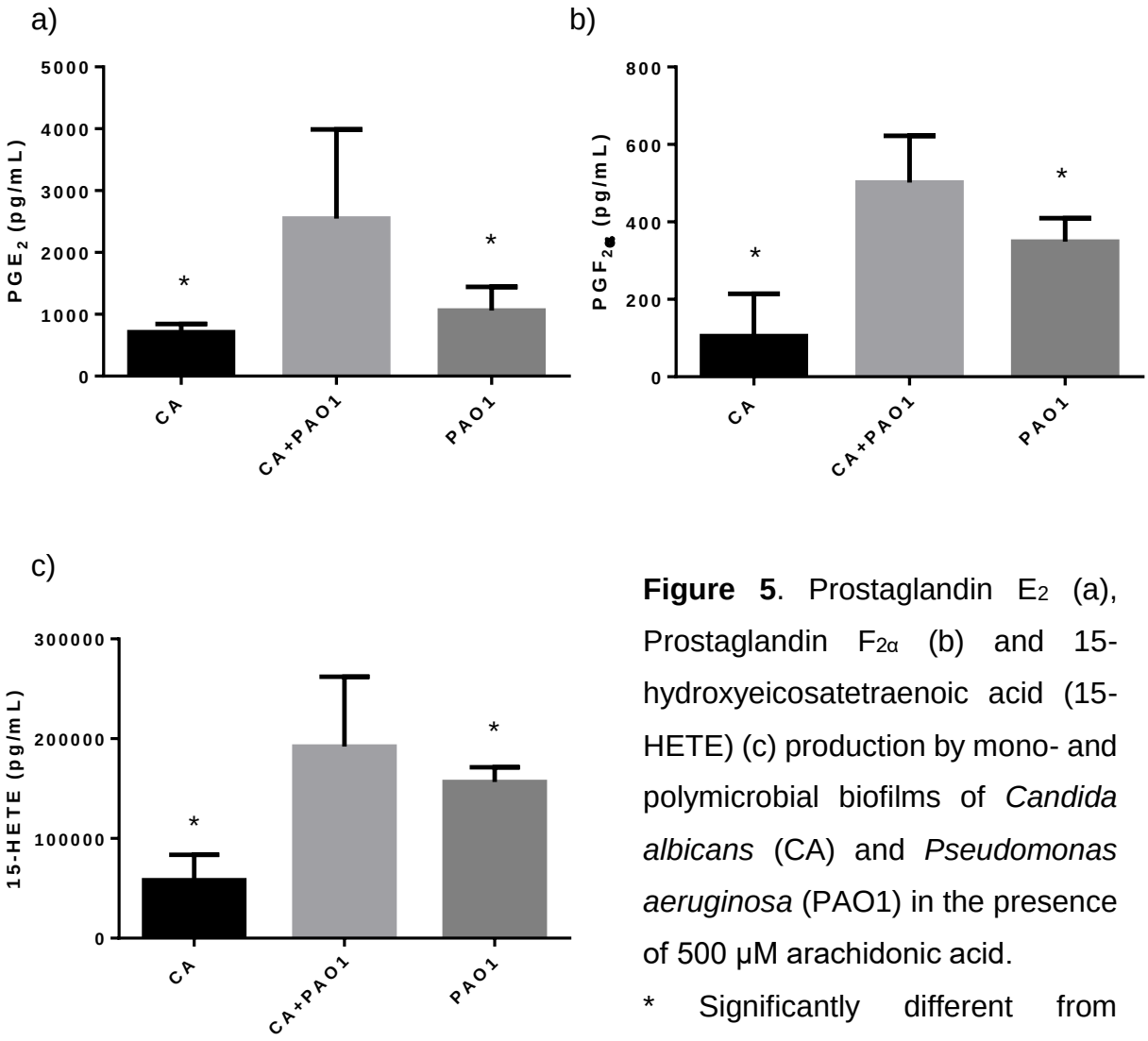

Figure 5. Prostaglandin E_2 (a), Prostaglandin $F_{2\alpha}$ (b) and 15-hydroxyeicosatetraenoic acid (15-HETE) (c) production by mono- and polymicrobial biofilms of *Candida albicans* (CA) and *Pseudomonas aeruginosa* (PAO1) in the presence of 500 µM arachidonic acid.
* Significantly different from polymicrobial biofilm ($P < 0.05$).

Interestingly, *P. aeruginosa* is also capable of producing significant quantities of all three these eicosanoids. Although *P. aeruginosa* has previously been shown to produce 15-HETE (Vance *et al*., 2004) as well as prostaglandins and prostaglandin-like molecules (Lamacka & Sajbidor, 1995), the production of PGE_2 and $PGF_{2\alpha}$ specifically has not been confirmed previously. Co-incubation of *C. albicans* and *P. aeruginosa* reveals a significant increase in PGE_2, $PGF_{2\alpha}$ and 15-HETE concentration compared to monomicrobial biofilms (Figure 5).

Especially the high level of PGE_2 raises questions of the effect on the host, as COX-2 inhibition, and concurrently PGE_2 production in the host, leads to increased clearance

of bacterial cells (Park *et al*., 2007; Sadikot *et al*., 2007). Whereas the role of PGE_2 production in mammalian systems have been well studied, limited information is available regarding the role of $PGF_{2\alpha}$ in infection and inflammation. Prostaglandin $F_{2\alpha}$ has, however, been implicated in playing roles in the human reproductive process, vasoconstriction, myocardial dysfunction, pain and renal dysfunction (Ricciotti & FitzGerald, 2011). In addition, F_2-series isoprostanes act as hallmarks of lipid peroxidation and acute inflammatory diseases. Significantly, *P. aeruginosa* produces a large amount of 15-HETE, which can be converted to lipoxins in the host with significant anti-inflammatory capabilities (Dennis & Norris, 2015). Although eicosanoids elicit their effect at nanomolar concentrations, localized production of these microbially produced eicosanoids may be able to elicit an effect (Funk, 2001). The combination of these eicosanoids by both species may ultimately increase the susceptibility of the host during co-infection.

2.4.5. Cross-reactivity of prostaglandin E_2 ELISA with prostaglandin E_2 isomers

Several studies on the production of PGE_2 by fungal species, including *C. albicans, C. dubliniensis, Cryptococcus neoformans, Saccharromyces cerevisiae* and *Saprolegnia parasitica* have relied on quantification by ELISA as well as mass spectrometry (MS) (Belmonte *et al*., 2014; Chikhalya, 2013; Ells, 2011; Erb-Downward & Noverr, 2007; Noverr *et al*., 2001). The use of ELISA relies on the principle of stereospecific PGE_2 stable derivative, bicyclo-PGE_2, being bound by a monoclonal antibody and has simplified the quantification of this eicosanoid tremendously. The low cross reactivity of this PGE_2 derivative specific assay conveys a large advantage in separating specific molecules from a large pool of enzymatic and non-enzymatic eicosanoids. However, manufacturers indicate that cross reactivity with other eicosanoid species are possible (Prostaglandin E_2 monoclonal EIA kit, Cayman Chemical Company). These cross-reactive substances include AA (< 0.01 %) prostaglandin E series molecules, such as PGE_1 (18.7 %) and PGE_3 (43 %), PGE_2 isomers, such as 8-*iso*-PGE_2 (2.5 %) as well as other prostanoids. The reader is referred to the product information for further specifications (Prostaglandin E_2 monoclonal EIA kit, Cayman Chemical Company)

The detection of PGE_2 from mono- and polymicrobial biofilms of *C. albicans* and *P. aeruginosa* using ELISA as well as the large production of other eicosanoids, raises

the question if eicosanoids with similar structure may cause false results in the ELISA used. For this reason, a number of PGE_2 isomers, namely 8-*iso*-PGE_2, 15(*R*)-PGE_2, 11β-PGE_2, *ent*-PGE_2, 15-keto-PGE_2 and 5-*trans*-PGE_2 were assayed to determine cross reactivity with the PGE_2 ELISA.

Table 1 indicates the results obtained when the cross-reactivity of several PGE_2 isomers were evaluated using PGE_2 ELISA. 8-*iso*-PGE_2 and 15-keto-PGE_2 corresponds to the product information provided by the manufacturer with slight variation possibly due to differences in assay conditions. Interestingly, 5-*trans*-PGE_2 showed high cross-reactivity (circa 25%). This indicates that the PGE_2 values obtained for a given sample may correspond to one or more PGE_2 isomers rather than PGE_2 alone.

Table 2. Comparison of supplier and experimental cross-reactivity of prostaglandin E_2 (PGE_2) isomers with PGE_2 enzyme linked immunosorbent assay (ELISA).

Isoprostane name	**% Cross reactivity (Cayman product information)**	**Calculated % Cross reactivity**
8-*iso*-PGE_2	2.5%	3.14%
15(*R*)-PGE_2	Not specified	0.16%
11β-PGE_2	Not specified	0.18%
ent-PGE_2	Not specified	0.02%
15-keto-PGE_2	<0.01%	0.02%
5-*trans*-PGE_2	Not specified	25.5%

2.4.6. Comparison of eicosanoid production by mono- and polymicrobial biofilms using LC-MS/MS

2.4.6.1. Arachidonic acid autoxidation interferes with metabolite detection

During *in vitro* studies, such as described here, AA is added to medium containing cells. Several studies have however described that AA can undergo non-enzymatic oxidation to a large range of eicosanoids (Jahn *et al*., 2008; Milne *et al*., 2015). These autoxidation products can normally be distinguished from enzymatic products through regioselective detection of metabolites, as autoxidation of AA results in the formation of raecemic eicosanoid metabolites (Gao *et al*., 2003). In contrast, the high specificity of enzymes usually catalyses the formation of regiospecific molecules. Arachidonic acid autoxidation products can however undergo epimerization *in vivo* and *in vitro* to produce eicosanoids, such as PGE_2, indistinguishable from authentic standards or enzymatic products (Milne *et al*., 2015). These products, as well as isomeric molecules of PGE_2 can produce false results in the detection and quantification of PGE_2 as well as other eicosanoids. In light of this information, the detection of specific AA enzymatic metabolites produced by *C. albicans* and *P. aeruginosa* mono- and polymicrobial biofilms were undertaken.

Extracellular eicosanoid profiles obtained by LC-MS/MS were compared to determine differential production of eicosanoids by mono- and polymicrobial biofilms of *C. albicans* and *P. aeruginosa.* For this, standards for AA, PGE_2 as well as HETEs were extracted similarly to sample preparation and identified by LC-MS/MS using a protocol used by Ells *et al*. (2011, 2013). Results obtained from control supernatants containing only AA incubated for the same duration as biofilms indicated that PGE_2, as well as 5-HETE, 8-HETE, 11-HETE, 12-HETE and 15-HETE could all be detected as autoxidation products of AA. In addition, profiles obtained from biofilm supernatants extracts yielded similar profiles. This indicated that the method of separation used was inadequate for separation of enzymatically produced eicosanoids from autoxidation products. This is of importance as previous research relied on this method of detection for PGE_2 in *C. albicans* (Ells *et al*., 2011, 2013). Due to the fact that authentic PGE_2 could be detected in AA control samples using the LC-MS/MS method proposed by Ells *et al*. (2013), another method proposed by Brose *et al*. (2011) was utilized. With

this method, isomers of PGE_2 could be separated. To possibly identify authentic PGE_2 in supernatant extracts of *C. albicans*, authentic PGE_2, as well as several PGE_2 isomers, including 8-*iso*-PGE_2, 15-keto-PGE_2, 5-*trans*-PGE_2, 11β-PGE_2, 15(*R*)-PGE_2 and *ent*-PGE_2 were extracted in a manner similar to biofilm supernatants to obtain retention times of PGE_2 and the isomers. As can be seen in Figure 6, PGE_2 isomers are detected in samples containing only AA in media incubated for 48 h (Figure 6b), including authentic PGE_2.

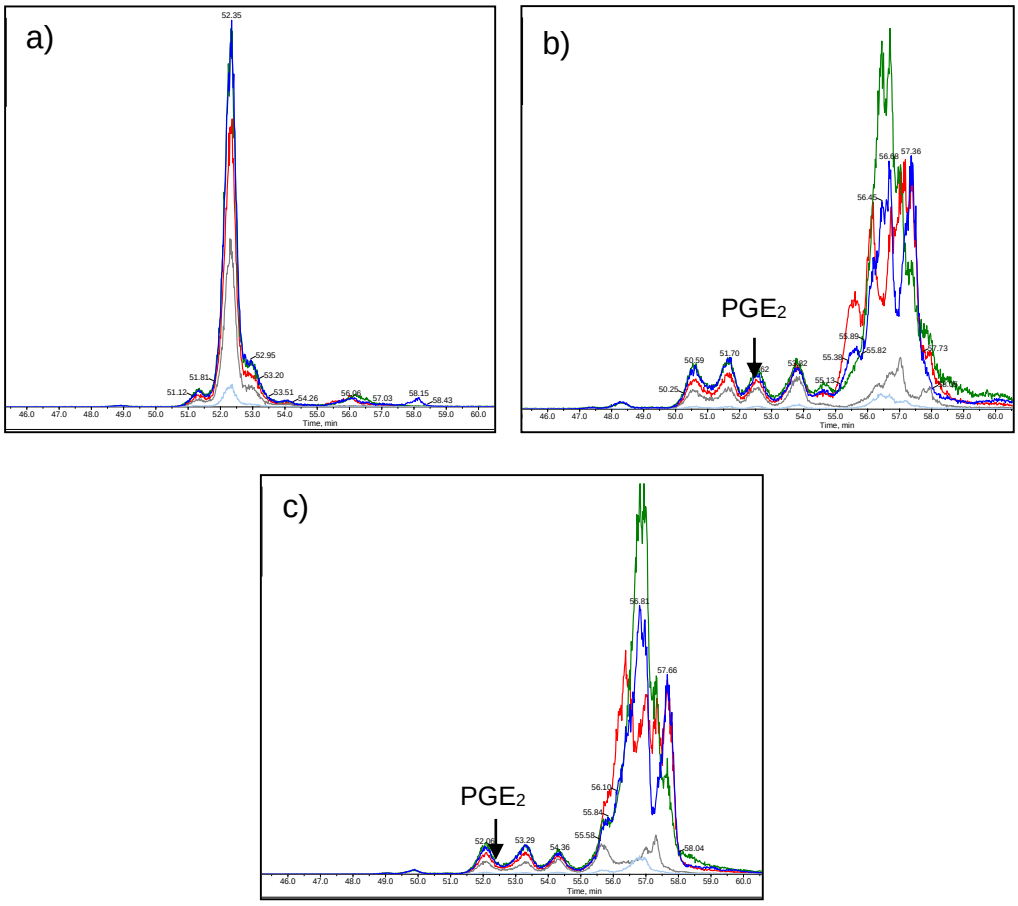

Figure 6. LC-MS/MS spectra (-MRM 5 pairs) using isomer separation method proposed by Brose *et al*. (2011) of Prostaglandin E_2 (PGE_2) standard (a); arachidonic acid (AA) in RPMI-1640 medium after 48h @ 37°C (b); Supernatant of *Candida albicans* with 500 µM AA after 48h @ 37°C (c). Arrows indicate expected PGE_2 retention times.

Further research is however needed to identify these compounds. A similar profile is obtained for extracted supernatant of *C. albicans* monomicrobial biofilms (Figure 6c). Due to the presence of PGE_2 by AA autoxidation, enzymatic PGE_2 production by

biofilms could not be identified. Extraction of PGE_2 isomers and separation using the method proposed by Ells *et al*. (2011) (not shown) indicated that PGE_2 isomers could give false identification of authentic PGE_2.

2.4.6.2. Intracellular prostaglandin E_2 detection

To decrease the occurrence of a large amount of autoxidation products possibly masking enzymatic products, intracellular PGE_2 extraction was performed on mono- and polymicrobial biofilms of *C. albicans* and *P. aeruginosa*. Additionally, the extraction of PGE_{2d4} with each sample indicates that the extraction protocol is sufficient for intracellular PGE_2 extraction. PGE_2 isomers, as mentioned above, as well as a range of HETEs, namely 8-HETE, 9-HETE, 15-HETE, 19-HETE and 20-HETE were also extracted using the same protocol. Prostaglandin E_2 isomers were readily detected after extraction, however, none of the HETEs could be detected after extraction, indicating that the intracellular extraction method used is insufficient for HETEs. In Figure 7a, PGE_2 was detected in polymicrobial intracellular extracts. In addition, authentic PGE_2 was detected in intracellular extracts of *C. albicans* monomicrobial biofilms (Figure 7b). This PGE_2 is not the result of AA autoxidation, as extraction of *C. albicans* monomicrobial biofilms without breaking the cells, did not contain PGE_2. Interestingly, low levels of PGE_2 was detected after intracellular extraction of monomicrobial biofilms of *P. aeruginosa* (Figure 7c). However, since AA may get trapped in the extracellular material produced by *P. aeruginosa*, formation of non-enzymatic PGE_2 cannot be totally discarded. Although dissimilarities was observed in respect of retention times between samples, this occurrence was not regarded as important, as the internal standard (PGE_{2d4}) was used as a reference for PGE_2 in each sample.

