The role of cryptococcal 3-hydroxy fatty acids in mediating interspecies interactions

Bonang Michael Mochochoko

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Bloemfontein

South Africa

Supervisor:

Dr. O.M. Sebolai

Co-supervisor:

Prof. C.H. Pohl

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DECLARATION

I hereby declare the work presented in the dissertation is as a result of my own independent investigations. In addition, I declare this dissertation has not been submitted, in full or part, to another institution for the granting of a M.Sc. degree. The successful completion of the dissertation has been made possible by a joint research grant from the National Research Foundation of South Africa and the University of the Free State, South Africa.

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Bonang Michael Mochochoko

Candidate: M.Sc. degree

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ETHICAL CONSIDERATION

University of the Free State's Ethics Committee has granted clearance to conduct all the studies presented in this dissertation. The designated ethics application reference number is Application No. ECUFS NR 05/2015.

TABLE OF CONTENTS

Note: The dissertation is written according to the reference style prescribed by the journal "Frontiers in Microbiology".

DISSERTATION OVERVIEW

CHAPTER 1:

LITERATURE REVIEW

CHAPTER 2:

MICROBE-TO-MICROBE INTERACTIONS

CHAPTER 3:

MICROBE-TO-HOST INTERACTIONS

DISSERTATION SUMMARY

VERHANDELING OPSOMMING

DISSERTATION OVERVIEW

Paul Kelsey previously defined life as “a process consisting of the orderly rearrangement of matter with the expenditure of energy”. Towards this end, these processes may include energy-dependent self-sustaining exercises such as replication. To date, it is not clear how life arose on Earth. According to Wikipedia the earliest physical evidence of microbial life is that of a microbial mat fossil (dated to be around 3.5 billion years old) that was discovered in Australia. However, it is clear that since the emergence of microbial life, microorganisms have had to evolve (driven either by mutations, genetic exchanges, even epigenetics) leading some to manifest unique physiological qualities that are absent in other microorganisms. Importantly, these qualities have allowed microbes to survive and flourish in different environments such as the open physical boundaries of the soil or confined space of the human body, for example.

In these different environments, microbes exist in communities, which are at the same time further defined by the presence of other microbial populations. Here, these microbes compete (directly or indirectly) for available resources within the space they share. Furthermore during an infection, the human body has to react to the collective behaviour of invading microbial cells. Thus, to outsmart competing cells, of either microbial- or human-origin, cells expend energy to produce secondary metabolites that can promote their own survival. It is therefore not surprising that the role of secondary metabolites has been extensively studied, more so in the context of understanding how they promote the survival of one microbial population to the detriment of the other. Towards this end, this dissertation is an attempt to advance our current knowledge concerning 3-hydroxy fatty acids, which were previously reported to be secreted to the extracellular environment of *Cryptococcus neoformans* UOFS Y-1378 cells during growth.

The dissertation is not structured in a classical way; and as such, it is composed of a

dissertation overview section, literature review section (Chapter 1) and two Research Chapters (Chapters 2 and 3), which are in publication format. A dissertation summary section is also included at the end of this document, which summarises all the work that is presented herein. A brief description of each Research Chapter is given below:

Chapter 2 focuses on the role of cryptococcal 3-hydroxy fatty acids in mediating the fate of cryptococcal cells during microbe-to-microbe interactions. The designed *in vitro* interaction takes place between *Cryptococcus* and *Pseudomonas* cells.

Chapter 3 evaluates the role of cryptococcal 3-hydroxy fatty acids in mediating the fate of cryptococcal cells during microbe-to-host interactions. The designed *in vitro* interaction occurs between *Cryptococcus* cells and murine-based macrophages.

CHAPTER 1:

LITERATURE REVIEW

1.1 MOTIVATION

It was previously reported that *Cryptococcus* (*C.*) *neoformans* UOFS Y-1378 cells produce 3-hydroxy fatty acids; and in particular 3-hydroxy C9:0, after extracting lipids from cells and analysing the extracts using a mass spectrometer (Sebolai *et al.*, 2007; Madu *et al.*, 2015). In order to determine the intracellular location of these molecules, cells (at different stages of the growth cycle) were sliced open during a transmission electron microscopy (TEM) study to expose the location of osmiophilic material, which represent lipids – including 3-hydroxy C9:0 (Sebolai *et al.*, 2008). It was noted that cells at an early stage of the growth cycle accumulated osmiophilic material around mitochondrial membranes, while cells at a late stage of the growth cycle, accumulated osmiophilic material at the site of the capsules (their mitochondria were devoid of osmiophilic material). This observation pointed towards the possible migration of osmiophilic material from the mitochondria, which is suggested to be the production site of 3-hydroxy fatty acids, towards the capsule.

Next, in order to definitely determine if 3-hydroxy fatty acids were present in the osmiophilic material, a polyclonal antibody, specific for 3(*R*)-hydroxy fatty acids, was reacted with cells during a TEM immuno-gold labelling assay. Here, it was shown that 3-hydroxy fatty acids were indeed contained within the osmiophilic material found on capsules of cryptococcal cells. More to the point, 3-hydroxy fatty acids were also shown to be present inside the characteristic spiky protuberances of *C. neoformans* UOFS Y-1378 capsules. Taken together, these findings suggested that these molecules were secreted to the extracellular environment of cells by being pushed or injected through the spiky protuberances, which were approximately 200 nm in diameter (Sebolai *et al.*, 2008). However, upon being released, the biological

function(s) of these molecules remains unknown. Is it possible that these molecules are synthesised and then secreted into the extracellular environment to promote the survival and/or pathogenesis of cryptococcal cells? To answer this question, it is first important to understand why interspecies interactions may lead to production of secondary metabolites such as cryptococcal 3-hydroxy fatty acids.

1.2 INTERSPECIES INTERACTIONS

Interspecies interactions are defined as those interactions that occur between members belonging to different species and are typically confined to a particular physical environment (Figure 1) (Comolli, 2014). In these interactions, the concerned species may have either direct contact or indirect contact i.e. through intermediaries such as materials present in the environment. For example, in a mutual symbiotic relationship, two microbial species (in indirect contact) can cooperate to break down complex molecules, which on their own the concerned individual species cannot (Hunter, 2006). On the other hand, interactions such as predation represent direct contact between the interacting species. In addition, in predation one species directly attacks another species and eventually derives energy from the biomass of the attacked species (Odum, 1959). Thus microbes that are often at the receiving end of antagonistic attacks have over time developed defence mechanisms, including production of secondary metabolites, in order to counter adverse effects exerted on them by aggressive microbes. At the same time, aggressive microbes can also deploy secondary metabolites in order to appropriate territorial dominance over susceptible microbes. Thus, these metabolites play a crucial secondary function that determines the fate of cells during interactions.

In the next section, special attention is given to antagonistic relations that involve *C. neoformans*, wherein its cells may assume the role of either a victim (such as in predation) or that of an aggressor (like in parasitism and competition).