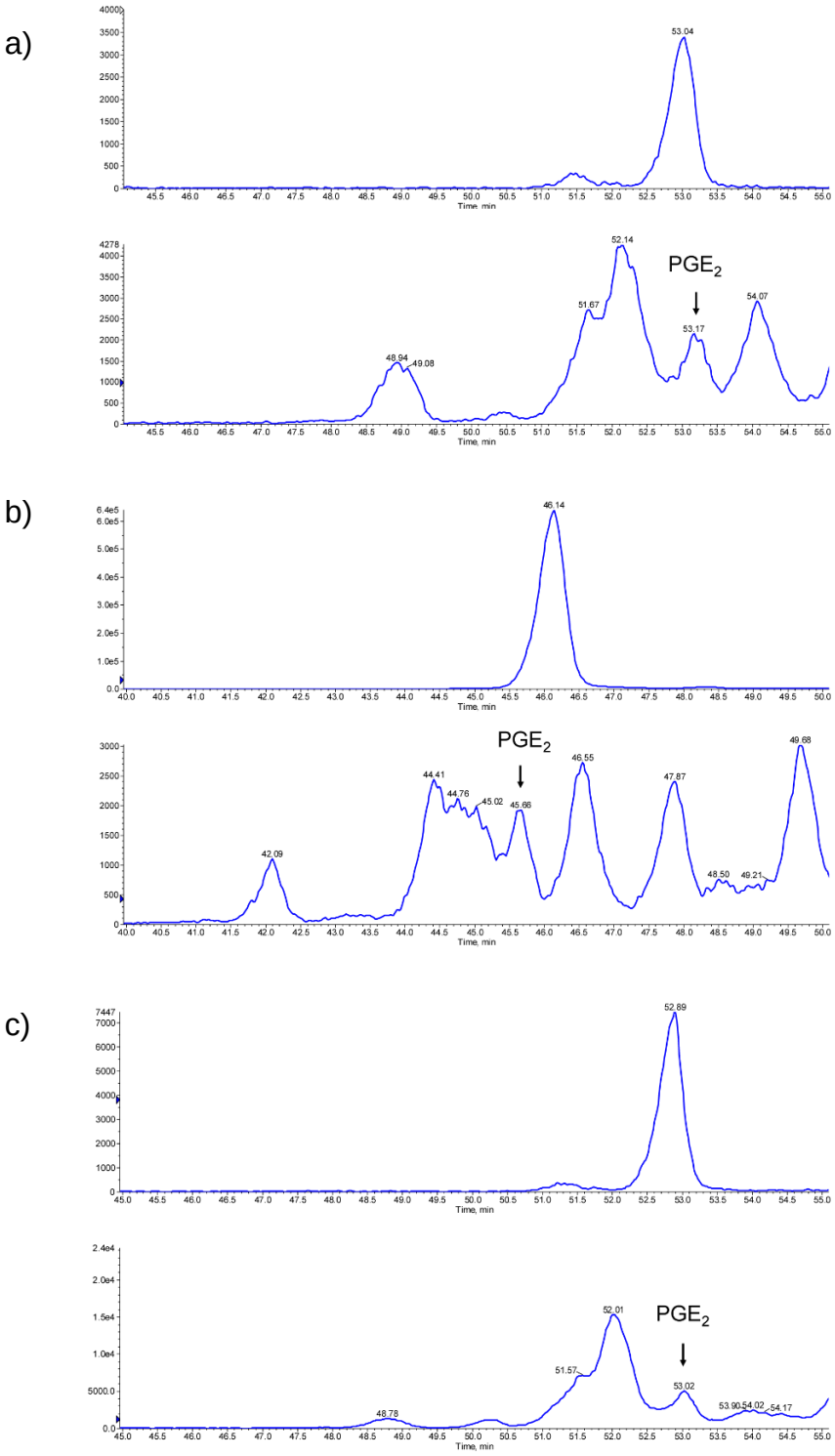

Figure 7. LC-MS/MS spectra of polymicrobial biofilm (a); *Candida albicans* (b) and *Pseudomonas aeruginosa* (c) monomicrobial biofilm intracellular prostaglandin E_2 (PGE_2). Top profiles indicate the dominant transition (355.2/315.2) of deuterated PGE_2 (PGE_{2d4}) spiked in each sample. Bottom profiles indicate dominant transition (351.1/315.2) corresponding to PGE_2 for each sample indicated by arrows. All transitions were present for PGE_{2d4} and PGE_2 respectively.

2.5. Conclusions

The results of this study confirms the production of various eicosanoids from exogenous AA by *C. albicans*. Additionally, it is evident from these results that *P. aeruginosa* produces significant amounts of eicosanoids. Also, the reactivity of these eicosanoids with PGE_2-, $PGF_{2\alpha}$- and 15-HETE-specific assays indicates that these eicosanoids are structurally similar to the specific metabolites tested. However, due to possible cross-reactivity, other eicosanoids distinct from PGE_2, $PGF_{2\alpha}$ and 15-HETE may be formed.

This study also indicates that caution should be taken when quantifying eicosanoid production of microorganisms *in vitro*, as AA autoxidation produces significant amounts of compounds structurally similar to possible enzymatic products. Prostaglandin E_2 production in *C. albicans* was confirmed by intracellular extraction. Additionally, PGE_2 could be identified in intracellular extracts of *P. aeruginosa*. Several PGE_2 isomers could also be detected using a LC-MS/MS method for PGE_2 isomer separation. This could indicate that quantification and non-isomer specific LC-MS/MS separation methods could give false results in terms of PGE_2 production *in vitro*.

The significant increase in amount of eicosanoids produced, including compounds structurally similar to PGE_2, $PGF_{2\alpha}$ and 15-HETE could dramatically influence the capability of hosts to clear infection of polymicrobial biofilms composed of *C. albicans* and *P. aeruginosa*. The PGE_2 produced by *P. aeruginosa* and *C. albicans* may initiate the T_h2 response, hampering clearance of pathogens from host infection sites. In addition, significant amount of 15-HETE is produced by mono- and polymicrobial biofilms. This 15-HETE may act as substrate for the formation of anti-inflammatory lipoxins. The effect of large amounts of PGE_2, $PGF_{2\alpha}$ and 15-HETE in combination has not been studied, and may alter pathogen clearance.

Further research should focus on identification of PGE_2 isomers present in *C. albicans* and *P. aeruginosa* biofilms. In addition, the effect of this eicosanoid production *in vivo* could provide important information regarding polymicrobial infection and the possible effect on the dynamics of growth of both species.

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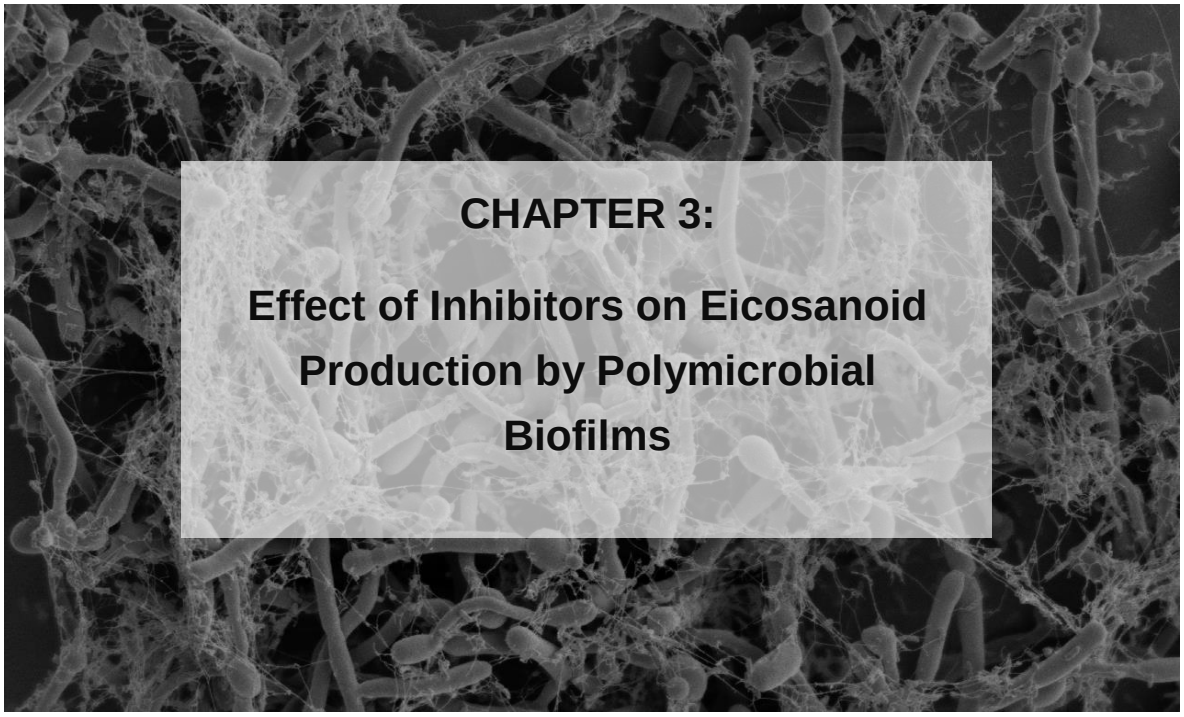

CHAPTER 3:

Effect of Inhibitors on Eicosanoid Production by Polymicrobial Biofilms

3.1. Abstract

Recently it has become evident that eicosanoids, oxygenated metabolites derived from arachidonic acid (AA), play significant roles in inflammation and infection. *Candida albicans* and *Pseudomonas aeruginosa* elicit the release of a large amount of AA from host cells during infection, with this abundance of AA utilized by hosts to produce a large number of eicosanoids, such as prostaglandin E_2 (PGE_2), which influence the ability of hosts to clear infection. *Candida albicans* and *P. aeruginosa* are frequently found together at infection sites with both having the ability to form biofilms, resistant to therapeutic intervention. Interestingly, *C. albicans* and *P. aeruginosa* are able to produce PGE_2, $PGF_{2\alpha}$ and 15-hydroxyeicosatetraenoic acid (15-HETE) from exogenous AA *in vitro*. In Chapter 2, an increase in eicosanoid production was observed by polymicrobial biofilms compared to monomicrobial counterparts. However, the mechanism of this eicosanoid formation is still unknown for these opportunistic pathogens. Multicopper oxidase and fatty acid desaturase homologs have however been implicated in *C. albicans* PGE_2 production. Several inhibitors, including acetylsalicylic acid (ASA), ammonium tetrathiomolybdate (ATM) and nordihydroguaiaretic acid (NDGA) inhibit PGE_2 production by *C. albicans*. To determine how eicosanoids are formed by polymicrobial biofilms of *C. albicans* and *P. aeruginosa*, these inhibitors were evaluated to determine their effect on eicosanoid production by monomicrobial and polymicrobial biofilms of *C. albicans* and *P. aeruginosa* through enzyme linked immunosorbent assay (ELISA), as well as on biofilm growth and morphology. This study indicated that PGE_2, $PGF_{2\alpha}$ and 15-HETE production by mono- and polymicrobial biofilms is sensitive to the inhibitors tested. The inhibitors also drastically affect the morphology of polymicrobial biofilms and ASA as well as NDGA also affect *P. aeruginosa* metabolic activity. In addition, the therapeutic potential in infection of the potent antioxidant NDGA is highlighted.

3.2. Introduction

Eicosanoids are a class of lipids involved in cellular signalling and possess important immunological functions in mammals (Dennis & Norris, 2015; Tehlivets *et al*., 2007). These eicosanoids are products of oxygenation reactions of 5,8,11,14-eicosatetraenoic acid (arachidonic acid, AA) (Marnett *et al.*, 1999). These modifications to AA can be due to enzymatic as well as non-enzymatic pathways (Jahn *et al*., 2008; Milne *et al*., 2015). Non-enzymatic reactions, which are the result of oxidative stress *in vivo*, cause the production of isoprostanes with varying biological capabilities. Both enzymatic and non-enzymatic products of AA regulate inflammation in mammalian hosts. One of the most important products formed is prostaglandin E_2 (PGE_2). In the host, cyclooxygenase enzymes (COX) are responsible for PGE_2 production (Marnett *et al.*, 1999; Murakami *et al*., 2003). Prostaglandin E_2 inhibits T_h1 and promotes T_h2 responses in hosts, inducing localized pro- or anti-inflammatory effects dependant on the host tissue affected (Romani, 2000). The commonly used non-steroidal anti-inflammatory drug (NSAID) acetylsalicylic acid (ASA), more commonly known as aspirin, irreversibly binds to COX, inhibiting the formation of PGE_2, and promoting the formation of 15-hydroxyeicosatetraenoic acid (15-HETE), which in turn acts as a precursor for anti-inflammatory eicosanoids such as lipoxins (Scneider & Brash, 2000; Vane & Botting, 2003). In addition to COX, various other enzymes are involved in AA metabolism, namely, lipoxygenases, multicopper oxidases, as well as monooxygenases (CYP450) (Dennis & Norris, 2015).

Two opportunistic pathogens that frequently co-infect hosts and possess the ability to form biofilms are *Candida albicans* and *Pseudomonas aeruginosa* (Bianchi *et al*., 2008; El-Azizi *et al*., 2004; Lindsay & Hogan, 2014; McAlester *et al*., 2008). Co-infection by these two microorganisms may increase morbidity and mortality in infections compared to monomicrobial infections (Azoulay *et al*., 2006; Hamet *et al*., 2012; Morales & Hogan, 2010; Roux *et al*., 2009; Xu *et al*., 2014). The interaction of *C. albicans* and *P. aeruginosa* can be categorised as antagonistic through physical interaction and secreted factors (Lindsay & Hogan, 2014). In addition, both elicit the release of a large amount of AA from host cells (Agard *et al*., 2013; Brash, 2001; Castro *et al*., 1994; Filler *et al*., 1994; König *et al*., 1996; Sadikot *et al*., 2007; Saliba *et al*., 2005). Cell wall components and phospholipase from *C. albicans* have been

shown to elicit the release of AA from host cells, as well as inducing the formation of PGE_2 (Smeekens *et al*., 2010). Interestingly, *C. albicans* is able to utilize the released AA as a carbon source as well as to produce a PGE_2 cross-reactive compound identified to be identical to PGE_2 produced by the host (Erb-Downward & Noverr, 2007; Noverr *et al*., 2001). Curiously, this PGE_2 production by *C. albicans* is inhibited by ASA, although *C. albicans* does not possess COX homologs (Alem & Douglas, 2005). However, other enzymes have been shown to possibly play a role in PGE_2 production by *C. albicans*, namely, a multicopper oxidase homolog (Fet3p), a fatty acid destaturase homolog (Ole2p), as well as monooxygenases (CYP450s) (Ells *et al*., 2011; Erb-Downward & Noverr, 2007; Krause *et al*., 2015).

Pseudomonas aeruginosa virulence factors, including exotoxin U, pyocyanin, the quorum sensing molecule 3-oxo-homoserine lactone and lipopolysaccharide also induce the release of AA from host cells and the formation of large amounts of PGE_2 (McDermott *et al*., 2013; Saliba *et al*., 2005; Smith *et al*., 2002). This Gram-negative bacterium is also able to produce prostaglandins and prostaglandin-like molecules (Lamacka & Sajbidor, 1995). In addition, *P. aeruginosa* possesses a secretable 15-lipoxygenase, capable of producing 15-HETE, similar to hosts, possibly affecting the clearance of *P. aeruginosa* during infection (Vance *et al*., 2004).

In Chapter 2, the production of eicosanoids from AA by biofilms of *C. albicans* and *P. aeruginosa* were evaluated. It was found that PGE_2-, $PGF_{2\alpha}$- and 15-HETE-cross-reactive compounds are produced. Although some information is available regarding the formation of PGE_2 by *C. albicans* and the possible enzymes involved in the formation of it, the enzymatic conversion of AA by polymicrobial biofilms of *C. albicans* and *P. aeruginosa* has not been studied. Therefore, the aim of this study is to elucidate the possible enzyme groups responsible for the metabolism of AA to form eicosanoids by *C. albicans* and *P. aeruginosa* polymicrobial biofilms *in vitro* through the use of various inhibitors, previously shown to inhibit *C. albicans* PGE_2 production.

3.3. Materials and methods

3.3.1. Strains used

Candida albicans strain CBS 8758 (SC5314) was used in the following studies and was maintained on yeast malt extract (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 also used. This strain was provided by Professor Hancock from the Department of Microbiology and Immunology at the University of British Columbia and revived according to American Type Culture Collection (ATCC) method from lyophilized strain ampoules, and maintained on nutrient agar (NA) (1 g/L malt extract, 2 g/L yeast extract, 5 g/L peptone, 8 g/L sodium chloride and 20 g/L agar) at 37 °C.

3.3.2. Formation of mono- and polymicrobial biofilms

3.3.2.1. Monomicrobial biofilm formation by Candida albicans

Candida albicans 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 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 then counted with a hemocytometer and diluted to 1 x 10^6 cells/mL in 20 mL filter sterilized (0.22 µm 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, 500 µM of AA (Sigma-Aldrich, USA) (Stock of 1 g in 25 mL of absolute ethanol reaching a concentration of 131.4 mM) was added to each petri dish containing medium plus cells (Ells *et al*., 2011). Petri dishes were incubated for 48 h at 37 °C to allow biofilm formation (Ramage *et al*., 2001). The final ethanol concentration in the biofilms before incubation was 0.38 %. Appropriate controls were included, including a cell free control (RPMI-1640 without AA) as well as a cell free control with RPMI-1640 and 500 µM AA.

3.3.2.2. Monomicrobial biofilm formation by Pseudomonas aeruginosa

Pseudomonas aeruginosa was grown on NA plates for 24 h at 37 °C and was 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). Cells were diluted to an optical density (OD_{600}) of approximately 0.05 in 20 mL filter sterilized RPMI-1640 medium and dispensed into 90 mm polystyrene petri dishes. In addition to the cells, 500 µM of AA was added to each petri dish containing medium plus cells. Petri dishes were incubated for 48 h at 37 °C to allow biofilm formation. The final ethanol concentration in the biofilms before incubation was 0.38 %. Appropriate controls were included. These controls were repeated for monomicrobial biofilms of *P. aeruginosa* as well as polymicrobial biofilms.

3.3.2.3. Polymicrobial biofilm formation by Candida albicans *and* Pseudomonas aeruginosa

The formation of polymicrobial biofilms follows the combination of monomicrobial biofilm formation of both *C. albicans* and *P. aeruginosa*. Briefly, *C. albicans* and *P. aeruginosa* were grown as described above, and diluted to 20 mL RPMI-1640 medium. The medium thus contains $1x10^6$ cells/mL *C. albicans* cells, as well as *P. aeruginosa* cells (approximately 0.05 OD_{600}). In addition to the cells in the medium, 500 µM of AA was added to each petri dish. Petri dishes were incubated for 48 h at 37 °C to allow biofilm formation. The final ethanol concentration in the biofilms before incubation was 0.38 %. Appropriate controls were included.

3.3.3. Effect of inhibitors

Prior to biofilm formation, 100 µM (Ells *et al*., 2011) of inhibitors namely, acetylsalicylic acid (dissolved in ethanol. Final ethanol concentration in medium 0.45 %), ammonium tetrathiomolybdate (dissolved in deionized water) and nordihydroguaiaretic acid (dissolved in ethanol. Final ethanol concentration in medium 0.30 %) were added (final concentration 100 µM). Biofilms were incubated for 48 h at 37 °C.

3.3.4. Influence of inhibitors on biofilm formation

3.3.4.1. Influence on metabolic activity

Biofilms in the presence of AA and inhibitors were prepared as described before in a 96-well plate (Corning Incorporated, Costar®, USA) with the volume of medium adjusted to 100 µL. The plate was incubated for 48 h 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. The XTT assay was performed according to Kuhn *et al.* (2003). Briefly, 50 µL 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. Following incubation, the optical density of each well was measured at 492 nm on a Labotec Spectramax M2 microplate reader (Molecular devices, USA) and data was obtained. Appropriate controls including RPMI-1640 containing only AA were included. This experiment was performed in triplicate.

3.3.4.2. Influence on biomass production

Biofilms were prepared as described before in petri dishes. After incubation, the individual biofilms were scraped off and washed twice with sterile PBS, where after the resuspended cells were filtered through pre-weighed filters (0.22 µm). The filters were dried at 37 °C overnight and the dry biomass of the mono- and polymicrobial biofilms determined (Thibane *et al*., 2010). This experiment was performed in triplicate.

3.3.4.3. Influence on morphology

Polymicrobial biofilms in the presence of AA and inhibitors were prepared as described above in flat bottom 6 well culture plates (Corning Incorporated, USA) in 3 mL medium. After incubation the supernatants were removed and 5 mm rectangular sections of the bottom of the wells were cut out, washed with PBS and fixed overnight with the primary fixative, 3 % (v/v) gluterdialdehyde (Merck, Germany) in phosphate buffer (pH 7.0). The cells were then washed with phosphate buffer (Hawser & Douglas, 1994) and

then fixed with the secondary fixative, 1 % (v/v) osmium tetroxide (Merck, Germany) for 1 h at room temperature. The biofilms were then sequentially dehydrated at 50 %, 70 % and 95 % ethanol for 15 min each and twice for 1 h with 100 % ethanol. After critical point drying (Samdri-795 Critical point dryer, Tousimis, USA), the disks were sputter coated with gold using a SEM coating system (Bio-Rad Microscience division, UK) and examined using a Shimadzu SSX-550 Superscan scanning electron microscope.

3.3.5. Influence of inhibitors on eicosanoid production

Monomicrobial and polymicrobial biofilms were prepared as described above. Supernatants were removed from biofilms and cells were removed with centrifugation (5750 *g* @ 4 °C for 5 minutes, Eppendorf, Germany). After centrifugation the supernatant was filtered (0.2 µm nitrocellulose filter). Extraction of eicosanoids were performed according to a modified protocol proposed by Cayman chemicals for PGE_2 purification for enzyme linked immunosorbent assay (ELISA). Briefly, supernatants were acidified by to a pH of approximately 4 with the addition of 1 M formic acid (Merck, Germany). Solid phase extraction (SPE) classic C18 cartridges (Waters, Ireland) were prepared with 5 mL methanol (Merck, Germany), followed by 5 mL deionized water. Samples (10 mL) were applied to cartridges and subsequently washed with 5 mL deionized water to remove impurities. Eicosanoids were then eluted from the SPE cartridges with 5 mL ethyl acetate containing 1 % methanol and collected in pre-washed poly top glass vials (Lasec, South Africa). The eluent was dried under a stream of N_2 and stored at -80 °C until use.

Samples were dissolved in eicosanoid affinity (EIA) buffer provided by the manufacturers and samples were assayed for PGE_2, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and 15-HETE using enzyme linked immunosorbent assay (ELISA) (Cayman Chemicals, USA) according to manufacturer's specifications. Samples were assayed in two dilutions in duplicate. This experiment was done in triplicate. Data was analysed according to manufacturer's specifications.

3.3.5. Statistical analysis

To evaluate significant differences between mono- and polymicrobial biofilms exposed to inhibitors compared to control biofilms (only AA without inhibitors), one-way analysis of variance (ANOVA) was used between data sets of mono- and polymicrobial biofilms ($P < 0.05$). In addition, this was followed by Dunnett's multiple comparisons test with an alpha value of 0.05 to determine significant differences between biofilms with only AA (control) and biofilms exposed to AA as well as inhibitors. Significant differences are indicated with ' * '.

3.4. Results and discussion

3.4.1. Effect of inhibitors on Candida albicans *and* Pseudomonas aeruginosa *mono- and polymicrobial biofilm formation*

Although the metabolism of eicosanoid in mammalian systems such as humans has been well studied, the same cannot be said for the production of eicosanoids by microorganisms. This gap in knowledge in eicosanoid production, such as the production of prostaglandins, by microorganisms, is because microbial cells rarely possess homologs of enzymes responsible for this conversion in animals. Previous studies have however indicated that several inhibitors decrease the production of PGE_2 by *C. albicans* (Ells *et al*., 2011; Erb-Downward & Noverr, 2007; Krause *et al*., 2015). These include ASA (Figure 1a), ammonium tetrathiomolybdate (ATM, Figure 1b) and nordihydroguaiaretic acid (NDGA, Figure 1c). These inhibitors are responsible for the inhibition of various enzyme groups. Acetylsalicylic acid acetylates COX enzymes in mammalian systems, inhibiting the production of prostaglandins and thromboxanes (Scneider & Brash, 2000; Vane & Botting, 2003). Ammonium tetrathiomolybdate finds important clinical use in copper metabolic disorders due to its copper chelating properties, and inhibits multicopper oxidases (De Brucker *et al*., 2013). The inhibitor NDGA, refined from the Creosote bush (*Larrea tridentate*), has a broad inhibitory effect, affecting lipoxygenase, multicopper oxidase and monooxygenase activity (Lü *et al*., 2010). This is due to the potent antioxidant activity of NDGA.

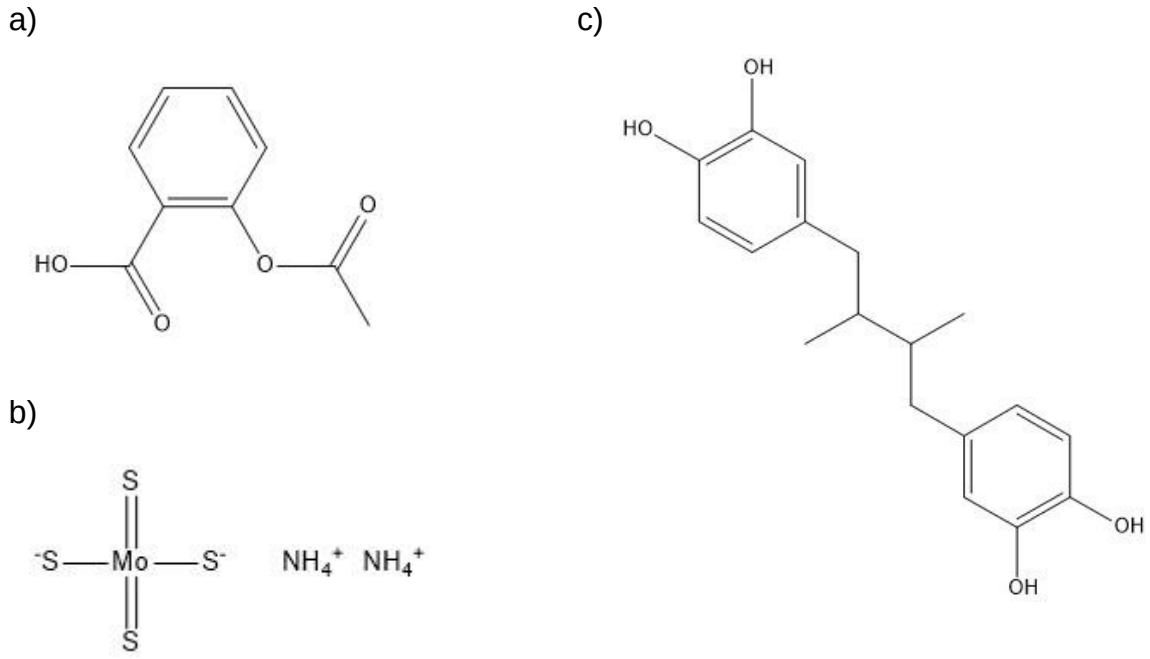

Figure 1. Structures of acetylsalicylic acid (a); ammonium tetrathiomolybdate (b); nordihydroguaiaretic acid (c).

It has previously been shown that these inhibitors affect the formation of PGE_2 in *C. albicans*, but do not have an effect on growth and metabolic activity at 100 µM, therefore, these inhibitors at 100 µM were utilized in this study (Ells *et al*., 2011). Figure 2 indicates the effect of ASA, ATM and NDGA in combination with AA on metabolic activity of *C. albicans* and *P. aeruginosa* mono- and polymicrobial biofilms compared to control biofilms without inhibitors. As can be seen, the inhibitors did not influence the metabolic activity (Figure 2a) or the biomass production (Figure 2b) of *C. albicans* monomicrobial biofilms or polymicrobial biofilms.

Interestingly, the inhibitor NDGA caused an almost 100 % decrease in metabolic activity of *P. aeruginosa* monomicrobial biofilms, although the biomass did not significantly differ between control and inhibitor-treated biofilms. In contrast, the COX-inhibitor ASA, significantly increased the metabolic activity of *P. aeruginosa* monomicrobial biofilms again without affecting the biomass production. Previous research indicated that salicylic compounds inhibits ATP synthesis in plant cells and is an electron uncoupler (Norman *et al*., 2004). A similar effect may occur in *P.*

aeruginosa, where the bacterial cells compensate for reduced ATP synthesis, caused by the disruption of electron flow through the electron transport chain, through increasing metabolic activity.

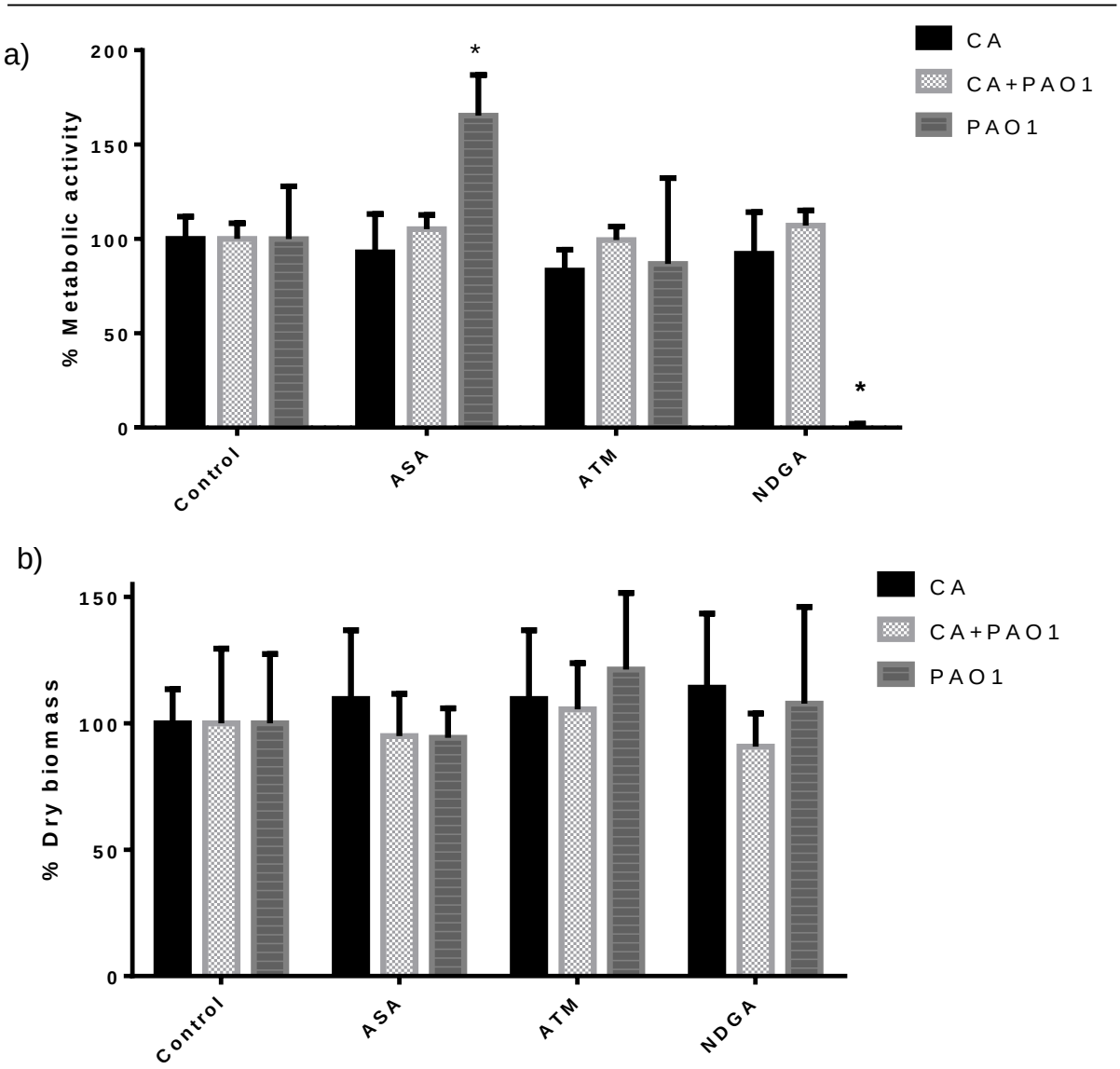

Figure 2. Percentage metabolic activity (a) and biofilm biomass (b) of mono- and polymicrobial biofilms of *Candida albicans* (CA) and *Pseudomonas aeruginosa* (PAO1) in the presence of acetylsalicylic acid (ASA), ammonium tetrathiomolybdate (ATM) and nordihydroguaiaretic acid (NDGA) compared to control biofilms. * Significantly different from control biofilms ($P < 0.05$).

Previous research indicates that ASA inhibits quorum sensing, virulence factors and biofilm formation in *P. aeruginosa* (El-Mowafy *et al*., 2014). However, the study utilized concentrations of ASA much higher (lowest concentration > 50 fold) than the 100 µM

used in this study. This indicates that although a high concentration of ASA may be inhibitory, lower concentrations may increase the metabolic activity of *P. aeruginosa.* Similar results were also seen for salicylic acid (Plyuta *et al*., 2013). This finding should be further investigated for possible clinical significance.

In addition to evaluating the effect of inhibitors on metabolic activity and biomass, the morphological effect of inhibitors on polymicrobial biofilms of *C. albicans* and *P. aeruginosa* was evaluated. In Figure 3, scanning electron micrographs of polymicrobial biofilms formed in the presence of AA and the inhibitors (ASA, ATM and NDGA) can be seen. The control biofilm consists of *C. albicans* cells, predominantly in the yeast morphology with a small amount of hyphae. In addition, *P. aeruginosa* cells can be observed entangled in extracellular matrix. The inhibition of filamentation by *C. albicans* cells usually observed in *C. albicans* monomicrobial biofilms may be due to the presence of *P. aeruginosa* as well as AA (Refer to Figure 2, Chapter 2).