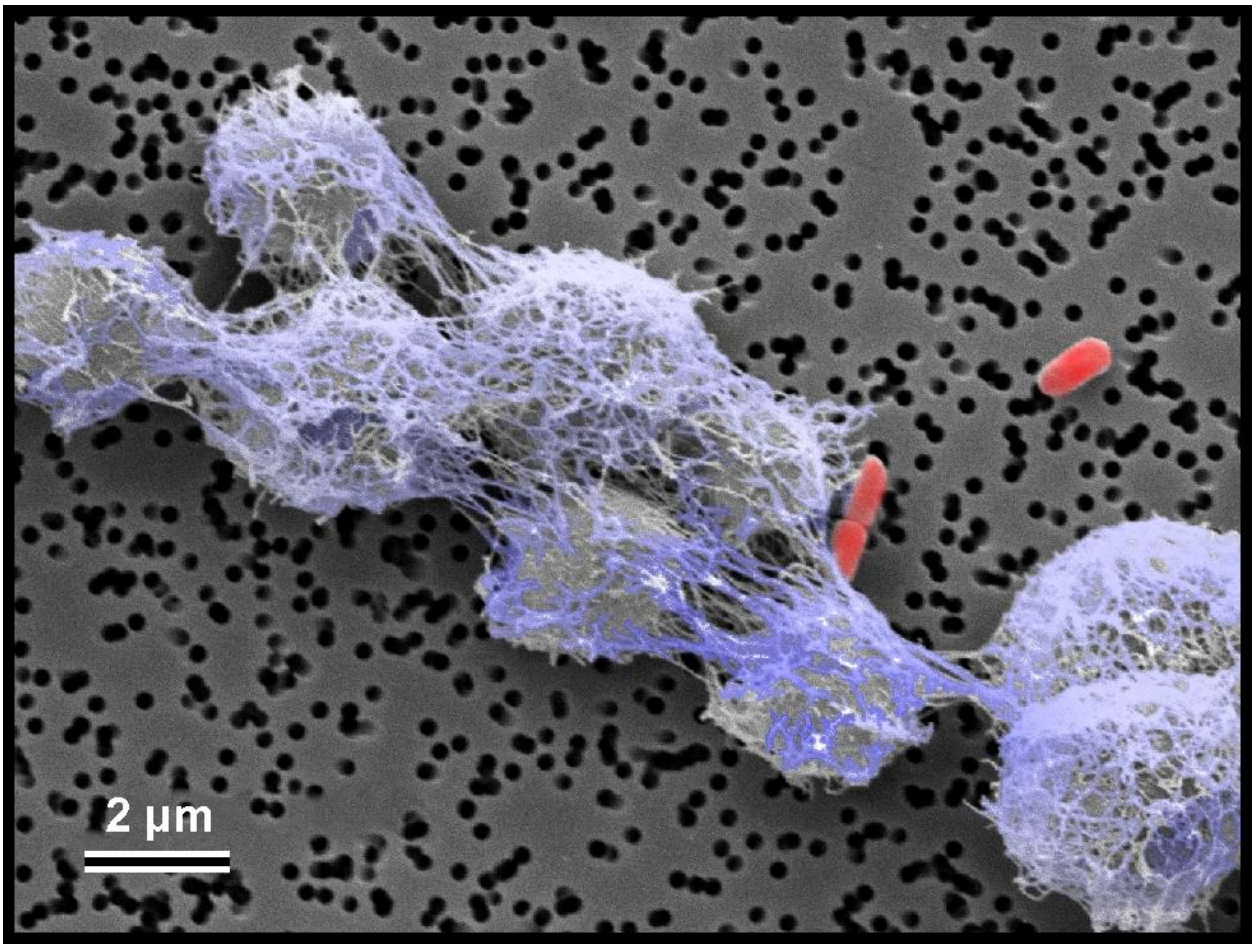


Figure 1. A scanning electron microscopy pictogram illustrating two different microbial species interacting i.e. *C. neoformans* UOFS Y-1378 cells depicted in blue and *Pseudomonas aeruginosa* PA 01 cells depicted in orange. Pictogram: Credit to Dr O.M. Sebolai.

1.3 *CRYPTOCOCCUS NEOFORMANS*

1.3.1 Description of *C. neoformans*

The basidiomycetous yeast *C. neoformans* was first identified in 1894 after Sanfelice isolated this yeast from fruit juice (Casavedall and Perfect, 1998). In the same year, two German physicians, Busse and Buske, gave the first description of *C. neoformans* as a human pathogen, after isolating the organism from a tibia lesion (Casadevall and Perfect, 1998). *Cryptococcus neoformans* isolates have traditionally been described as varieties i.e. *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii*, and not as separate individual species (Chayakulkeeree and Perfect, 2008; Kwon-Chung, 2011). These varieties have also been serotyped, based on their unique capsular antigens, as either serotype A (represents *C. neoformans* var. *grubii*) or serotype D (represents *C. neoformans* var. *neoformans*) (Chen *et al.*, 2010). Recently a hybrid strain, AD (*C. neoformans* var. *grubii-neoformans*; that is probably a diploid or aneuploid organism), has also been identified (Table 1) (Enache-Angoulvant *et al.*, 2007). Formerly, *C. neoformans* was thought to constitute a species complex together with its closely related *C. gattii* relative. However, *C. gattii* is now recognised as a separate individual species, distinct from *C. neoformans* based on "biochemistry, ecology, genetics and phylogenetic diversity, including differences underscored by how the two species interact with immune cells" as previously pointed out by Kwon-Chung and Varma (2006). Towards this end, our discussion will therefore be limited to *C. neoformans*.

Cryptococcus neoformans var. *neoformans* (serotype D) in particular, is mainly limited to central Europe. While on the other hand, *C. neoformans* var. *grubii* (serotype A) has a universal distribution (Viviani *et al.*, 2006). Importantly, it is worthwhile to also note that *C. neoformans*

var. *grubii* is the most prevalent serotype that is frequently isolated from patients, especially those with an impaired cell-mediated immunity mainly due to HIV infection (Day, 2004). This serotype accounts for approximately 95% of all *C. neoformans* infections (Hull and Heitman, 2002).

In general, when cultivated on 2% malt agar, *C. neoformans* colonies appear to be white to cream in colour. Yeast cells are globose to ovoid in shape, and are between 2.5 μm to 10 μm in diameter (Kwon-Chung, 2011). Heterothallism is usually observed when compatible mating types are cultivated together in a nitrogen-poor mycological agar media. Towards this end, V8 juice can successfully be employed to induce formation of basidial structures (Kwon-Chung *et al.,* 1982). *Cryptococcus neoformans* is not known to ferment any sugars, thus this species is greatly dependent on actively respiring mitochondria to produce cellular energy (Kwon-Chung, 2011).

Table 1.

Classification of *Cryptococcus neoformans* (*C. neoformans*) (Day, 2004; Chayakulkeeree and Perfect, 2008).