Interestingly, the inhibition of filamentation of *C. albicans* in the presence of AA and *P. aeruginosa* is abolished in the biofilm grown in the presence of ASA with *C. albicans* showing profuse hyphae even in the presence of large numbers of *P. aeruginosa* cells. This lack of inhibition of filamentation of *C. albicans* by *P. aeruginosa* may indicate that the *P. aeruginosa* inhibitory factors, such as the quorum sensing molecule 3-oxo-homoserine lactone and pyocyanin, may be inhibited by ASA. Recent work by EL-Mowafy *et al*. (2014) also found that high concentrations (33 mM) of ASA inhibited 3-oxo-homoserine lactone and pyocyanin production. Significantly, a dramatic increase in the amount of *P. aeruginosa* cells is seen in the presence of ASA as compared to the control biofilm. In addition, a decrease in extracellular material is observed by *P. aeruginosa* in the presence of ASA. This confirms previous observations, where ASA inhibited the production of alginate, the principle component of *P. aeruginosa* extracellular material (Dergez *et al*., 2014). This inhibition of extracellular material may increase susceptibility of biofilms to immune attack and antimicrobial agents.

A significant influence on morphology can be seen for the biofilms grown in the presence of NDGA. No *C. albicans* hyphae can be seen and yeast cells are all attached to one another, indicating that not only is filamentation inhibited, but also cell-separation during budding of the yeast. This might me due to the inability to degrade the septum between mother and daughter cells (Song *et al*., 2008). This effect of

NDGA on *C. albicans* morphology has not previously been observed. This morphotype of *C. albicans* cells is similar to the *C. albicans* cells deficient in the RAM (regulation of Ace2p transcription factor and polarized morphogenesis) signalling network, indicating that the RAM signalling network in *C. albicans* may be affected by NDGA (Song *et al*., 2008). In addition, very little *P. aeruginosa* cells are observable in the presence of NDGA. Interestingly, a large amount of extracellular material is observed.

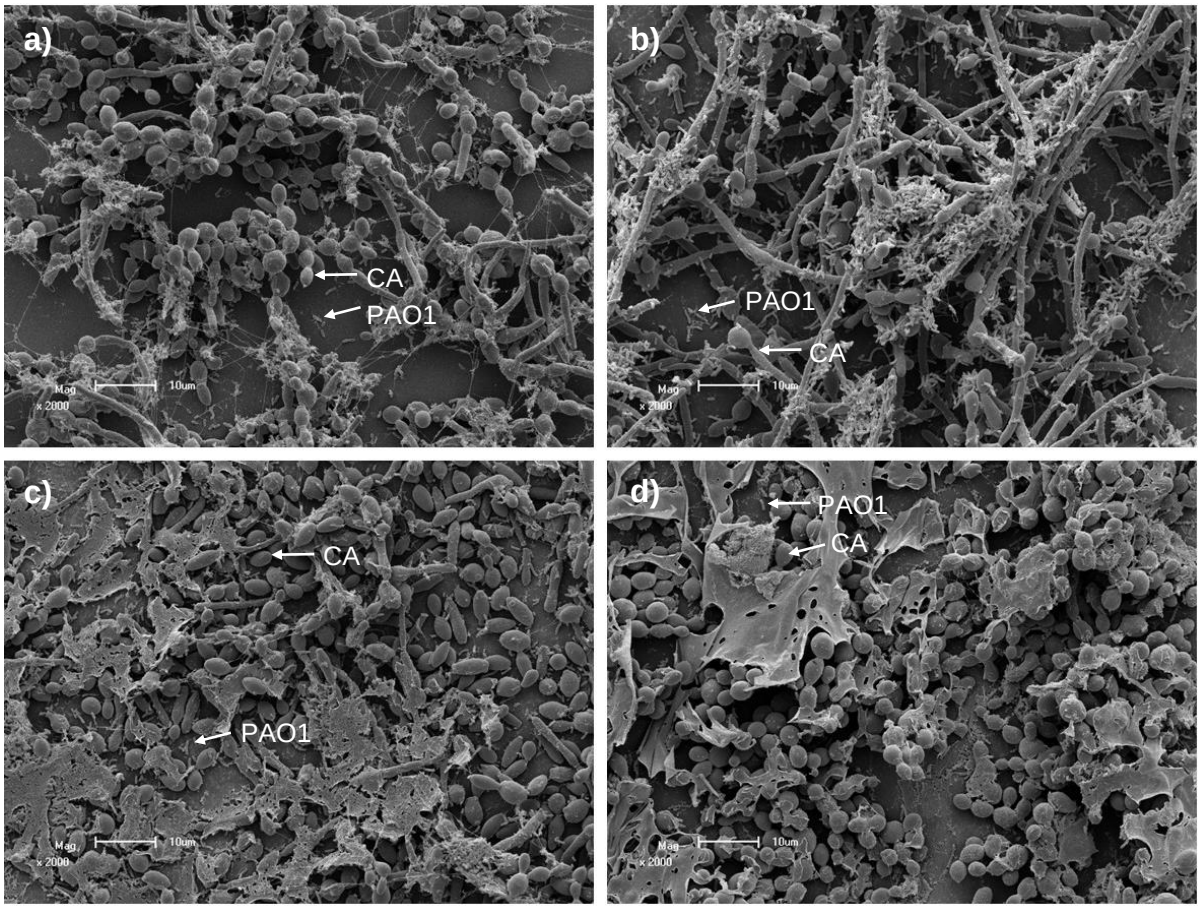

Figure 3. Scanning electron micrograph of *Candida albicans* (CA) and *Pseudomonas aeruginosa* (PAO1) polymicrobial biofilms exposed to arachidonic acid (AA) as well as eicosanoid synthesis inhibitors for 48 h. Scale bars represent 10 µm. Control with only AA (a); acetylsalicylic acid (b); ammonium tetrathiomolybdate (c); nordihydroguaiaretic acid (d).

3.4.2. Effect of inhibitors on eicosanoid production by Candida albicans *and* Pseudomonas aeruginosa *mono- and polymicrobial biofilms*

3.4.2.1. Effect of acetylsalicylic acid

Figure 4 indicates the amount of PGE_2, $PGF_{2\alpha}$ and 15-HETE respectively of mono- and polymicrobial biofilms of *C. albicans* and *P. aeruginosa* in the presence of ASA. A significant decrease in *C. albicans* PGE_2 production by ASA has previously been reported (Ells *et al.*, 2011). In the present study, PGE_2 production by *C. albicans* monomicrobial biofilms was also significantly inhibited (67% +/- 9.3). However, $PGF_{2\alpha}$ production was increased. In mammalian systems, the conversion of AA to the unstable intermediate PGH_2 by COX, acts as substrate for synthases, to form PGE_2 and $PGF_{2\alpha}$ (Murakami *et al*., 2003; Tam, 2013). As ASA inhibits COX in mammalian systems, inhibition thereof would yield a decrease in $PGF_{2\alpha}$ as well. This phenomenon is not seen with *C. albicans*, indicating $PGF_{2\alpha}$ production is not dependent on the same enzyme(s) as PGE_2 production. Interestingly, 15-HETE production by *C. albicans* was not significantly affected, indicating the production of 15-HETE by *C. albicans* is also via another pathway that may differ drastically from mammalian systems.

A significant increase in PGE_2, $PGF_{2\alpha}$ and 15-HETE production is observed by *P. aeruginosa* monomicrobial biofilms in the presence of ASA. This phenomenon has not been described before. The increase in eicosanoid production might be due to the increased metabolic activity observed for *P. aeruginosa* monomicrobial biofilms. Acetylsalicylic acid did not significantly affect the production of PGE_2 in polymicrobial biofilms of *C. albicans* and *P. aeruginosa*, although a significant increase (over 200 %) in 15-HETE production as well as in $PGF_{2\alpha}$ by polymicrobial biofilms was observed, possibly indicating the combined contribution of the two microbes, resulting in a shift in eicosanoid profile in the presence of ASA. As ASA may reach much higher concentrations *in vivo* during treatment, these phenomena need to be evaluated with physiologically relevant concentrations of ASA.

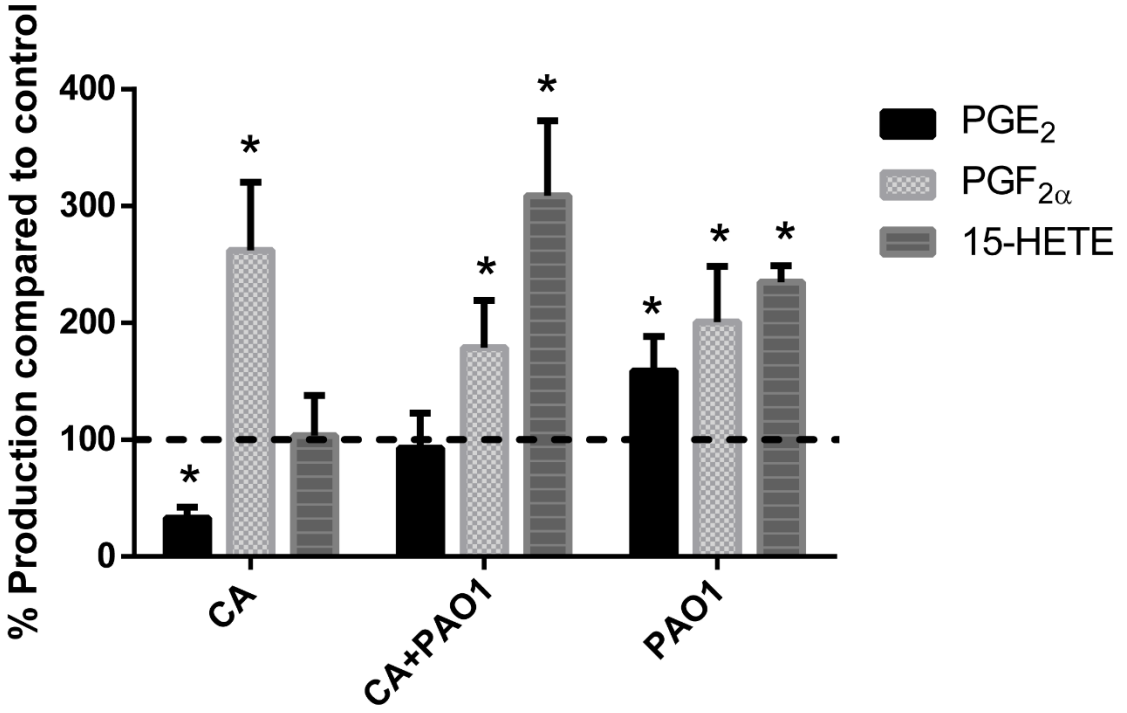

Figure 4. Eicosanoid production of mono- and polymicrobial biofilms in the presence of acetylsalicylic acid. Percentage production of prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and 15-hydroxyeicosatetraenoic acid (15-HETE) by mono- and polymicrobial biofilms of *Candida albicans* and *Pseudomonas aeruginosa* in the presence of acetylsalicylic acid compared to control biofilms. * Significantly different from control biofilms.

3.4.2.2. Effect of ammonium tetrathiomolybdate

Prostaglandin E_2 production by *C. albicans* monomicrobial biofilms displayed a significant decrease (circa 87% inhibition) in the presence of ATM (Figure 5). This confirms previous research indicating that a multicopper oxidase homolog (Fet3p) possibly plays a role in *C. albicans* PGE_2 production (Ells *et al*., 2011; Erb-Downward & Noverr, 2007). In addition, ATM abolished 15-HETE production by *C. albicans* suggesting that similar copper dependent enzyme(s) may also be involved in 15-HETE production in this yeast. Interestingly, a dramatic increase (over 10 fold) is seen in the amount of $PGF_{2\alpha}$. This increase in *C. albicans* $PGF_{2\alpha}$ is not due to increased autoxidation of AA extracellularly in the growth medium, as control sample values of only extracted AA and inhibitors are subtracted from sample values. De Brucker *et al*.

(2013) revealed that the combination of 10 mM ATM with the antimycotic amphotericin B, significantly increased the effect of amphotericin B against *C. albicans* biofilms. This is due to the fact that ATM inhibits superoxide dismutase in *C. albicans*, hampering the ability of *C. albicans* to relieve oxidative stress in cells caused by the formation of superoxide by amphotericin B. This could possibly indicate that oxidative stress in *C. albicans* cells in biofilms may be increased in the presence of ATM. The increased reactive oxygen species could non-enzymatically react with intracellular AA, producing F_2-isoprostanes, which may cross-react with the $PGF_{2\alpha}$ specific ELISA (Jahn *et al*., 2008; Milne *et al*., 2015).

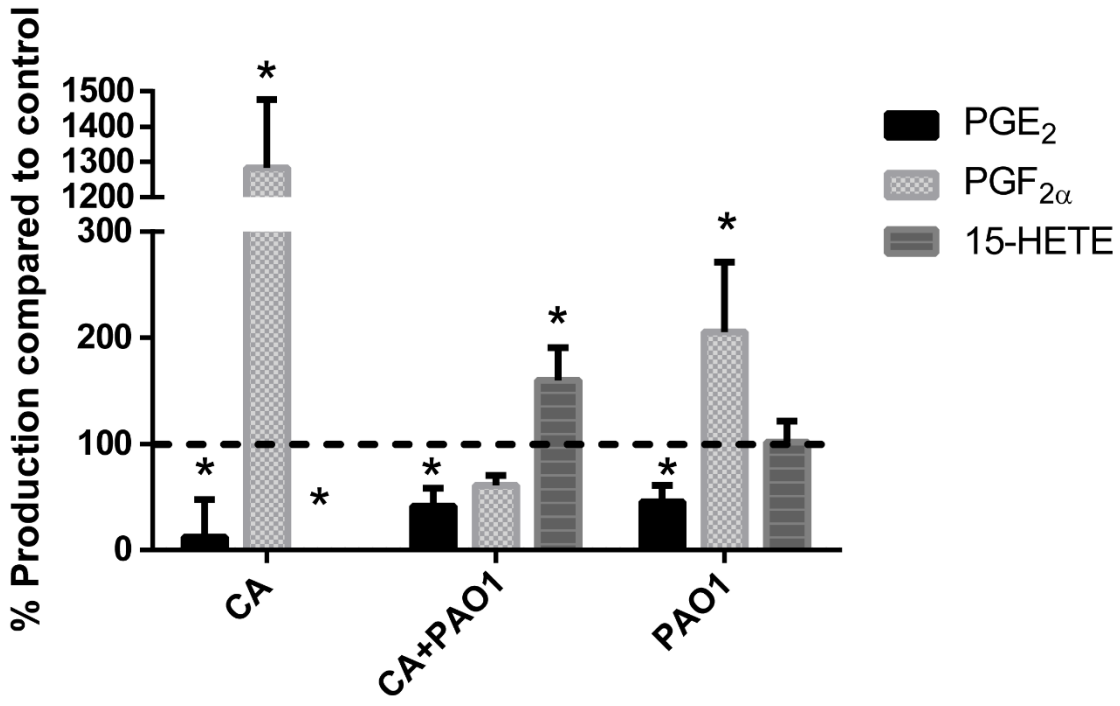

Figure 5. Eicosanoid production of mono- and polymicrobial biofilms in the presence of ammonium tetrathiomolybdate. Percentage production of prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and 15-hydroxyeicosatetraenoic acid (15-HETE) by mono- and polymicrobial biofilms of *Candida albicans* and *Pseudomonas aeruginosa* in the presence of ammonium tetrathiomolybdate compared to control biofilms. * Significantly different from control biofilms.

Pseudomonas aeruginosa monomicrobial biofilm PGE_2 production was also significantly inhibited (over 50 %), accompanied by an increase in $PGF_{2\alpha}$, similar to *C. albicans* monomicrobial biofilms. However, 15-HETE production did not differ significantly as is expected for a product of 15-LOX. In the case of polymicrobial biofilms, a significant reduction in PGE_2 (circa 60 %) concentration is observed in the presence of ATM. However, in contrast to the effect on monomicrobial biofilms, this was accompanied by a significant increase in 15-HETE without a significant effect on $PGF_{2\alpha}$ concentration. This indicates the complexity of AA metabolism responsible for eicosanoid formation by polymicrobial biofilms. The use of copper chelating tetrathiomolybdate in bacterial infections was recently assessed, indicating that copper chelation suppresses the increase in inflammatory mediators, such as TNF-α among others, brought on by LPS in mice (Wei *et al*., 2011). The results above indicate that copper chelating compounds may not only affect the production of inflammatory mediators in hosts, but may also affect microbial eicosanoid production, possibly altering inflammation, and therefore, the ability of the host to clear pathogens during *C. albicans* and *P. aeruginosa* infection.

3.4.2.3. Effect of nordihydroguaiaretic acid

Nordihydroguaiaretic acid, an inhibitor of multiple enzyme classes, including lipoxygenase, multicopper oxidase and CYP450, significantly decreased PGE_2 production by *C. albicans* monomicrobial biofilms (Fig 6), confirming previous observations (Ells *et al*., 2011). This decrease was accompanied by an almost complete inhibition of 15-HETE production. A decrease in $PGF_{2\alpha}$ production is also observed, however, this decrease was not significant according to statistical analysis ($p = 0.07$, *t*-test), possibly due to high standard deviations observed in analysis of $PGF_{2\alpha}$ production. Production of PGE_2 by *P. aeruginosa* monomicrobial biofilms was significantly inhibited, and 15-HETE production was almost completely inhibited. All three eicosanoids tested were significantly inhibited in polymicrobial biofilms, with 15-HETE production abolished, as with monomicrobial biofilms.