Species detail			**Ecology**	
Name	**Variety**	**Serotype**	**Geography**	**Environmental source**
C. neoformans	*grubii*	A	Worldwide	Pigeon excreta; Soil
	neoformans	D	Mainly Europe	Pigeon excreta; Soil
	grubii-neoformans	A-D	Unknown	Unknown

1.3.2 *Cryptococcus neoformans* interactions

Although the first environmental isolation of *C. neoformans* was made from fruit juice, this fungus has consistently been isolated from the soil, which is its suggested natural habitat, and primarily soil that is contaminated with bird droppings (Steenburgen and Casadevall, 2003). In this environment, *C. neoformans* cells interact with other organisms, often in a struggle to establish territorial dominance due to limited space to grow, as well as materials to extract energy from. In a case of a predatory relationship, cryptococcal cells can fall prey to foraging amoebae like *Acanthamoeba castellanii* via direct attack (Figure 2) (Steenburgen and Casadevall, 2003). Amoebae are said to have evolved efficient strategies to recognise, internalise and kill internalised cryptococcal cells (Bottone *et al.,* 1994). Subsequently, amoebae can then extract energy from killed cells to support growth. The fitness of a microbe to withstand an assault is determined by the quality of its defensive armour. Therefore, the selective pressure that is exerted upon cryptococcal cells has led cells to the evolution and production of a protective structure called the capsule. The capsule is a polysaccharide layer that surrounds the cell wall (Zaragoza *et al.,* 2009), thus forming a barrier to the extracellular space and assists the cell to perceive its environment (Pommerville, 2010). Towards this end, the capsule is reported to have the capability to alter the phagocytic machinery of macrophages (Kozel and Gotschlich, 1982; Vecchiarelli, 2000) - thus assisting cells to maintain self-preservation. Moreover, it has been reported that the capsule can enlarge up to 50 μm in size when interacting with phagocytic cells (Figure 3) (Casadevall and Perfect, 1998). As a result of the above, it is logical to reason that the enlarged capsule can enable cells to evade amoebal predation. Essential, the capsule is composed by two large polysaccharide molecules viz. glucuronoxylomannan and galactoxylomannan, and to a smaller extend, mannoproteins (Zaragoza *et al.,* 2009).

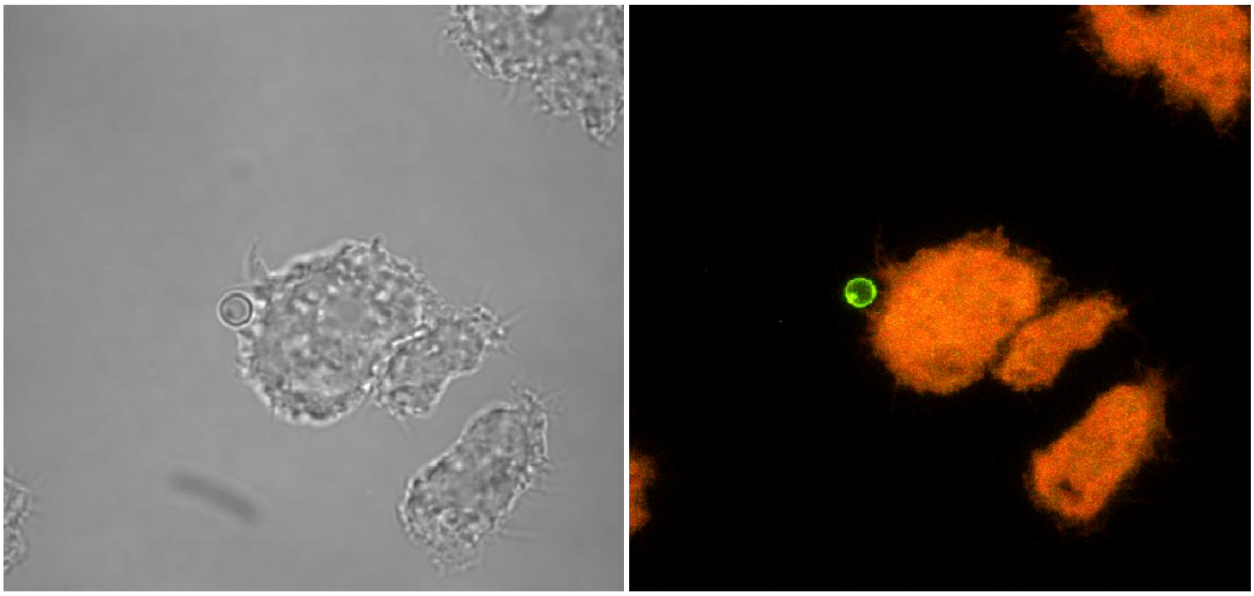


Figure 2. An interactive moment between a cryptococcal cell and amoeba cells captured under normal light (left) and fluorescent light (right). The photomicrograph depicts a cryptococcal cell (green) about to be phagocytised by amoebal cell (orange). 1000x magnification. Part of the image was published in Madu *et al.*, 2015 and has been used here with permission.

Another dynamic that involves cryptococcal cells is parasitism, wherein cells can take up residency inside hostile macrophages. However, unlike with obligate parasites such as viruses, cells are not dependent on host cells for replication. Thus, *C. neoformans* is regarded as a facultative parasite or facultative intracellular pathogen that invades macrophages (Del Poeta, 2004).