The inhibition of enzymatic activity of microbial, as well as mammalian systems, are due to the significant antioxidant properties of the polyphenolic structure of NDGA (Lü *et al*., 2010). This antioxidant activity is due to its ability to scavenge reactive oxygen

species. In addition, NDGA inhibits 5-lipoxygenase activity, inhibiting the formation of leukotrienes. The results above in terms of eicosanoid production inhibition, the dramatic effect on *C. albicans* morphology, as well as the inhibition of *P. aeruginosa* metabolic activity *in vitro*, indicate the therapeutic potential of NDGA during infection. The use of NDGA clinically is however not feasible yet, due to the toxicity of the compound (Lü *et al*., 2010). Modification of this compound to reduce toxicity may provide a powerful tool in treatment of infection.

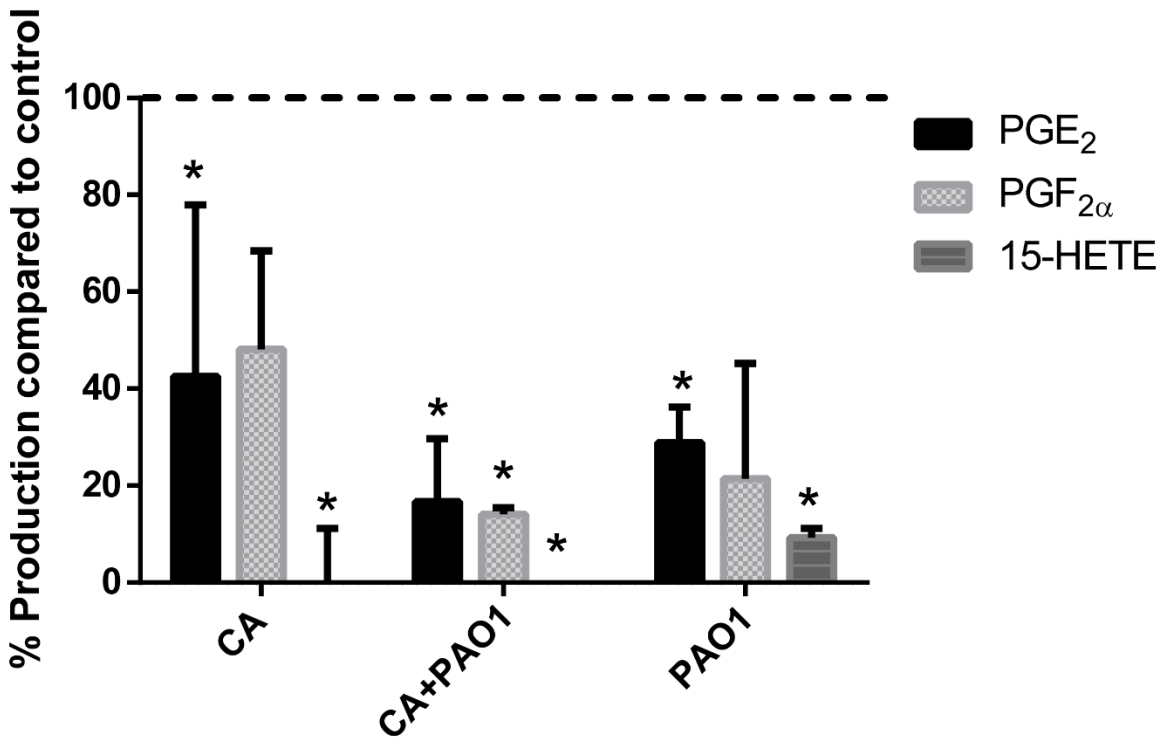

Figure 6. Eicosanoid production of mono- and polymicrobial biofilms in the presence of nordihydroguaiaretic acid. Percentage production of prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and 15-hydroxyeicosatetraenoic acid (15-HETE) by mono- and polymicrobial biofilms of *Candida albicans* and *Pseudomonas aeruginosa* in the presence of nordihydroguaiaretic acid compared to control biofilms. * Significantly different from control biofilms.

3.5. Conclusions

The identification of immune modulating compounds produced by microorganisms, previously thought to be restricted to animals, may lead to alternative therapeutic options for treating infections. In Chapter 2, previous uncharacterized interactions between *C. albicans* and *P. aeruginosa* in terms of eicosanoid production *in vitro* was assessed. However, the mechanism of eicosanoid production, specifically PGE_2, $PGF_{2\alpha}$ and 15-HETE in *C. albicans* and *P. aeruginosa* is not known. This study confirms previous observations that *C. albicans* PGE_2 production is dependent on ASA-sensitive enzymes. In addition, results obtained suggest that prostaglandin synthesis for PGE_2 and $PGF_{2\alpha}$ may be due to different enzyme groups. The copper chelating agent ATM, is also shown to possibly shift eicosanoid metabolism in *C. albicans* monomicrobial biofilms, possibly promoting the formation of $PGF_{2\alpha}$ cross-reactive compounds.

This study also reports the production of eicosanoids by *P. aeruginosa* that are sensitive to ASA, as well as ATM and NDGA. This study is also the first to report that 100 µM ASA increases metabolic activity, as well as eicosanoid production by *P. aeruginosa* biofilms, possibly increasing the virulence of this bacterium, although previous research indicates a reduction in virulence at higher concentrations. In addition, the use of inhibitors in polymicrobial biofilm eicosanoid production indicate that a complex interaction between various enzyme groups may catalyse the formation of PGE_2, $PGF_{2\alpha}$ and 15-HETE. Also, NDGA dramatically affects *C. albicans* cell morphology in polymicrobial biofilms *in vitro*, with this potent antioxidant also significantly decreasing eicosanoid production by polymicrobial biofilms.

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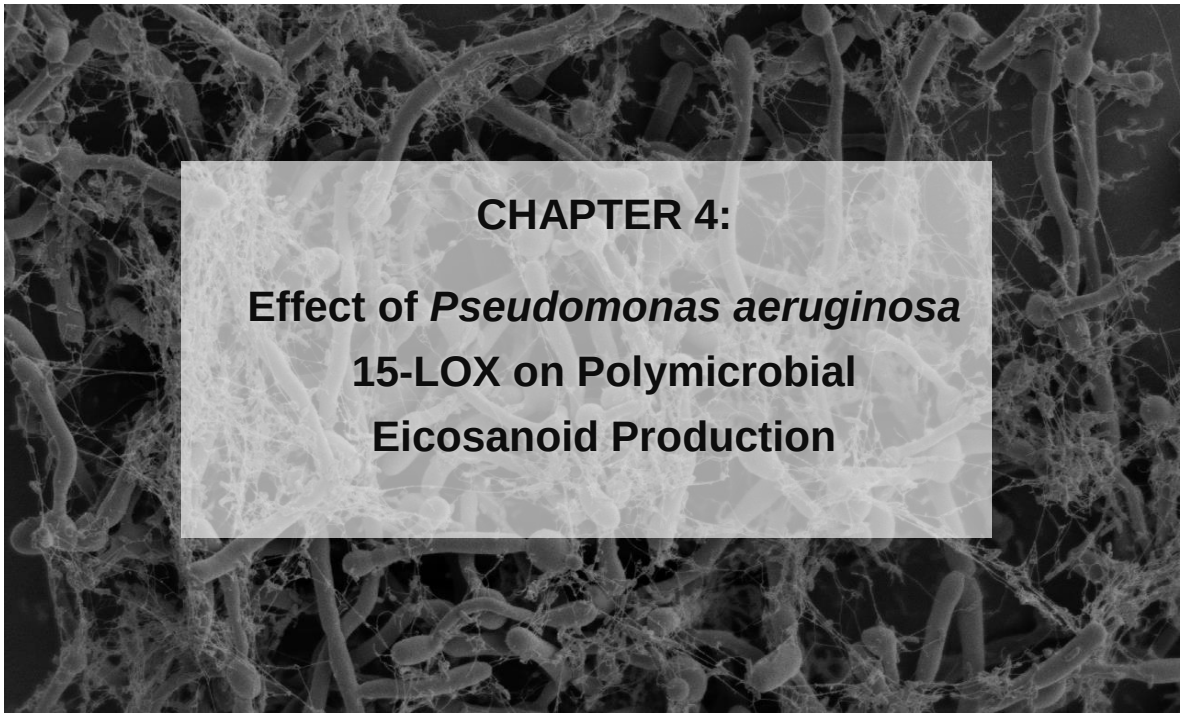

CHAPTER 4:

Effect of *Pseudomonas aeruginosa* 15-LOX on Polymicrobial Eicosanoid Production

4.1. Abstract

Pseudomonas aeruginosa and *Candida albicans* are two opportunistic pathogens frequently found together at infection sites. *Candida albicans* and *P. aeruginosa* interact with each other through physical interaction and secreted factors. The Gram-negative bacterium as well as *C. albicans* liberate arachidonic acid (AA) from host phospholipids during infection. This AA is utilized by the host to form a large range of oxygenated metabolites termed eicosanoids. These eicosanoids regulate inflammation, and ultimately the ability to clear infection. *Pseudomonas aeruginosa* and *C. albicans* are also able to utilize AA to produce eicosanoids, such as prostaglandin E_2 (PGE_2), $PGF_{2\alpha}$ and 15-hydroxyeicosatetraenoic acid (15-HETE). *Pseudomonas aeruginosa* accomplishes the production of 15-HETE through a secretable 15-lipoxygenase (LoxA), capable of converting AA to 15-HETE, similar to host cells. As 15-HETE can be further modified to form anti-inflammatory lipoxins with the potential to affect the ability of the host to clear infection, it is necessary to determine the influence in infection, as well as the role in interkingdom interaction with *C. albicans*. Therefore, this study aimed to evaluate the role of LoxA in eicosanoid production by monomicrobial biofilms of *P. aeruginosa*, as well as in polymicrobial biofilms with *C. albicans*. A *P. aeruginosa* strain deficient in LoxA created with Tn5-mini transposon mutagenesis was used in this study to determine the role of LoxA. Through the use of molecular techniques the presence of the disruption construct in the promotor region of *P. aeruginosa loxA* was confirmed and enzyme-linked immunosorbent assay (ELISA) was used to evaluate eicosanoid production. No significant difference in growth of *P. aeruginosa* was observed when comparing *loxA*::Tn5 with the wild type (PAO1). It was determined that the mutant strain was still capable of producing 15-HETE, possibly indicating that the method for the creation of the disruption construct may not be efficient in terminating transcription. Further research is needed to evaluate this aspect of interaction between *C. albicans* and *P. aeruginosa*.

4.2. Introduction

Pseudomonas aeruginosa, a Gram-negative opportunistic pathogen, frequently infects immunocompromised individuals (Pier, 1985; Rinzan, 2009; Tan *et al*., 1999). The ability of this bacterium to cause disease is due to a large amount of virulence factors, encoded by one of the largest bacterial genomes (6.3 Mbp) (Stover *et al*., 2000). A large number of these virulence factors are released via a type II as well as a type III secretion system, producing a number of hydrolytic enzymes (Ball *et al*., 2002; Hogardt *et al*., 2004). In addition, quorum sensing molecules, phenazine compounds and the ability to form biofilms add to the virulence arsenal of *P. aeruginosa* (Drenkard, 2003; Gibson *et al*., 2009). These virulence factors not only play roles during infection, but also play extensive roles in interkingdom interactions with microorganisms, such as the yeast *Candida albicans* (Lindsay & Hogan, 2014; Morales & Hogan, 2010; Rinzan, 2009). The interaction between these two microorganisms is largely antagonistic, promoting the growth of *P. aeruginosa* with suppression of fungal growth. Research into physical interaction indicated that *P. aeruginosa* colonizes and kills *C. albicans* hyphae, partly accomplished through the phenazine compound pyocyanin (Brand *et al*., 2008; Kerr *et al*., 1999). In addition, the *P. aeruginosa* quorum sensing molecule 3-oxo-dodecanoylhomoserine lactone inhibits the yeast to hyphal switch in *C. albicans* (McAlester *et al*., 2008). Ethanol production by *C. albicans*, extracellular DNA, iron sequestration and bacterial cell wall components all play a role in this interkingdom interaction (Bandara *et al*., 2013; Chen *et al*., 2014; Morales *et al*., 2013; Purschke *et al*., 2012; Sapaar *et al*., 2014; Xu *et al*., 2008).

During infection, *P. aeruginosa* and *C. albicans* elicit the release of large amounts of arachidonic acid (AA) from host phospholipids (Castro *et al*., 1994; Saliba *et al*., 2005). In mammalian systems, this AA is subjected to enzymatic modification to produce various immune modulating oxygenated derivatives, termed eicosanoids (Dennis & Norris, 2015; Tam, 2013). The main product of enzymatic conversion of AA in host cells is prostaglandins, more specifically, prostaglandin E_2 (PGE_2), cyclooxygenase (COX) (Marnett *et al*., 1999; Murakami *et al*., 2003). The production of these compounds, such as PGE_2, can promote pro-inflammatory effects in hosts. In addition, a large amount of eicosanoids may be formed through the action of various other

enzymes, such as monooxygenases (CYP450s) and lipoxygenases, the actions of which regulate inflammation during infection and ultimately affect the ability of the host to clear infection (Dennis & Norris, 2015; Rodriguez *et al*., 2014). Lipoxygenases (LOX) are dioxygenases, catalysing the insertion of oxygen into fatty acids (Brodhun & Feussner, 2011; Kuhn & O'Donnell, 2006). The carbon number at which this insertion is accomplished, designates the type of LOX, for example, a 15-LOX acts upon carbon 15 of AA. The action of 15-LOX promotes the formation of 15-hydroxyeicosatetraenoic acid (15-HETE) from AA, which can act as substrate for lipoxin formation, with dramatic anti-inflammatory capabilities (Serhan, 2002).

Interestingly, *P. aeruginosa* possesses a secretable 15-LOX (LoxA), homologous to host 15-LOX, with the ability to convert AA to 15-HETE (Vance *et al*., 2004). The role of this microbially produced 15-LOX during infection has not been addressed and may alter the ability of the host to clear infection. In Chapter 2 and 3 the interaction of *P. aeruginosa* with *C. albicans* in terms of eicosanoid production was addressed, with the possible involvement of various enzyme groups in the production of PGE_2, $PGF_{2\alpha}$ and 15-HETE. The aim of this study is to possibly elucidate the role of *P. aeruginosa loxA* in the interaction of *P. aeruginosa* and *C. albicans* in terms of eicosanoid production.

4.3. Materials and methods

4.3.1. Strains used

Pseudomonas aeruginosa PAO1 (wild type) and *loxA*::Tn5 (*loxA* mutant) were used in this study. These strains was provided by Professor Hancock from the Department of Microbiology and Immunology at the University of British Columbia and revived according to American Type Culture Collection (ATCC) method from lyophilized strain ampoules, and maintained on nutrient agar (NA) (1 g/L malt extract, 2 g/L yeast extract, 5 g/L peptone, 8 g/L sodium chloride and 20 g/L agar) at 37 °C. The mutant strain (*loxA*::Tn5) was created by Tn5-luxCDABE mini transposon mutagenesis (Lewenza *et al*., 2005). In addition, *Candida albicans* strain CBS 8758 (SC5314) was used in the polymicrobial biofilms with *P. aeruginosa* and was maintained on yeast malt extract (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.

4.3.2. Confirmation of loxA *disruption construct in* Pseudomonas aeruginosa loxA*::Tn5*

Pseudomonas aeruginosa PAO1 and *loxA*::Tn5 were streaked out on NA and incubated for 24 h at 37 °C. After incubation, cells were inoculated into 5 mL of nutrient broth and incubated for 24 h @ 37 °C with shaking (150 rpm). Cells were collected through centrifugation and genomic DNA was extracted with a Zymo Research Fungal/Bacterial DNA MiniPrep™ (The Epigenetics Company, USA). Extraction of DNA was confirmed with gel-electrophoresis (0.8 % agarose, 90 V, 35 min).

The Tn5::*luxCDABE::Tet* mini transposon is characterized by the *luxCDABE* fluorescent gene on the O-end, as well as a tetracycline resistance (Tet) gene at the I-end (Lewenza *et al*., 2005). As this transposon can integrate in a forward or a reverse orientation, the orientation of the insert needed to be determined. For this, three sets of primers were designed (Table 1).

Table 1. Primer sets designed for amplification of *Pseudomonas aeruginosa* *loxA*::Tn5.

Primer set	Primer direction	Sequence	Length	GC-content (%)	Tm (°C)
1	Forward	TCAACCGTACCCACGAGCT	19	57	55
	Reverse	CATAAAGGCCGCCTTTAGAA	20	45	53
2	Forward	TCAACCGTACCCACGAGCT	19	57	55
	Reverse	TATCGTCGCCGCACTTATGA	20	50	56
3	Forward	TCAACCGTACCCACGAGCT	19	57	55
	Reverse	GATGGCATTCCATACCAGCA	20	50	55

The first set of primers (Primer set 1) was designed for amplification if the transposon's O-end was inserted at the promotor side of the *loxA* gene (IDT, USA). The forward primer is 277 bp upstream of the *loxA* gene, whilst the reverse primer is designed to bind to the *luxC* gene in the transposon insert (O-end). The second primer set (Primer set 2) was designed to accomplish amplification if the transposon was inserted in reverse orientation, where the I-end is at the side of the *loxA* promotor region. The forward primer is identical to described before, whereas the reverse primer will bind in

the tetracycline resistance gene at the I-end of the transposing element. The third set of primers (Primer set 3), is designed to amplify the *loxA* wild type gene. Once again, the forward primer is as described before, whereas the reverse primer is 365 bp into *loxA* (from the promotor side).