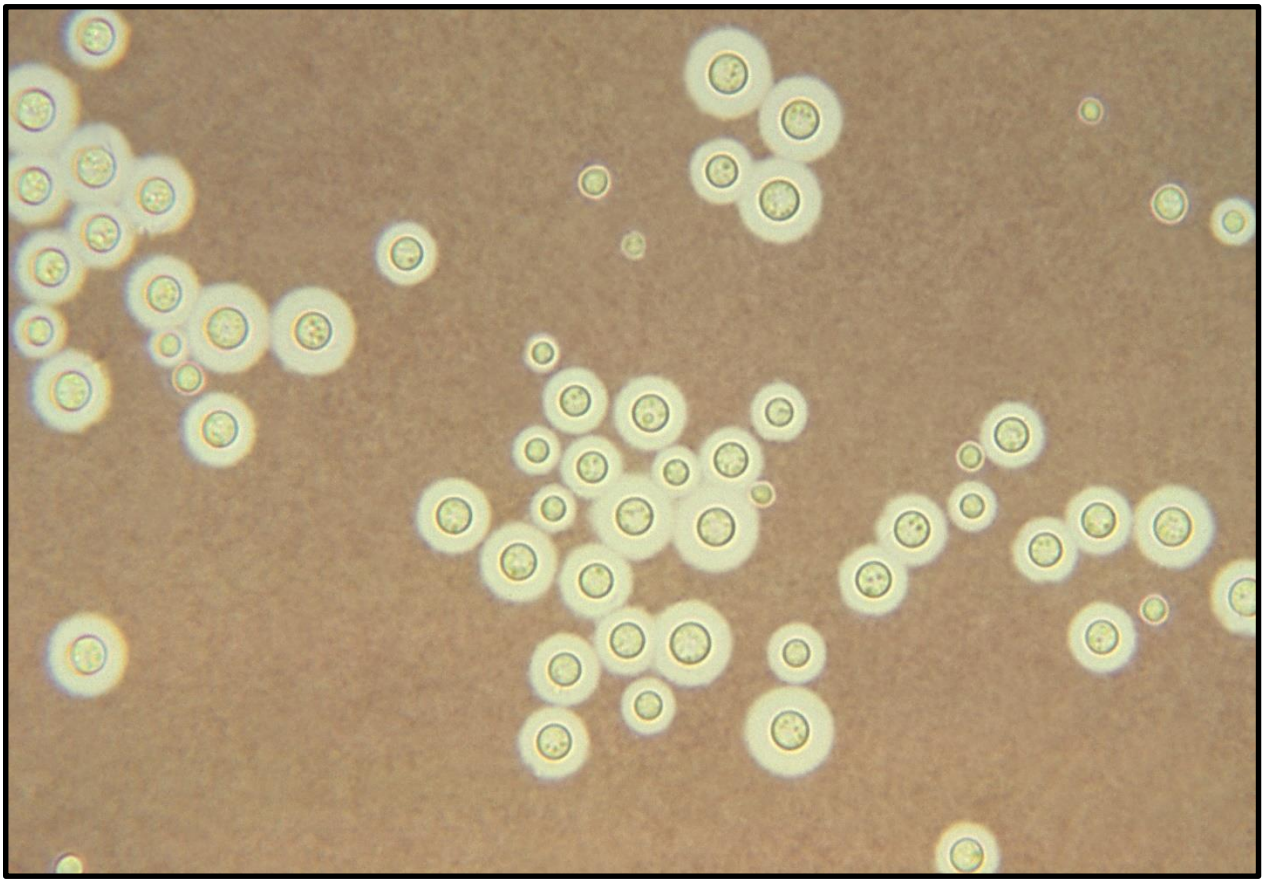


Figure 3. An Indian ink preparation. The pictogram depicts characteristic thick capsules of encapsulated *C. neoformans* cells. The pictogram is in public domain, and was obtained from Wikipedia. Pictogram: Credit to CDC/ Dr. Leanor Haley.

To reach the macrophages, cryptococcal cells are first inhaled as airborne infectious propagules from the environment that eventually lodge in the lungs (Figure 4) (Casadevall and Perfect, 1998). The relatively small size of the cells, usually due to poor encapsulation, allows cells to easily lodge within the alveoli. Upon lodging in the alveolar space, these invading cells are directly attacked by macrophages in an attempt to clear the infection. Macrophages, much like amoebae, can kill internalised microbes by phagocytosis, which is a receptor-mediated process that is governed by a balance between pro- and anti-signal molecules that promote or inhibit the process of phagocytosis (Voelz *et al.*, 2009; Freeman and Grinstein, 2014).

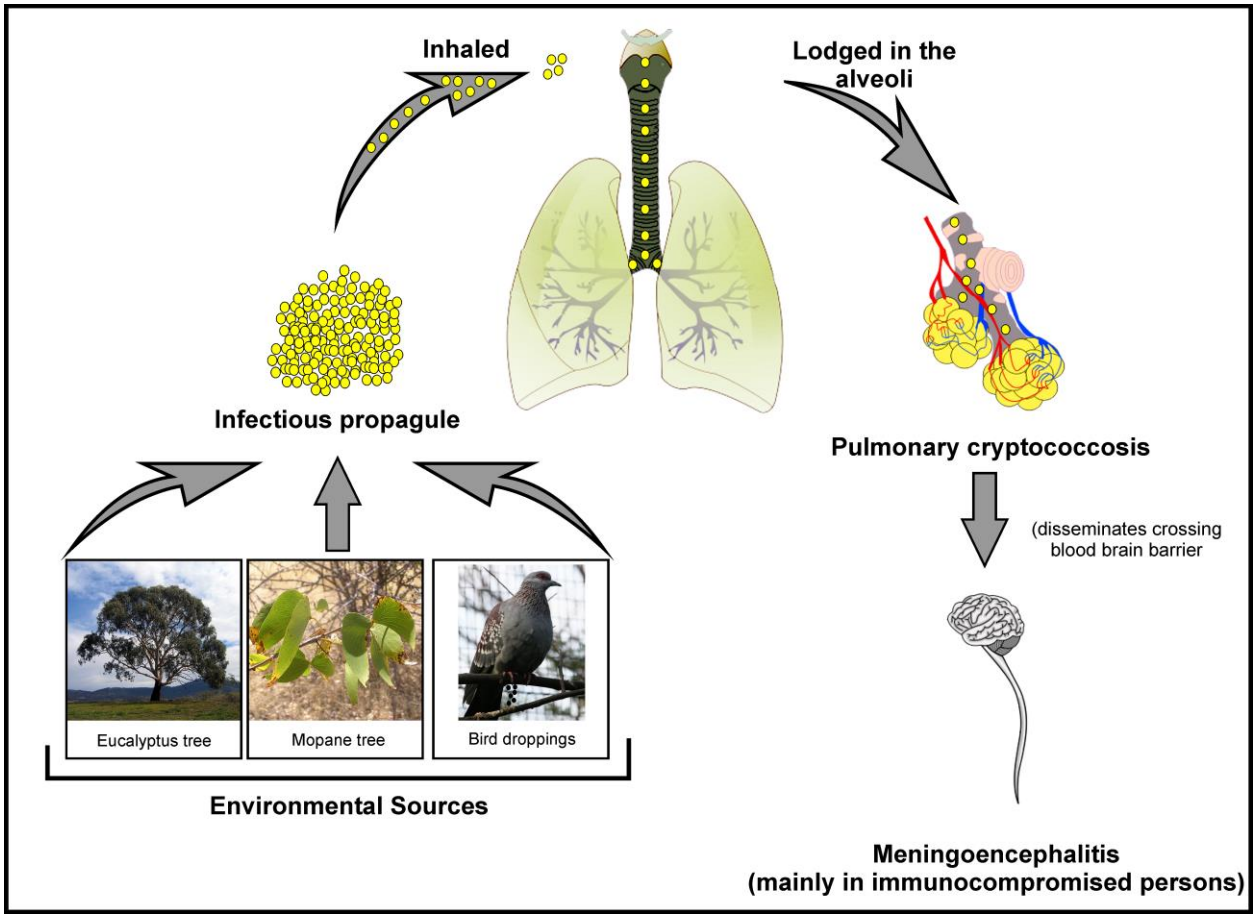


Figure 4. A schematic representation of cryptococcal infection. Infection usually starts with inhalation of an infectious propagule leading to a diseased-state either in the lungs or, in disseminated cases, in other body parts. The scheme was constructed using pictures obtained from Wikipedia. All images are in the public domain. Bird: Credit to Rklawton; Mopane tree: Credit to Teo Gomez; Eucalyptus tree: Credit to Alexander110; Lungs: Credit to Rastrojo; Alveoli: Credit to Rastrojo; Brain: Credit to Grm wnr.

However, cryptococcal cells have developed a way to manipulate macrophages, wherein after being internalised, cells can establish an intracellular lifestyle i.e. parasitism, within the infected host body without killing the macrophages (Voelz *et al.*, 2009). More interestingly, upon being internalised, cryptococcal cells (unlike some pathogenic bacteria) have adapted to proliferate inside macrophages regardless of the prevailing harsh environment (Horwitz, 1983;

Levitz *et al.,* 1999; Voelz and May, 2010). Usually in a susceptible host i.e. in an immuno-compromised individual, cryptococcal cells can spread to other organs such as the brain, using infected macrophages as vehicles in a so-called "Trojan-Horse" model. The model, is the best available theory that attempts to explain how cryptococcal cells can "under cloak" travel inside macrophages, cross the blood-brain barrier and exit the infected macrophages without eliciting an immune response to cause inflammation of the brain (Voelz *et al.,* 2009; 2010).