Primer set 1 and 2 were used for the *loxA* mutant and primer set 3 was used for the PAO1 wild type strain in a polymerase chain reaction (PCR) (2720 Thermal Cycler, Applied Biosystems, USA) following the parameters in Table 2.

Table 2. Polymerase chain reaction parameters for amplification of *Pseudomonas aeruginosa loxA*.

Temperature (°C)	Time	No. of cycles
95	2 min	1X
94	30 sec	25X
50	30 sec	
72	2 min	
72	5 min	1X

After amplification, amplicons were visualized with gel electrophoresis. Restriction digest was performed using *Sac*II (Fermentas) to further confirm successful amplification of the *loxA* wild type gene. A 10 µL reaction was carried out containing 5 µL DNA, 0.5 µL restriction enzyme (*Sac*II). The *loxA* gene contains 2 restriction sites for *Sac*II with 714 bp, 883 bp, 102bp or 102bp, 612 bp or 985 bp as possible fragments.

PCR products were prepared for Sanger sequencing through a PCR cleanup reaction. Briefly, 5 µL DNA together with 0.5 µL exonuclease and 1 µL alkaline phosphatase was incubated at 37 °C for 15 minutes, followed by 15 minutes at 85 °C to deactivate the enzymes. Amplicons for the wild type and mutant were sequenced using the BigDye terminator v. 3.1. kit according to manufacturer's specifications. Appropriate controls were included. Sequences were compared to a *loxA* reference sequence as well as available sequences for the Tn5::*luxCDABE::Tet* insert through available sequence data on the NCBI database.

4.3.3. Effect of disruption construct on Pseudomonas aeruginosa *planktonic growth*

Pseudomonas aeruginosa PAO1 and *loxA*::Tn5 were streaked out on NA and incubated for 24 h at 37 °C. After incubation, cells were inoculated into 5 mL of nutrient broth and incubated for 24 h @ 37 °C with shaking (150 rpm). These tubes served as the pre-inoculum. *Pseudomonas aeruginosa* cells were diluted to an optical density (OD_{600}) of 0.01 in 50 mL NB in 500 mL side arm flasks and incubated at 37 °C. The OD was read at 600 nm every hour until early stationary phase was reached (13 hours). This was done in triplicate for each *P. aeruginosa* strain. Values obtained were plotted as $\ln(OD_{600})$ and the maximum growth rate for each replicate was determined.

4.3.4. Formation of mono- and polymicrobial biofilms

4.3.4.1. Monomicrobial biofilm formation by Pseudomonas aeruginosa

Pseudomonas aeruginosa was grown on nutrient agar NA plates for 24 h at 37 °C and was inoculated into 5 mL Nutrient broth and incubated at 37 °C for 24 h with shaking (150 rpm). Cells were diluted to an optical density (OD_{600}) of approximately 0.05 in 20 mL filter sterilized (0.22 µm nitrocellulose filter, Merck Millipore, Ireland) RPMI-1640 medium (Sigma-Aldrich, USA) and dispensed into 90 mm polystyrene petri dishes. In addition to cells, 500 µM AA (Sigma-Aldrich, USA) (stock of 1 g in 25 mL of absolute ethanol reaching a concentration of 131.4 mM) was added to each petri dish containing medium plus cells. Petri dishes were incubated for 48 h at 37 °C to allow eicosanoid production. The final ethanol concentration in the biofilms before incubation was 0.38 %. Appropriate controls were included, consisting of a cell free control (RPMI-1640 medium without AA) as well as RPMI-1640 medium with AA.

4.3.4.2. Polymicrobial biofilm formation by Candida albicans and Pseudomonas aeruginosa

Candida albicans 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 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 then counted with a hemocytometer and

diluted to 1 x 10^6 cells/mL in 20 mL filter sterilized (0.22 µm nitrocellulose filter, Merck Millipore, Ireland) RPMI-1640 medium (Sigma-Aldrich, USA). In addition, *P. aeruginosa* cells (approximately 0.05 OD_{600}) was added to the medium and dispensed into 90 mm polystyrene petri dishes. Additionally, 500 µM of AA was added to each petri dish. Petri dishes were incubated for 48 h at 37 °C to allow biofilm formation. The final ethanol concentration in the biofilms before incubation was 0.38 %. Appropriate controls were included.

4.3.5. Comparison of biofilm growth between Pseudomonas aeruginosa *PAO1 and* loxA*::Tn5*

4.3.5.1. Influence on biofilm metabolic activity

Biofilms in the presence of AA and inhibitors were prepared in a 96-well plate (Corning Incorporated, Costar®, USA), as described previously, with the volume of medium adjusted to 100 µL. The plate was incubated for 48 h 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. The XTT assay was performed according to Kuhn *et al.* (2003). Briefly, 50 µL 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. Following incubation, the absorbance of each well was measured at 492 nm on a Labotec Spectramax M2 microplate reader (Molecular devices, USA) and data was obtained. Appropriate controls including medium containing only AA were included. This experiment was performed in triplicate.

4.3.5.2. Influence on biofilm biomass production

Biofilms were prepared as described before in petri dishes. After incubation, the individual biofilms were scraped off and washed twice with sterile PBS, where after the resuspended cells were filtered through pre-weighed filters (0.22 µm). The filters

were dried at 37 °C overnight and the dry biomass of the mono- and polymicrobial biofilms determined (Thibane *et al*., 2010). This experiment was performed in triplicate.

4.3.6. Effect of loxA *on eicosanoid production by mono- and polymicrobial biofilms*

Biofilms were prepared as described above and incubated for 48 h after addition of 500 µM AA. Supernatants were removed from biofilms and cells were removed with centrifugation (5750 *g* @ 4 °C for 5 minutes). After centrifugation the supernatant was filtered (0.2 µm nitrocellulose filter). Extraction of eicosanoids were performed according to a modified protocol proposed by Cayman chemicals for PGE_2 purification for enzyme linked immunosorbent assay (ELISA). Briefly, supernatants were acidified by to a pH of approximately 4 with the addition of 1 M formic acid (Merck, Germany). Solid phase extraction (SPE) classic C18 cartridges (Waters, Ireland) were prepared with 5 mL methanol (Merck, Germany), followed by 5 mL dH_2O. Samples (10 mL) were applied to cartridges and subsequently washed with 5 mL deionized water to remove impurities. Eicosanoids were then eluted from the SPE cartridges with 5 mL ethyl acetate containing 1 % methanol and collected in pre-washed poly top glass vials (Lasec, South Africa). The eluent was dried under a stream of N_2 and stored at -80 °C until use.

Samples were dissolved in eicosanoid affinity (EIA) buffer provided by the manufacturers and samples were assayed for PGE_2, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and 15-HETE using enzyme linked immunosorbent assay (ELISA) (Cayman Chemicals, USA) according to manufacturer's specifications. Samples were assayed in two dilutions in duplicate. This experiment was done in triplicate. Data was analysed according to manufacturer's specifications.

4.3.7. Statistical analysis

To evaluate significant differences between *P. aeruginosa loxA*::Tn5 and PAO1, one-way analysis of variance (ANOVA) was used ($P < 0.05$). In addition, this was followed by Tukey's multiple comparisons test with an alpha value of 0.05 to determine significant differences between *P. aeruginosa loxA*::Tn5 and PAO1. Significant differences are indicated with ' * '.

4.4. Results and discussion

4.4.1. Confirmation of loxA::Tn5 *disruption construct*

In order to assess the influence of the *P. aeruginosa* LoxA on eicosanoid production by monomicrobial and polymicrobial biofilms, the absence of LoxA is required. Therefore, a *P. aeruginosa* strain deficient in functional *loxA* (*loxA*::Tn5) was obtained. The Tn5::*luxCDABE*::*Tet* mini transposable element used to construct *loxA*::Tn5 is characterized by the *luxCDABE* fluorescent reporter set of genes on the O-end, as well as a tetracycline resistance (Tet) gene at the I-end (Lewenza *et al*., 2005). To confirm that the disruption construct is situated in the *loxA* gene, a primer specific to the region 500 bp upstream of *loxA* was designed. This primer (forward) would bind and initiate replication of both the wild type (PAO1) and *loxA*::Tn5 strain. As the disruption construct (Tn5::*luxCDABE*::*Tet*) could potentially integrate in the forward orientation (*luxC* at promotor end) or the reverse orientation (Tet-resistance gene at promotor end), two reverse primers, specific for *luxC* and tetracycline resistance gene was designed respectively. Figure 1 indicates gel-electrophoresis using the two primers sets discussed above, indicating successful amplification of the *loxA* gene (~1700 bp, lane 2). The amplicon in lane 3 (~1300 bp) confirmed that the Tn5-transposon inserted in a reverse orientation, as no amplification is observed for primers specific for *luxC* (lane 4).

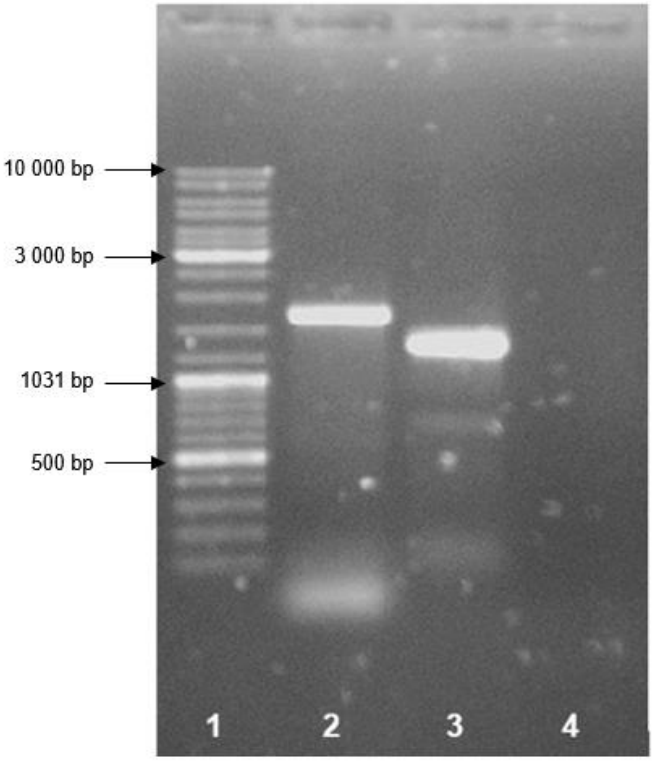

Figure 1. Image of gel electrophoresis (0.8 % agarose, 90 V) indicating orientation of the Tn5-mini transposon in reverse in *Pseudomonas aeruginosa loxA*. Lane 1 – DNA marker (ThermoFisher Scientific, O'GeneRuler DNA Ladder Mix); Lane 2 - amplification of wild type *loxA*. Lane 3 - amplification using reverse primer specific for tetracycline resistance gene in the Tn5::*luxCDABE*::*Tet* construct. Lane 4 - amplification using reverse primer specific for *luxC* in Tn5::*luxCDABE*::*Tet* construct.

The polymerase chain product obtained from the primer pair specific for the *loxA* gene was further digested with *Sac*II to confirm that *loxA* was indeed amplified with this method. Figure 2 indicates the gel-electrophoresis obtained after *Sac*II digestion, indicating correct amplification with fragment sizes of ~883 bp and 714 bp with *Sac*II digestion. However, the expected 102 bp band is not visible.

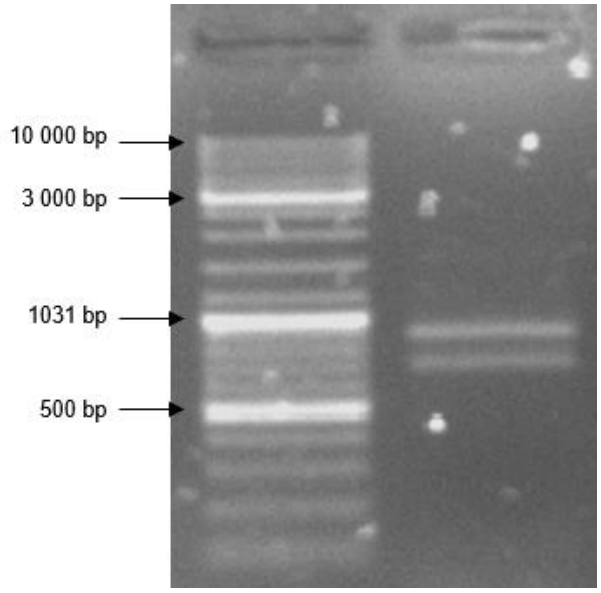

Figure 2. Image of gel electrophoresis (0.8 % agarose, 90 V) after the *loxA* PCR product was digested with SacII (right lane). The left lane represents a DNA fragment size ladder (ThermoFisher Scientific, O'GeneRuler DNA Ladder Mix).

The *loxA*::Tn5 PCR product was subjected to Sanger sequencing, and the sequence analysed with Geneious® ver.7 software (Biomatters Ltd., Auckland, New Zealand). Sequence data revealed that the Tn5::*luxCDABE*::*Tet* mini transposon as well as a large portion of the plasmid vector backbone integrated into the promotor region of the *loxA* gene (Figure 3).

4.4.2. Comparison of planktonic growth between Pseudomonas aeruginosa *PAO1 and* loxA*::Tn5*

To determine the effect of *P. aeruginosa loxA* on eicosanoid production and interaction in polymicrobial biofilms, the growth of the wild type (PAO1) was compared to the mutant (*loxA*::Tn5). Growth of both strains were evaluated (Figure 4) until early stationary phase and maximum growth rates were calculated. The growth of *P. aeruginosa loxA*::Tn5 did not differ significantly (*t*-test) from *P. aeruginosa* PAO1. Additionally, no significant difference in dry biomass of monomicrobial biofilms of *P. aeruginosa* strains, or polymicrobial biofilms of *P. aeruginosa* strains with *C. albicans* was observed (Figure 5a). This was also seen when comparing metabolic activity of both strains of *P. aeruginosa* in mono- and polymicrobial biofilms with *C. albicans* (Figure 5b). Therefore, the *loxA*::Tn5 transposon mutagenesis did not influence *P. aeruginosa* growth or metabolic activity.

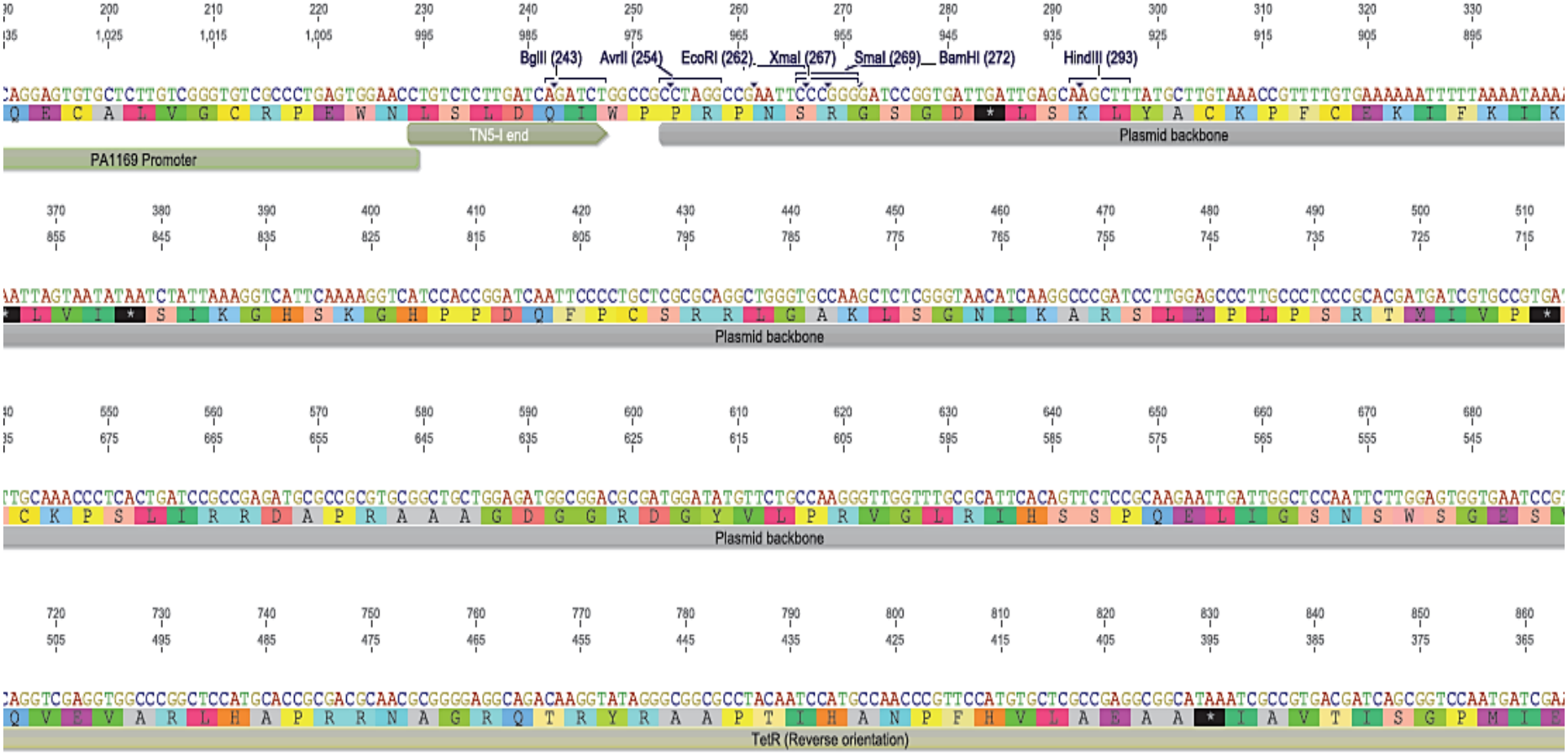

Figure 3. Representation of Tn5::*luxCDABE*::Tet mini transposon ligation in the promotor region of the *loxA* gene of *Pseudomonas aeruginosa.*

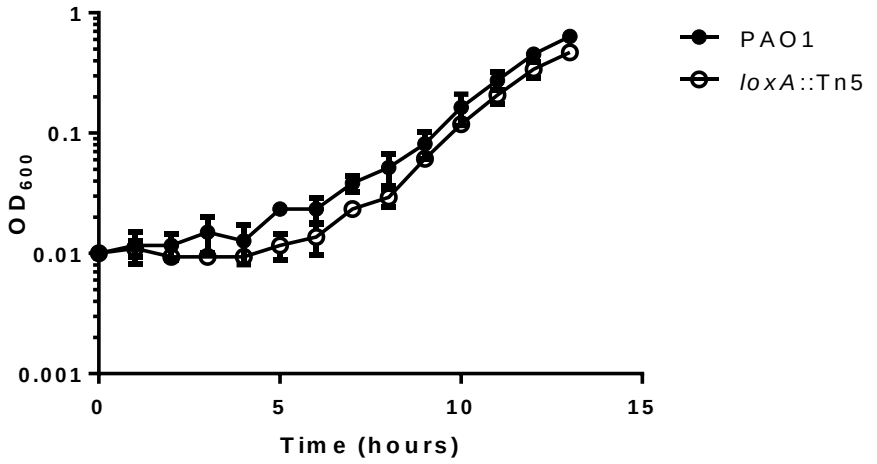

Figure 4. Comparison of growth of *Pseudomonas aeruginosa* wild type (PAO1) and *loxA* mutant (*loxA*::Tn5). The y-axis represents optical density (600 nm) over time (hours).

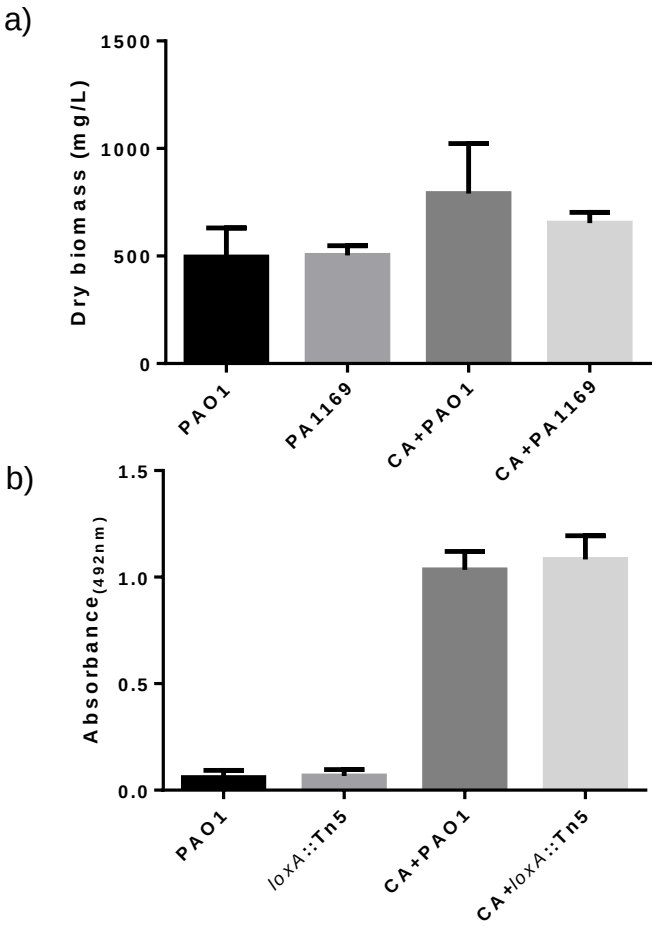

Figure 5. Comparison of a) dry biofilm biomass and b) metabolic activity of *Pseudomonas aeruginosa* wild type (PAO1) and *loxA* mutant (*loxA*::Tn5) mono- and polymicrobial biofilms with *Candida albicans* in the presence of 500 µM arachidonic acid.

* Significantly different from *P. aeruginosa* PAO1.

4.4.3. Effect of loxA *on eicosanoid production by* Pseudomonas aeruginosa *PAO1 and* loxA*::Tn5 mono- and polymicrobial biofilms with* Candida albicans

Figure 6 indicates the production of eicosanoids by monomicrobial and polymicrobial biofilms of *P. aeruginosa* PAO1 and *loxA*::Tn5 with *C. albicans*. Evaluation of eicosanoid production by monomicrobial and polymicrobial biofilms of *P. aeruginosa loxA*::Tn5 indicated that PGE_2 production by monomicrobial biofilms of *P. aeruginosa loxA*::Tn5 did not differ significantly from *P. aeruginosa* wild type (PAO1). In addition, the disruption of *loxA* did not significantly influence the PGE_2 production by polymicrobial biofilms of *P. aeruginosa* with *C. albicans*. Therefore, as expected, the secretable lipoxygenase produced by *P. aeruginosa* does not influence the production of PGE_2 by mono- and polymicrobial biofilms. Surprisingly, significant differences in $PGF_{2\alpha}$ production by *P. aeruginosa loxA*::Tn5 is however observed, with an increase in $PGF_{2\alpha}$ observed by the *P. aeruginosa loxA*::Tn5 compared to wild type monomicrobial biofilms. In addition, a reduction in $PGF_{2\alpha}$ production is observed in polymicrobial biofilms compared to the *P. aeruginosa* wild type counterpart. This indicates that *loxA* may possibly influence $PGF_{2\alpha}$ production by mono- and polymicrobial biofilms of *P. aeruginosa* and *C. albicans* although the mechanism remains unclear. Interestingly, no significant difference in 15-HETE production is observed by monomicrobial biofilms of *P. aeruginosa loxA*::Tn5 and PAO1 (wild type), possibly indicating that the *loxA* gene is not sufficiently disrupted to inhibit expression and that partial expression may still continue with the disrupted promotor region. Due to this, the evaluation of the effect of *P. aeruginosa loxA* on eicosanoid production by monomicrobial and polymicrobial could not conclusively be identified with the use of *P. aeruginosa loxA*::Tn5. However, previous research suggests that differential expression may be obtained by overexpression of *loxA*, which could be useful in this aspect of interaction (Lewenza *et al*., 2015). Interestingly, a significant decrease in 15-HETE production is observed by polymicrobial biofilms of *P. aeruginosa loxA*::Tn5 with *C. albicans* compared to PAO1. The reason for this is however unclear.

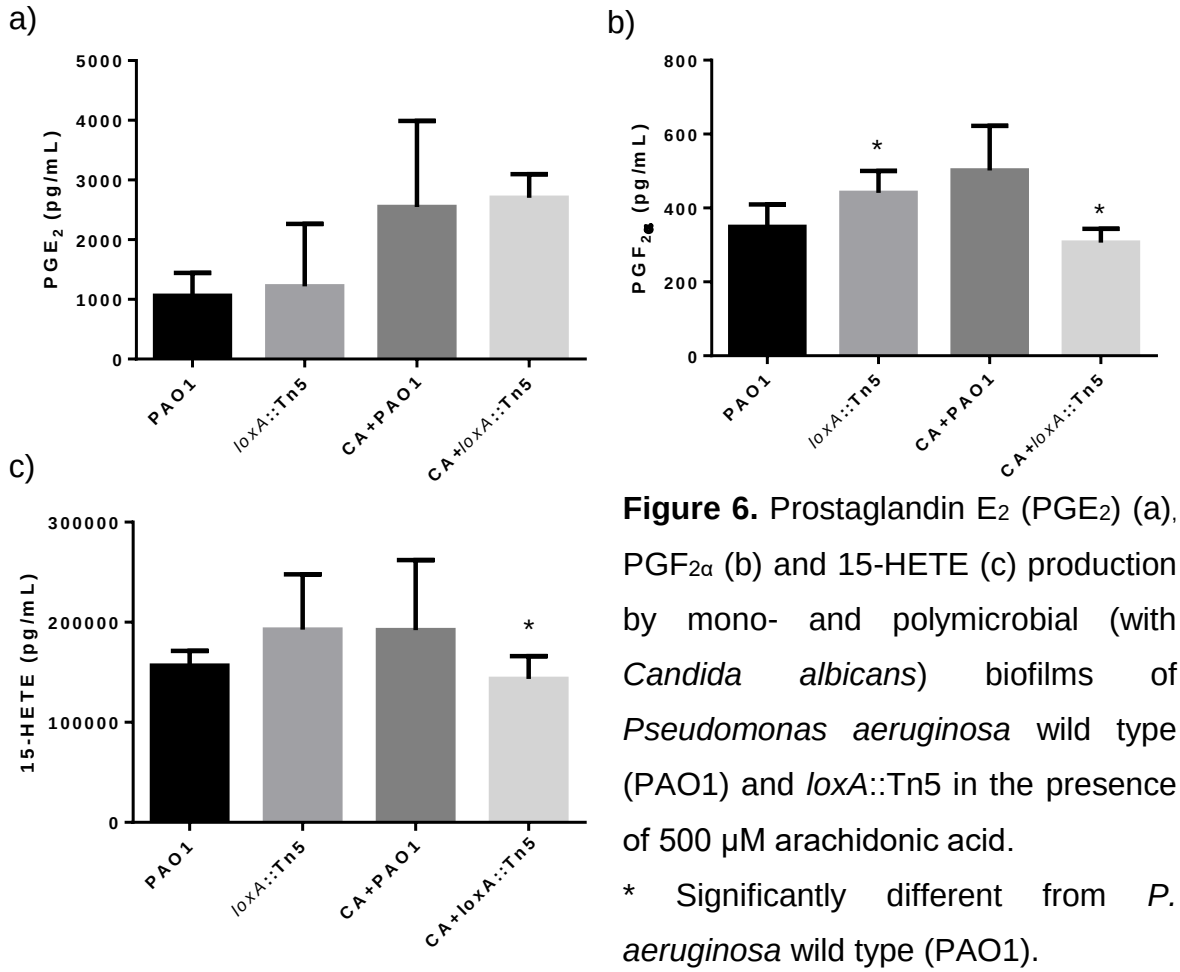

Figure 6. Prostaglandin E_2 (PGE_2) (a), $PGF_{2\alpha}$ (b) and 15-HETE (c) production by mono- and polymicrobial (with *Candida albicans*) biofilms of *Pseudomonas aeruginosa* wild type (PAO1) and *loxA*::Tn5 in the presence of 500 µM arachidonic acid.

* Significantly different from *P. aeruginosa* wild type (PAO1).

4.5. Conclusions

The secretable 15-lipoxygenase and the subsequent production of 15-HETE, may alter host-pathogen interaction, influencing the ability of the host to clear infection. In addition, *loxA* may also play a role in the interaction in polymicrobial biofilms, especially in terms of eicosanoid production. The disruption of *loxA* in *P. aeruginosa loxA*::Tn5 in the promotor region was confirmed. In addition, evaluation of *P. aeruginosa loxA*::Tn5 indicated no significant difference in growth compared to the *P. aeruginosa* wild type (PAO1). Although PGE_2 production by mono- and polymicrobial biofilms was not significantly affected, differential production of $PGF_{2\alpha}$ was observed for *P. aeruginosa loxA*::Tn5 compared to PAO1, indicating the possible involvement of *loxA* in $PGF_{2\alpha}$ production by mono- and polymicrobial biofilms of *P. aeruginosa*.

Unexpectedly, 15-HETE production by monomicrobial biofilms of *P. aeruginosa* did not differ significantly, indicating that *loxA* may not be sufficiently disrupted to alter expression. Interestingly, a significant decrease in 15-HETE production is observed when *P. aeruginosa* is co-incubated with *C. albicans*, however the reason for this is unclear. Further research into this aspect of *P. aeruginosa* virulence with overexpression of *loxA* may find value.

4.6. References

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General Discussion & Conclusions

5.1. Eicosanoid production by mono- and polymicrobial biofilms

It is known from *in vivo* studies that the oxygenated products of arachidonic acid (AA) play extensive roles in infection (Dennis & Norris, 2015). The ability of *C. albicans* and *P. aeruginosa* to produce these immunomodulatory compounds suggest that this can form part of their virulence arsenal. Previous research identified the production of prostaglandin E_2 (PGE_2) by *C. albicans* (Erb-Downward & Noverr, 2007; Noverr *et al*., 2001). In this study, this production of PGE_2 by monomicrobial biofilms was confirmed. In addition, *C. albicans* was shown to produce significant quantities of $PGF_{2\alpha}$ and 15-hydroxyeicosatetraenoic acid (15-HETE). Although *P. aeruginosa* was previously shown to produce prostaglandins and prostaglandin-like molecules, this study indicates the production of the specific prostaglandin species, PGE_2, $PGF_{2\alpha}$ by monomicrobial biofilms (Lamacka & Sajbidor, 1995). In addition, a large amount of 15-HETE is produced by *P. aeruginosa*, validating the presence of a secretable 15-lipoxygenase produced by the bacterium (Vance *et al*., 2004). The present research is also the first to provide evidence that polymicrobial biofilms produce significantly higher amounts of PGE_2, $PGF_{2\alpha}$ and 15-HETE. The increased production is of specific importance, as the inhibition of COX-2, responsible for the production of large amounts of PGE_2 in mammalian systems, was shown to increase the clearance of pathogens such as *P. aeruginosa* (Park *et al*., 2007; Sadikot *et al*., 2007). This increase in PGE_2 may then indicate the reduced ability of hosts to clear infective particles and may contribute to increased morbidity during polymicrobial infection (Morales & Hogan, 2010). The exact role of $PGF_{2\alpha}$ in infection is however unknown, and may also play a role in clearance of pathogens (Ricciotti & FitzGerald, 2011). In addition, this large amount of 15-HETE produced may also contribute to pathogen clearance, as 15-HETE may act as substrate for lipoxin formation in mammalian systems, with dramatic anti-inflammatory capabilities (Dennis & Norris, 2015; Serhan, 2002). Although eicosanoids elicit their effect at nanomolar concentrations, localized production of these microbially produced eicosanoids may be able to elicit an effect (Funk, 2001). Further research *in vivo* is however needed to fully understand the effect of microbial eicosanoid production.

5.2. Interference with eicosanoid identification and quantification

Due to the fact that the identification and quantification of PGE_2 by fungal species such as *C. albicans*, *C. dubliniensis*, *Cryptococcus neoformans* and *Saprolegnia parasitica* has relied on PGE_2 specific ELISA and LC-MS/MS (Belmonte *et al*., 2014; Chikhalya, 2013; Ells, 2011; Erb-Downward & Noverr, 2007; Noverr *et al*., 2001), this study also evaluated these techniques with the use of PGE_2 isomers. This study reports that PGE_2 isomers, such as 5-*trans*-PGE_2, or a combination of isomers could cross-react with PGE_2 specific ELISA and give an inaccurate representation of *in vitro* PGE_2 production. This is of importance as *in vitro* incubation techniques promote the formation of AA autoxidation products that could falsely be identified as enzymatic products, due to the structural similarity of these non-enzymatic produced eicosanoids (Jahn *et al*., 2008; Milne *et al*., 2015). In addition, an LC-MS/MS method utilized by Ells *et al*. (2011) was used to identify and quantify PGE_2, and proved to be insufficient in accurately determining authentic PGE_2 in supernatants, as PGE_2 was identified in control samples containing only AA and devoid of cells. Therefore, a more sensitive separation technique with LC-MS/MS was utilized, previously proposed by Brose *et al*. (2011). With this method, PGE_2 isomers could be successfully separated from authentic PGE_2, although these isomers, as well as PGE_2, could be detected in control supernatant samples. To possibly remove the large amount of autoxidation products, intracellular PGE_2 extraction was performed. With this method, PGE_2 could be identified in *C. albicans* and *P. aeruginosa* monomicrobial biofilms, as well as polymicrobial biofilms with the use of deuterated PGE_2, added to samples prior to extraction. The production of PGE_2 by *C. albicans* was shown to not be the result of AA autoxidation, as unbroken *C. albicans* samples did not contain PGE_2.