The capacity of cryptococcal cells to avoid immuno-processing is attributed to the capsules. Somehow, the selective pressure that is initially exerted on cryptococcal cells in the soil by amoebae has, at the same time, allowed cells to display the same defensive behaviour when under attack by macrophages in the host body. The logical explanation for this is that a cryptococcal cell would perceive both the two hostile phagocytic cells i.e. macrophage and amoeba, as being one and the same thing – as it was previously pointed out by Feldmesser *et al.,* (2001). Unfortunately this defensive behaviour i.e. capsule production, has also translated into this microbe establishing itself as a successful human pathogen, more so in susceptible hosts (Levitz and Boekhout, 2006). While capsules may be critical in shielding cells from macrophages and advance their course in manifesting a diseased-state in susceptible hosts, much is still unknown about other mechanisms, including secondary metabolites that are secreted, which may enable this pathogen to survive encounters with other hostile cells in nature. Luberto *et al.,* (2003) reported on a secondary metabolite that can assist cryptococcal cells to evade immuno-processing. In the study, they showed that cryptococcal cells could secrete a protein called anti-phagocytic protein 1, which is reported to use the complement system to block the macrophage phagocytosis process in the presence or absence of the capsule (Luberto *et al.,* 2003; Hull, 2011).

In the context of competition, a dominant microbe can successfully wage a chemical assault by secreting secondary metabolites into the surrounding extracellular environment in order to out-compete other microbes (Hunter, 2006), and in respect of immunity, to out-smart macrophages by impairing their function (Luberto *et al.,* 2003). These secondary metabolites are usually delivered as cargo into the surrounding extracellular environment contained in lipid-based extracellular vesicles or "fatty carry bags" that transport them across the membrane as well as the dense matrix of the cell wall (Rodrigues *et al.,* 2007; Wolf *et al.,* 2014). The transported cargo or secondary metabolites may be in the form of proteins, carbohydrates or even lipids; and typically they do not play a role in the primary metabolism i.e. replication, of the concerned microbe. A classic example of secondary metabolites is quorum-sensing molecules. In *C. neoformans,* one such molecule has been identified, namely pantothenic acid (Albuquerque *et al.,* 2014). It is reported that this molecule can coordinate the expression of certain genes in response to population density and/or the presence of other microbes – in order to promote the survival of this fungus. In the context of infection, when threshold concentrations of pantothenic acid are reached, cryptococcal cells can release glucuronoxylomannan into the environment, which may exert deleterious effects on macrophages (Buchanan and Murphy, 1998; Vecchiarelli *et al.,* 2003; Ellenbroek *et al.,* 2004; Yauch *et al.,* 2006).

In the motivation section, it has highlighted that cryptococcal cells produce 3-hydroxy fatty acids. In species like *Candida albicans,* 3-hydroxy fatty acids have successfully been implicated to act as virulence factors that promote the survival of *Candida* cells (Deva *et al.,* 2000; Ciccoli *et al.,* 2005; Nigam *et al.,* 2010). Therefore, is it possible that these molecules would like-wise promote the survival of cryptococcal cells? In the next section, the available

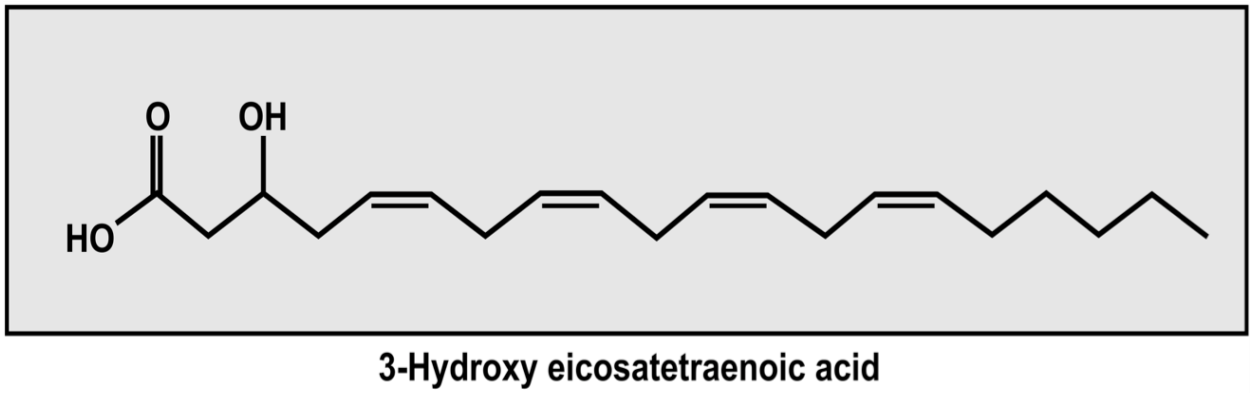
literature regarding the biological functions of 3-hydroxy fatty acids during interspecies interactions is interrogated.

1.4 3-HYDROXY FATTY ACIDS

1.4.1 Definition, biosynthesis and occurrence

3-Hydroxy fatty acids are lipid-based molecules, and as such they are defined based on their amphiphilic quality, which is evidenced when immersed in aqueous environments (Kock and Botha, 1998). 3-Hydroxy fatty acids are characterised by a hydroxyl group on the beta carbon or carbon 3 when counting from the carboxyl group (Figure 5). Sebolai *et al.*, (2012) suggested that the oxygen molecule (that forms part of the hydroxyl group) that is inserted in the fatty acid chain originates from water. It is the first three carbons of the molecule (i.e. from carboxyl carbon to the hydroxyl carbon) that constitute the polar head that, in turn, contributes to the molecule's amphiphilic quality. In general, 3-hydroxy fatty acids are regarded as secondary metabolites, with no apparent function in the primary metabolism of microbes (Tsitsigiannis and Keller, 2007). These molecules are considered to be produced via an incomplete enzymatic pattern similar to mitochondrial beta-oxidation, wherein the mitochondrial enzyme, 3-hydroxyacyl-CoA dehydrogenase, is reported to poorly metabolise 3-D hydroxyacyl-CoA enantiomer (Venter *et al.*, 1997; Sebolai *et al.*, 2012). As a result of the latter, the D-enantiomer initially accumulates inside the mitochondria (Finnerty, 1989), and is eventually excreted out. However, Jones *et al.*, (2011) importantly pointed out that these molecules rarely occur in significant quantities under normal physiological conditions (Jones *et al.*, 2011). Thus emphasising that cells expend energy to produce these molecules when in need.

In addition, these molecules are well distributed across the microbial kingdom – occurring in some pathogenic bacterial species and fungal species, including non-pathogenic species (Kock *et al.*, 2007). In these species, 3-hydroxy fatty acids may exist in a complex form - where they may be linked to other macromolecules (polysaccharides) such as in the lipopolysaccharide of Gram-negative bacteria or in a simple, free form – as was previously reported in *C. neoformans* UOFS Y-1378 (Sebolai *et al.*, 2007).