5.3. Effect of inhibitors on biofilm formation and eicosanoid production

To possibly determine which enzyme groups play a role in monomicrobial and polymicrobial eicosanoid production, inhibitors, which have previously been identified to inhibit PGE_2 production by *C. albicans*, were evaluated. The inhibitors tested did not significantly affect biofilm biomass or metabolic activity of monomicrobial and polymicrobial biofilms of *C. albicans*. However, an increase in metabolic activity of monomicrobial biofilms of *P. aeruginosa* was observed with 100 µM acetylsalicylic acid

(ASA) without significantly affecting biomass, although previous research at higher concentrations indicated repression of quorum sensing and phenazine production by *P. aeruginosa* (El-Mowafy *et al*., 2014). This effect was reflected in the morphology of polymicrobial biofilms, with an increase in *P. aeruginosa* cell numbers compared to control biofilms. In addition, we speculate the inhibition of *P. aeruginosa* quorum sensing molecule 3-oxo-homoserine lactone, which inhibits yeast to hyphal switch by *C. albicans*, as an increase in hyphae production was observed by *C. albicans* in the presence of ASA (McAlester *et al*., 2008). Additionally, less extracellular material (EXM) was observed, confirming previous observations with higher concentrations of ASA (Dergez *et al*., 2014). Although no significant effect on *C. albicans* metabolic activity and biomass were evident in this study, a reduction in *C. albicans* PGE_2 production was observed, confirming results previously obtained, indicating that PGE_2 production is partly dependant on ASA-sensitive enzyme(s) (Ells *et al*., 2011). Prostaglandin synthesis in mammals, such as PGE_2 and $PGF_{2\alpha}$ is dependent on an initial conversion by cyclooxygenase (COX) enzymes, sensitive to ASA (Murakami *et al*., 2003; Scneider & Brash, 2000; Tam, 2013; Vane & Botting, 2003). As such, ASA treatment in mammalian systems inhibits both PGE_2 and $PGF_{2\alpha}$. The production of prostaglandins by *C. albicans* does not follow this pathway, as $PGF_{2\alpha}$ production was increased in the presence of ASA. This indicates that PGE_2 and $PGF_{2\alpha}$ production by *C. albicans* may follow different pathways for synthesis. In addition, 15-HETE production was unaffected, indicating that the production thereof is not dependant on cyclooxygenase-like enzymes. In the case of *P. aeruginosa*, a significant increase in PGE_2, $PGF_{2\alpha}$ and 15-HETE was observed, possibly correlating with the increased metabolic activity. The production of eicosanoids by polymicrobial biofilms indicated a possible additive effect of monomicrobial eicosanoid production.

The copper chelating compound, ammonium tetrathiomolybdate (ATM), did not affect metabolic activity or biofilm biomass of monomicrobial or polymicrobial biofilms, although a decrease in hyphae production by *C. albicans* and an increase in EXM was observed in polymicrobial biofilm morphology. *Candida albicans* PGE_2 production was inhibited, confirming previous observations and possibly indicating the involvement of copper-containing enzymes in the production of PGE_2 by *C. albicans* (Ells *et al.*, 2011). Interestingly, *C. albicans* 15-HETE production was abolished by ATM, indicating the involvement of copper-containing enzymes in the production of 15-HETE by this

fungus. A large increase in $PGF_{2\alpha}$ production is observed by *C. albicans*, indicating a possible “shift” in eicosanoid production towards the formation of $PGF_{2\alpha}$. Previous research indicates that ATM could increase the amount of reactive oxygen species (ROS) of *C. albicans* intracellularly by inhibiting superoxide dismutase (De Brucker *et al*., 2013). This may lead to the production of F_2-series isoprostanes generated by these radicals, which may possibly cross-react with the $PGF_{2\alpha}$-specific ELISA utilized in this study and generate the high values obtained (Jahn *et al*., 2008; Milne *et al*., 2015). *Pseudomonas aeruginosa* PGE_2 production was also significantly inhibited by ATM, indicating the involvement of copper-containing enzymes in its production, similarly to *C. albicans*. In contrast to *C. albicans*, 15-HETE production is not significantly affected by ATM in *P. aeruginosa*. This may validate that a lipoxygenase plays a role in the production of 15-HETE by *P. aeruginosa* (Vance *et al*., 2004). Similarly to *C. albicans* however, is the significant increase in $PGF_{2\alpha}$ production by *P. aeruginosa*, again possibly indicating a shift in eicosanoid production from PGE_2 towards $PGF_{2\alpha}$. Polymicrobial PGE_2 production is also inhibited by ATM as with monomicrobial counterparts. Although an additive effect of eicosanoid production by monomicrobial biofilms to form polymicrobial biofilms is speculated in the case of ASA, this cannot be said for ATM. This is due to the fact that a significant increase in 15-HETE production is observed, and $PGF_{2\alpha}$ production is not significantly affected. This is in contrast to monomicrobial counterparts, demonstrating that polymicrobial eicosanoid production may generate drastically different eicosanoid profiles compared to monomicrobial counterparts, and signifies the complexity of interaction during co-incubation, even in terms of eicosanoid production.

In the case of the antioxidant, nordihydroguaiaretic acid (NDGA), a dramatic effect on *C. albicans* morphology was observed, with cells possibly unable to separate, and only yeast morphology observed (Lü *et al*., 2010). However, metabolic activity and biomass of *C. albicans* monomicrobial and polymicrobial biofilms were unaffected. Although polymicrobial biofilm metabolic activity and biomass were unaffected, *P. aeruginosa* monomicrobial metabolic activity was significantly decreased with no significant effect on biofilm biomass. In addition, little *P. aeruginosa* cells were visible in polymicrobial biofilms, with a large amount of EXM. This could indicate that the biofilm biomass observed for *P. aeruginosa* may mostly reflect EXM, rather than metabolically active cells. Eicosanoid production by both monomicrobial and polymicrobial biofilms was

significantly inhibited. This may be due to the strong antioxidant activity of NDGA inhibiting multiple enzymatic groups (Lü *et al*., 2010). The eicosanoid production, together with the effect on *C. albicans* morphology in polymicrobial biofilms and effect on *P. aeruginosa* metabolic activity, highlights the therapeutic potential of antioxidants in treatment of infection. Further research is important to further investigate these phenomena caused by NDGA.

5.4. Role of *Pseudomonas aeruginosa* 15-lipoxygenase

Through the use of a *P. aeruginosa* strain deficient in the secretable 15-lipoxygenase (*loxA*::Tn5) normally present in the wild type (PAO1), the identification of the role of the *P. aeruginosa* secretable lipoxygenase in monomicrobial and polymicrobial biofilm eicosanoid production was attempted (Lewenza *et al*., 2005). However, large amounts of 15-HETE is still produced by *P. aeruginosa loxA*::Tn5, although the presence of a disruption construct in the *loxA* promotor region was confirmed. This may indicate that the disruption of the promotor region may be insufficient in eliciting differential expression. Therefore, further research is needed to identify the possible role of 15-lipoxygenase in polymicrobial interaction, as the method used in the present study was insufficient.

Although further research is needed, especially taking the influence of the host environment into consideration, the present *in vitro* study highlights the possible increased virulence of interkingdom interaction between *C. albicans* and *P. aeruginosa* by the significantly higher production of immunomodulatory eicosanoids. In addition, the eicosanoid production observed is sensitive to the inhibitors tested, indicating the involvement of several enzymatic groups in the production of these eicosanoids. Further research into this may lead to possible therapeutic intervention during both monomicrobial and polymicrobial infection.

5.5. References

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SUMMARY

Interkingdom interactions between microorganisms facilitate the growth and survival of microbial communities, and the understanding of these interactions can be beneficial to mankind. These interactions may also be detrimental to human health, with the combination of virulence factors promoting the survival of microbial populations during infection. A relevant model for this, is the interaction between *Candida albicans* and *Pseudomonas aeruginosa*. These two microorganisms are frequently found together at infection sites. During infection, *C. albicans* and *P. aeruginosa* elicit the release of arachidonic acid (AA), utilized by the host to form immunomodulatory compounds termed eicosanoids. Among these is prostaglandins, for example, prostaglandin E_2 (PGE_2). Prostaglandin E_2 can inhibit T_h1 and promote T_h2 responses. In combination to other eicosanoids, they modulate inflammation in hosts, ultimately affecting the ability of the host to clear infection. *Candida albicans* and *P. aeruginosa* are also able to produce immunomodulatory eicosanoids from exogenous AA. This study confirms the production of PGE_2 by *C. albicans* monomicrobial biofilms, together with the production of $PGF_{2\alpha}$ and 15-hydroxyeicosatetraenoic acid (15-HETE) with the use of enzyme-linked immunosorbent assay (ELISA) and LC-MS/MS for PGE_2 confirmation. The production of these eicosanoids is also reported here by *P. aeruginosa* monomicrobial biofilms. This study is the first to identify authentic PGE_2 production by *P. aeruginosa* biofilms. In addition, polymicrobial biofilms were shown to produce significantly more eicosanoids that monomicrobial counterparts, possibly contributing to the increased morbidity during co-infection by these pathogens.

Although the pathways and enzymes involved in eicosanoid production by mammalian systems have been well studied, the production of eicosanoids by microorganisms requires much research. This is due to the fact that microorganisms frequently don't possess homologs to mammalian enzymes responsible for eicosanoid production. Therefore, inhibitors previously identified to inhibit *C. albicans* PGE_2 production, were evaluated in terms of their effect on eicosanoid production by monomicrobial and polymicrobial biofilms of *C. albicans* and *P. aeruginosa*. The inhibitors used are acetylsalicylic acid (ASA, a cyclooxygenase inhibitor), ammonium tetrathiomolybdate (ATM, inhibition of copper-dependant enzymes) and nordihydroguaiaretic acid

(NDGA, a potent antioxidant inhibiting various enzyme classes). A possible “shift” in eicosanoid production by *C. albicans* is seen in the presence of ASA as well as ATM. This phenomenon is also seen for *P. aeruginosa* in the presence of ATM. Interestingly, ASA increased eicosanoid production by *P. aeruginosa*. The anti-oxidant NDGA decreased eicosanoid production by monomicrobial, as well as polymicrobial biofilms. Different profiles for eicosanoid production obtained between monomicrobial and polymicrobial biofilms in the presence of ASA and ATM were observed suggest the complex interaction of *C. albicans* and *P. aeruginosa* in terms of eicosanoid production. In addition, the inhibitors caused dramatic alterations in polymicrobial biofilm morphology. Interestingly, although these inhibitors did not affect *C. albicans* metabolic activity or biofilm biomass, ASA caused a significant increase in *P. aeruginosa* metabolic activity. In addition, *P. aeruginosa* metabolic activity was significantly inhibited by NDGA. The possible clinical relevance of these findings warrant further investigation, as the use of inhibitors, such as the ones used in the present study, could possibly affect virulence and the ability of hosts to clear infection. This study also evaluated the role of a secretable 15-lipoxygenase produced by *P. aeruginosa* capable of converting AA to 15-HETE, although further research and methodology is needed to elucidate its role.

This study is the first to investigate the production of eicosanoids by polymicrobial biofilms of *C. albicans* and *P. aeruginosa*. The increased production of these eicosanoids compared to monomicrobial counterparts, suggest that the microbially produced eicosanoids may possibly play a role in pathogen-pathogen interaction, as well as host-pathogen interaction. This may ultimately affect the ability of the host to clear infection. In addition, with the use of inhibitors, the possible involvement of various enzymatic groups can be speculated during polymicrobial eicosanoid production. Further research into this interaction may provide valuable insight into polymicrobial eicosanoid production and may contribute to possible therapeutic intervention strategies during monomicrobial and polymicrobial infection.

KEYWORDS: Arachidonic acid, biofilms, *Candida albicans*, eicosanoids, *in vitro*, inhibitors, interaction, polymicrobial, *Pseudomonas aeruginosa*

OPSOMMING

Interkoninkryk-interaksies tussen mikro-organismes fasiliteer die groei en oorlewing van mikrobiese populasies en begrip van hierdie interaksies kan vir die mensdom voordelig wees. Hierdie interaksies kan ook nadelig wees vir menslike gesondheid, waar die kombinasie van virulensiefaktore die oorlewing van mikrobiese populasies tydens infeksie bevorder. ‘n Relevante model vir hierdie is die interaksie tussen *Candida albicans* en *Pseudomonas aeruginosa*. Hierdie twee mikro-organismes word gereeld saam gevind tydens infeksie. Gedurende infeksie veroorsaak *C. albicans* en *P. aeruginosa* die vrystelling van arachidoonsuur (AA), wat gebruik word deur die gasheer vir die produksie van immuunmodulerende verbindings, waarna verwys word as eikosanoïede. Prostaglandiene, insluitend prostaglandien E_2 (PGE_2) vorm deel van hierdie groep. Prostaglandien E_2 kan die T_h1-reaksie inhibeer en die T_h2-reaksie bevorder. In kombinasie met ander eikosanoïede, kan hierdie verbindings die inflammasie van die gasheer moduleer, wat uiteindelik die vermoë van die gasheer om infeksie te beveg affekteer. *Candida albicans* en *P. aeruginosa* kan ook hierdie immuunmodulerende eikosanoïede vanaf eksterne AA produseer. Die huidige studie bevestig die produksie van PGE_2 deur *C. albicans* mono-mikrobiese biofilms, sowel as die produksie van $PGF_{2\alpha}$ en 15-hidroksie-eikosatetraenoësuur (15-HETE) deur die gebruik van ‘n ensiem-gebonde immuunadsorberende toets (ELISA) en LC-MS/MS vir die bevestiging van PGE_2. Die produksie van hierdie eikosanoïede deur *P. aeruginosa* mono-mikrobiese biofilms word ook hier berig. Die huidige studie is die eerste om PGE_2-produksie deur *P. aeruginosa* biofilms the rapporteer. Bykomend word aangetoon dat poli-mikrobiese biofilms aansienlik meer eikosanoïede produseer in vergelyking met die mono-mikrobiese biofilms. Hierdie verhoogde produksie kan moontlik aanleiding gee tot die verhoogde morbiditeit gedurende ko-infeksie deur hierdie patogene.

Alhoewel die weë en ensieme wat ‘n rol speel in die produksie van eikosanoïede deur soogdiere goed bestudeer is, kan dieselfde nie gesê word vir die produksie van eikosanoïede deur mikro-organismes nie. Dit is as gevolg van die feit dat mikro-organsismes selde homoloë van soogdier-ensieme vir die produksie van eikosanoïede besit. As gevolg hiervan, was inhibitore, wat voorheen bewys is om PGE_2-produksie deur *C. albicans* te inhibeer, geëvalueer ten opsigte van hul effek op

eikosanoïed-produksie deur mono- en poli-mikrobiese biofilms. Die inhibitore wat gebruik is, sluit asetielsalisiensuur (ASA, ‘n sikloöksigenase inhibitor), ammonium-tetratiomolibdaat (ATM, ‘n inhibitor van koper-afhanklike ensieme) en nordihidroguaiariensuur (‘n antioksidant wat ‘n verskeidenheid ensiemklasse inhibeer) in. ‘n Moontlike verskuiwing van ekosanoïed-produksie deur *C. albicans* in die teenwoordigheid van ASA en ATM is waargeneem. Hierdie verskuiwing is ook gesien in die geval van *P. aeruginosa* biofilms in die teenwoordigheid van ATM. ‘n Toename in eikosanoïed-produksie deur *P. aeruginosa* in die teenwoordigheid van ASA is waargeneem. Die anti-oksidant NDGA het eikosanoïed-produksie deur mono- en poli-mikrobiese biofilms inhibeer. Verskillende profiele vir eikosanoïed-produksie deur mono- en poli-mikrobiese biofilms wat met ASA en NDGA behandel is, is verkry, wat ‘n komplekse interaksie tydens ko-inkubasie aandui. Die inhibitore het ook dramatiese verskille in poli-mikrobiese biofilm morfologie veroorsaak. Alhoewel die metaboliese aktiwiteit en biofilm biomassa vand *C. albicans* nie beïnvloed deur die inhibitore was nie, was daar ‘n verhoging in die metabolise aktiwiteit van *P. aeruginosa* biofilms in die teenwoordigheid van ASA. Die metabolise aktiwiteit van hierdie bakteriese biofilms was ook aansienlik verlaag deur NDGA. Die kliniese effek van hierdie bevindings sal verder evalueer moet word, aangesien die inhibitore wat gebruik is in hierdie studie moontlik die virulensie van die patogene, sowel as die vermoë van die gasheer om ontslae te raak van infeksie kan beïnvloed. Hierdie studie het ook die rol van die 15-lipoksigenase van *P. aeruginosa* geëvalueer, maar verdere navorsing is nodig om hierdie faset van interaksie te verstaan.

Die huidige studie is die eerste van sy tipe om die produksie van eikosanoïede deur poli-mikrobiese biofilms van *C. albicans* en *P. aeruginosa* te evalueer. Die verhoogde produksie hiervan deur poli-mikrobiese biofilms dui moontlik aan dat mikrobiese eikosanoïede ‘n rol kan speel gedurende patogeen-patogeen interaksies, sowel as patogeen-gasheer interaksies. Dit kan moontlik die vermoë van die gasheer om patogene te verwyder beïnvloed. Die studie het ook resultate gegee wat lei tot die moontlike identifikasie van ensieme wat ‘n rol kan speel in die produksie van eikosanoïede deur poli-mikrobiese biofilms. Verdere navorsing kan belangrike inligting wat voordelig kan wees in kliniese behandeling tydens infeksie van *C. albicans* en *P. aeruginosa* bied.