3-Hydroxy eicosatetraenoic acid

Figure 5. A chemical structure of a typical 3-hydroxy fatty acids (3-hydroxy eicosatetraenoic acid (with a hydroxyl group on the beta carbon). The hydrocarbon chain can at times be branched, saturated and linked to other macromolecules. Pictogram: Credit to Dr O.M. Sebolai.

1.4.2 Biological functions during interspecies interactions

The presence of unwanted fungal species in the fermentation process can often lead to food spoilage, including food poisoning (Sjogren *et al.*, 2003). Thus to maintain the integrity of fermentation products, microbes such as Lactic acid bacteria (LAB) have a long history of being used as bio-preservatives. The ability of LAB to exclude unwanted microbes from the fermentation process is attributed to production of antimicrobial agents, and in particular 3-hydroxy fatty acids. To demonstrate this point, in their study, Sjogren and co-workers showed

that in response to the presence of unwanted fungal species during fermentation, LAB secrete a number of 3-hydroxy fatty acid molecules, which in turn, inhibited the growth of unwanted fungal species (Sjogren *et al.*, 2003). These 3-hydroxy fatty acids were identified by mass spectrometry as 3-hydroxy decanoic acid, 3-hydroxy-5-*cis*-dodecenoic acid, 3-hydroxy dodecanoic acid, and 3-hydroxy tetradecanoic acid.

In addition to the above, microbe to microbe interactions, 3-hydroxy fatty acids have also been implicated in mediation of microbe to host interactions. For example, 3-hydroxy oxylipins (as part of Gram negative bacteria endotoxin) can be deliberately shed in the host body leading to development of sepsis, more so at high concentrations (Rietschel *et al.*, 1994; Annane *et al.*, 2005). Upon shedding, the endotoxin is reported to trigger an immune response that is characterised by production of pro-inflammatory cytokines (Annane *et al.*, 2005). These cytokines acting together with mediator molecules (cyclooxygenase 2, phospholipase A_2 and nitric oxide synthase) through specific G-protein-coupled receptors promote inflammation and can cause widespread endothelial injury, among others (Dinarello, 2000; Annane *et al.*, 2005).

3-Hydroxy oxylipins also occur as mycolic acids in *Mycobacterium tuberculosis*, which is the aetiological agent of tuberculosis (Rao *et al.*, 2006). Mycolic acids are 3-hydroxy oxylipins that are further defined by long alpha alkyl branched chains (Takayama *et al.*, 2005). Under normal physiological conditions, once this bacterium is detected by Toll-like receptors (through pathogen-associated molecular patterns i.e. mycolic acids), it is subsequently internalised and phagocytosed by macrophages. However, these molecules can at the same time allow this bacterium to subvert the course of immunological development by surviving the harsh internal environment of macrophages. To the point, it has been reported that the hydrophobic nature of 3-

hydroxy fatty acids provides protection against the chemical assault that is exerted by host bactericidal agents (Vander Beken *et al.,* 2011). In addition, *Mycobacterium's* 3-hydroxy fatty acids can lead to an upsurge of pro-inflammatory cytokines, which could lead to formation of severe lung lesions (Rao *et al.,* 2006; Riley, 2006).

Nigam *et al.,* (1999) were the first to provide evidence on the influence of fungal 3-hydroxy fatty acids on mammalian cells (Nigam *et al.,* 1999). These authors reported that 3-hydroxy fatty acids acted as a strong chemotactic agent - the potency of which is comparable with those of leukotriene B4 or fMet-Leu-Phe. In addition, these molecules affected signal transduction processes, via a G-protein receptor, in human neutrophils and tumour cells in multiple ways possibly. Work later done in the Nigam laboratory further uncovered and described a novel aspirin-sensitive mode of infection that is mediated by 3-hydroxy fatty acids of *Candida albicans* (Ciccoli *et al.,* 2005). Ciccoli *et al.,* (2005) found that this yeast converts arachidonic acid, released from infected or inflamed host cells, to a 3-hydroxy fatty acid via incomplete mitochondrial action. This 3-hydroxy fatty acid, stereo-chemically similar to arachidonic acid, then acts as substrate for the host cyclooxygenase-2 (COX-2), leading to the production of potent pro-inflammatory 3-hydroxy prostaglandin E_2 (3-OH-PGE_2). This novel compound, via the PGE_2 receptor 3, could signal the expression of IL-6 gene and raise cAMP levels via the EP 4 receptor. These results lead this group of researchers to conclude that these compounds have strong biological activities similar to and in some cases even more potent than those of the normally produced mammalian eicosanoids.

In 2007, the presence of these molecules in a fungal pathogen called *Cryptococcus neoformans* were reported (Sebolai *et al.,* 2007). In this fungus, these molecules were intimately

associated with the principal virulence factor *of C. neoformans* viz. the capsule (Casadevall and Perfect, 1998). However, the biological function(s) of these molecules have not been elucidated. Could these molecules be acting in concert with the capsule, during infection, to prevent phagocytosis or could they act to inhibit growth of other microbes occupying the same space to appropriate territorial dominance?

1.5 PURPOSE

With the preceding discussion providing a background and context for the studies presented in the dissertation, the aims became:

1. To establish the role of cryptococcal 3-hydroxy fatty acids when cells are interacting with *Pseudomonas* cells (Chapter 2); and
2. To establish the role of cryptococcal 3-hydroxy fatty acids when cells are interacting with host cells (Chapter 3).

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CHAPTER 2:

MICROBE-TO-MICROBE INTERACTIONS

This study was performed by the candidate, and has been submitted to the journal "Medical Mycology" for publication. As a result, repetition of some information could not be avoided.

2.1 ABSTRACT

Previous studies reported on a 3-hydroxy fatty acid (3-hydroxy C9:0) that is secreted into the surrounding environment of *Cryptococcus* (*C.*) *neoformans* UOFS Y-1378. Towards this end, it was sought to determine if this molecule possessed any anti-microbial quality. More to the point, the effects of this molecule on *Pseudomonas* (*P.*) *aeruginosa* were examined. *Pseudomonas aeruginosa* cells were revealed to have a dose-dependent response profile i.e. 12% growth reduction at 0.2 mM and 32% growth reduction at 1 mM when compared to non-treated cells. Corollary, a dose-dependent reduction in pyocyanin production viz. 80% reduction at 0.2 mM and 92% reduction at 1 mM was also observed. Cell growth inhibition was achieved through membrane function impairment possibly through incorporation of this saturated molecule into the bilayer leading to a rigid membrane. As a result, treated cells could traffic significantly less adenylate kinase into the extracellular environment compared to non-treated cells. In turn the loss of membrane functions further manifested in a dose-dependent accumulation of ROS in drug-treated cells. The data presented herein assigns an anti-microbial quality to this 3-hydroxy fatty acid metabolite. It is, therefore, reasonable to conclude that these molecules would allow *C. neoformans* UOFS Y-1378 to appropriate an environmental advantage over other microbes in nature.

Key words: 3-Hydroxy fatty acids, 3-hydroxy C9:0, Anti-microbial quality, *Cryptococcus, Pseudomonas*.

2.2 INTRODUCTION

The respiratory tract often serves as a portal for microbes to access hospitable environments within the body that are rich in nutrients (Fernstrom *et al.,* 2013). Towards this end, microbes have to overcome effective and complex defence mechanisms such as the phagocytic process in the lungs (Janeway *et al.,* 2001), in order to flourish. One organism that frequently colonises the lungs is the opportunistic bacterium, *Pseudomonas (P.) aeruginosa* (Rella *et al.,* 2012). *Pseudomonas* infections typically start with: 1) aspiration of cells from the upper respiratory tract more so in patients on mechanical ventilation or, in some instances 2) through bacteraemia with cells finally reaching the lungs (Rella *et al.,* 2012). Another microbe that causes pneumonia is the fungus *Cryptococcus (C.) neoformans* (Lin and Heitman, 2006), and its infection usually begins with the inhalation of airborne infectious basidiospores from the environment, which can then be lodged in the lungs (Casadevall and Perfect, 1998).

In the lung environment, populations occupying the same space would have to compete due to the dynamics in nutrient availability. Thus as a matter of natural course, such microbes would develop mechanisms for: 1) appropriating territorial dominance, and 2) nutrient scavenging (Czaran and Hoekstra, 2001; Hogan and Kolter, 2004). One classical mechanism is the secretion of secondary metabolites such as quorum sensing molecules, which upon release can stimulate transcription of specific genes from neighbouring cells leading to production of factors that control population density or even repel competitors (Williams, 2007; Strateva and Mitov, 2011; Albuquerque *et al.,* 2014). For this, it was sought to investigate if cryptococcal secondary metabolites, and in particular 3-hydroxy fatty acids, may inhibit the growth of *Pseudomonas* cells when sharing the same environment. The latter is on the basis that it was previously shown that these molecules are secreted to the extracellular environment of *C.*

neoformans UOFS Y-1378 cells (Sebolai *et al.*, 2008), possibly to promote the survival of cryptococcal cells. However, to test the biological function(s) of these molecules i.e. act as antimicrobial agents – studies to test their effects on *Pseudomonas* cells were set up.

2.3 MATERIALS AND METHODS

Strains used and cultivation

Pseudomonas aeruginosa PA01 (a commonly used and metabolically versatile opportunistic *Pseudomonas* strain) and *C. neoformans* UOFS Y-1378 (a pathogenic strain that has been shown to produce 3-hydroxy fatty acids), which are kept as cultures at the University of the Free State, were used in this study. *Pseudomonas aeruginosa* was cultivated on nutrient agar (5 g/l peptic digest of animal tissue, 3 g/l beef extract, 15 g/l agar; Sigma-Aldrich, South Africa) at 37^{o}C while *C. neoformans* was grown on yeast-malt-extract agar (3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone, 10 g/l glucose, 16 g/l agar; Merck, South Africa) at 30^{o}C.

A loopful (0.001 ml loop) of *Pseudomonas* cells and *Cryptococcus* cells were separately collected from their respective agar plates and each loopful inoculated into corresponding sterile 50 ml centrifuge tubes (Fisher-Scientific, United Kingdom), containing 25 ml of broth. For *Pseudomonas*, nutrient broth (15 g/l peptone, 3 g/l yeast extract, 6 g/l sodium chloride and 1 g/l glucose; Sigma-Aldrich) was used while for *Cryptococcus* Difco-yeast nitrogen base (YNB) broth (6.7 g/l YNB and 40 g/l glucose; Becton, Dickson and Company, United States) was used. Respective cells were allowed to reach their mid-logarithmic phase while shaking ((180 rpm at 37^{o}C for *Pseudomonas*) and (160 rpm at 30^{o}C for *Cryptococcus*)), after which cells were washed twice using phosphate buffered saline (PBS; Oxoid, South Africa), and then separately

standardised in 25 ml of fresh YNB broth. For bacterial standardisation, a formula by Jacobsen *et al.,* (2011) was used. *Pseudomonas* cells (number of CFU/ml) were calculated as follows: number of CFU/ml = OD_{600} x 2.5 x 10^8. The optical density (OD) was read using a spectrophotometer (Biochrom EZ Read 800 Research, United Kingdom). *Cryptococcus* cells were standardised using a haemocytometer (Marienfeld, Germany). Standardised cells were kept on ice prior to use.

Survival assay of *P. aeruginosa* co-cultured with *C. neoformans*

It was sought to determine if *Cryptococcus* cells could dominate *Pseudomonas* cells when co-cultured. For this, a 100-µl suspension of *P. aeruginosa* (1x 10^6 CFU/ml in YNB media) and a 100-µl suspension of *C. neoformans* (1x 10^6 CFU/ml in YNB media) were aliquoted into the same well of a microtitre plate (Greiner Bio-One, Germany) in order to prepare a co-culture. Subsequently, the microtitre plate was incubated at 30ºC for 24 h as previously detailed by Rella *et al.,* (2012). Following incubation, the contents of each well were spotted onto Luria-Bertani (LB) agar plates (Merck; 5g/l yeast extract, 10 g/l tryptone, 10 g/l sodium chloride, 15 g/l agar), and cells were immediately spread with a sterile glass "hockey stick" to create a uniform lawn. The LB plates were chosen because they are reported to not support the growth of *C. neoformans* (Rella *et al.,* 2012). The plates were grown overnight at 30ºC. The following day, images of LB plates (using a Canon digital camera) were taken in order to visually grade the amount of pyocyanin secreted (depicted by a green pigment) onto LB plates. Secondly, cells were scraped from the surfaces of the same LB agar plates and suspended in 25 ml of PBS. The *Pseudomonas* cell count was again determined at the end of the experiment using the formula: number of CFU/ml = OD_{600} x 2.5 x 10^8 (Jacobsen *et al.,* 2011). As a control, *Pseudomonas*-alone cells (i.e. not co-cultured with *C. neoformans* cells) were also seeded into wells of a microtiter plate (30 ºC

for 24 h) and were spread (after 24 h) on LB plates. The plates were subsequently incubated overnight at 30^{o}C. Following this: 1) digital images of LB plates showing pyocyanin pigmentation were taken, and 2) final cell counts of *Pseudomonas* cells were also calculated using the formula shown above.

In order to visualise interactive moments between *Pseudomonas* cells and *Cryptococcus* cells, material from a 6 h co-culture microtitre plate was prepared for scanning electron microscopy (SEM) according to the method of Van Wyk and Wingfield (Van Wyk and Wingfield, 1991). This material was chemically fixed using sodium-phosphate-buffered 3% glutaraldehyde (Merck) and similarly buffered osmium tetroxide (Merck) followed by dehydration in a graded ethanol (Merck) series, critical-point dried (Biorad Microscience Division, England), mounted on stubs, and sputter coated with gold (confer electron conductivity) using a SEM coating system (Biorad Microscience Division). Preparations were examined using a Shimadzu Superscan SSX 550 scanning electron microscope (Japan).

Analytical 3-hydroxy fatty acid standard

A commercial standard was used to determine the direct effect of 3-hydroxy fatty acids on *Pseudomonas* cells since *C. neoformans* UOFS Y-1378 cells were determined to secrete minimal amounts of 3-hydroxy fatty acids (Madu *et al.,* 2015). Thus the analytical compound (3-hydroxy C9:0; obtained from Laradon Fine Chemicals (Sweden)), was completely dissolved in water (at ambient temperature and pressure) to yield a 2 mM stock solution. This compound was tested at 0.2 mM, which is the estimated physiological concentration secreted by *C. neoformans* UOFS Y-1378 (Madu *et al.,* 2015) and 1 mM, which was used as an upper limit concentration.

Direct effect of 3-hydroxy fatty acid on *P. aeruginosa* growth and pyocyanin production

Pseudomonas aeruginosa cells were standardised to 1 x 10^6 CFU/ml in fresh RPMI-1640 media (Sigma-Aldrich), following which 100 µl of this cell suspension was added to a sterile microtitre plate. Aliquots of 100 µl of the drug, at twice the desired final concentrations, i.e. 0.2 mM and 1 mM, were dispensed into wells. The plates were incubated for 24 h at 37^{o}C and then OD_{620} readings taken at 0 h, 6 h, 12 h, 18 h and 24 h. In order to qualitatively inspect the effect of this molecule on pyocyanin production, the contents of each well (representing the different experimental conditions i.e. non-treated cells, cells treated with 0.2 mM and 1 mM; after a 24-h incubation period) were immediately spread with a sterile bent glass "hockey stick" to create a uniform lawn on LB plates and grown overnight at 30^{o}C. Pyocyanin production was measured by visually grading the degree of pigmentation on each LB agar plate.

In a separate experiment, pyocyanin production was quantified according to a method previously described by Essar *et al.*, (1990). In brief, *Pseudomonas* cells (non-treated cells, cells treated with 0.2 mM and 1 mM) were standardised to 1 x 10^6 CFU/ml and cultivated in 5 ml of RPMI-1640 media contained in 15 ml centrifuge tubes (Becton-Dickinson Labware, United States) for 24 h at 37^{o}C. Following this incubation period, the supernatant fraction was first separately collected by centrifugation (6000 rpm for 10 min) from the 5 ml cultures (representing the different experimental conditions). Next, pyocyanin was extracted using 5 ml of chloroform (Merck). Following this, pyocyanin was re-extracted using 1.5 ml of 0.2 M hydrochloric acid (Merck) from the chloroform layer. The absorbance of the collected top fraction (which had a slight pink colour) was measured. Pyocyanin concentration was calculated as previously detailed by Essar *et al.*, (1990) by multiplying the obtained optical density (OD_{520nm}) reading by a factor of 17.052, and expressing the answer in µg/ml (Essar *et al.*, 1990).

Direct effect of 3-hydroxy fatty acids on *P. aeruginosa* ultrastructure

Material (24 h old cells – initially standardised to 1 x 10^6 CFU/ml in RPMI-1640 media, (non-treated cells, and 3-hydroxy C9:0-treated cells (i.e. 0.2 mM and 1 mM))) for scanning electron microscopy (SEM) was separately pooled into 1.5 ml plastic tubes (Merck). The material was prepared for SEM viewing as above.

For nano-scanning auger microscopy (Nano-SAM) examination**,** the same SEM stubs were re-coated with gold and re-examined using a nano-scanning Auger microscope in SEM mode linked to Argon (Ar^+) etching as described by Swart *et al.,* (2012). Cells (from each experimental condition) were then examined with a PHI 700 Nanoprobe (Japan) equipped with SEM and Scanning Auger Microscopy (SAM) facilities. For the SEM and SAM analyses in the field emission electron, gun used was set as: 2.788 A filament current; 3.56 kV extractor voltage and 175 µA extractor current. A 25 kV, 1 nA electron beam was obtained with these settings for the Auger analyses and SEM imaging. The electron beam had a diameter of 12 nm. The electron gun unit had an upper pressure of 8.7E-10 Torr and the pressure of the main chamber was 4.4E-10 Torr. Aperture A was used for all the measurements. For SEM the field of view (FOV) was 2 µm. Four (4) cycles per survey, 1 eV per step and 50 ms per step were used to obtain Auger point analyses. The Ar^+ ion sputtering gun, which the Nanoprobe was also equipped with, was set at: 2 kV beam voltage, 5 µA ion beam current and a 1 x 1 mm rraster area, giving a sputter rate of 15 nm/min.

3-Hydroxy fatty acids killing mode of action

In order to determine if membrane function was maintained after challenging cells with 3-hydroxy C9:0, a Toxilight® Bioassay (Lonza Rockland, Inc., United States) was performed

according to the manufacturer's instructions. This assay quantitatively measures the release of adenylate kinase from cells with damaged membranes into the extracellular environment. A 100-µl suspension of test cells standardised to 1 x 10^6 CFU/ml in RPMI-1640 media was aliquoted into microtiter plate wells. Next, aliquots of 100 µl of the drug, at twice the desired final concentrations i.e. 0.2 mM and 1 mM, were also dispensed into the same wells, and the plate was incubated at 37ºC for 24 h. Non-treated cells were also included for referencing. After this incubation period, the supernatant was collected (6000 rpm for 10 min) and reacted with the Toxilight reagent in a sterile white 96-well flat-bottom microtitre plate (Greiner Bio-One) for 5 minutes. The emitted light intensity was measured using a Fluoroskan Ascent FL (Thermo-Scientific, USA) microplate reader, which converts logarithmic signals to relative luminescence units.

In a separate experiment, accumulation of reactive oxygen species (ROS) was also measured using a fluorescent dye, 2',7-dichlorofluorescin diacetate (DCFHDA; Sigma-Aldrich). Cells for this experiment were prepared, treated with the test drug and grown under similar conditions as the Toxilight® Bioassay cells. After a 24 h incubation period, 10 µl of DCFHDA (1µg/ml) was reacted with 90 µl of cells in a sterile black 96-well flat-bottom microtitre plate (Greiner Bio-One) for 30 minutes in the dark at room temperature. The induced fluorescence was measured (485nm; ex / 535nm; em) using a Fluoroskan Ascent FL (Thermo-Scientific, United States) microplate reader.

Statistical analyses

All experiments were performed in triplicate. Where appropriate, a student *t*-test was conducted to determine the statistical significance of data between the different experimental conditions. *P* values equal or below 0.05 were regarded as being statistically significant.

2.4 RESULTS AND DISCUSSION

C. neoformans dominates *P. aeruginosa* and 3-hydroxy fatty acids inhibit *P. aeruginosa*

In this study, it was shown that when *C. neoformans* and *P. aeruginosa* cells were co-cultured (Figure 1), cryptococcal cells exerted territorial dominance over *Pseudomonas* cells. To illustrate this point, there was a significant reduction ($p = 0.05$) in *Pseudomonas* cell numbers from when *Pseudomonas* were not co-cultured with *Cryptococcus* cells to when they were co-cultured with cryptococcal cells (Figure 2A). Typically in a co-culture, the different microbial populations compete for nutrient availability (in this case, the limited amount provided in the YNB broth). To this end, a dominant microbe out-competes and inhibits the other through successfully waging a chemical assault. Based on the aforementioned finding, it is reasonable to conclude that the observed cryptococcal dominance (depicted by the significantly low *Pseudomonas* cell numbers from the co-cultured experiment) might be due to the secretion of 3-hydroxy fatty acids into the extracellular environment.

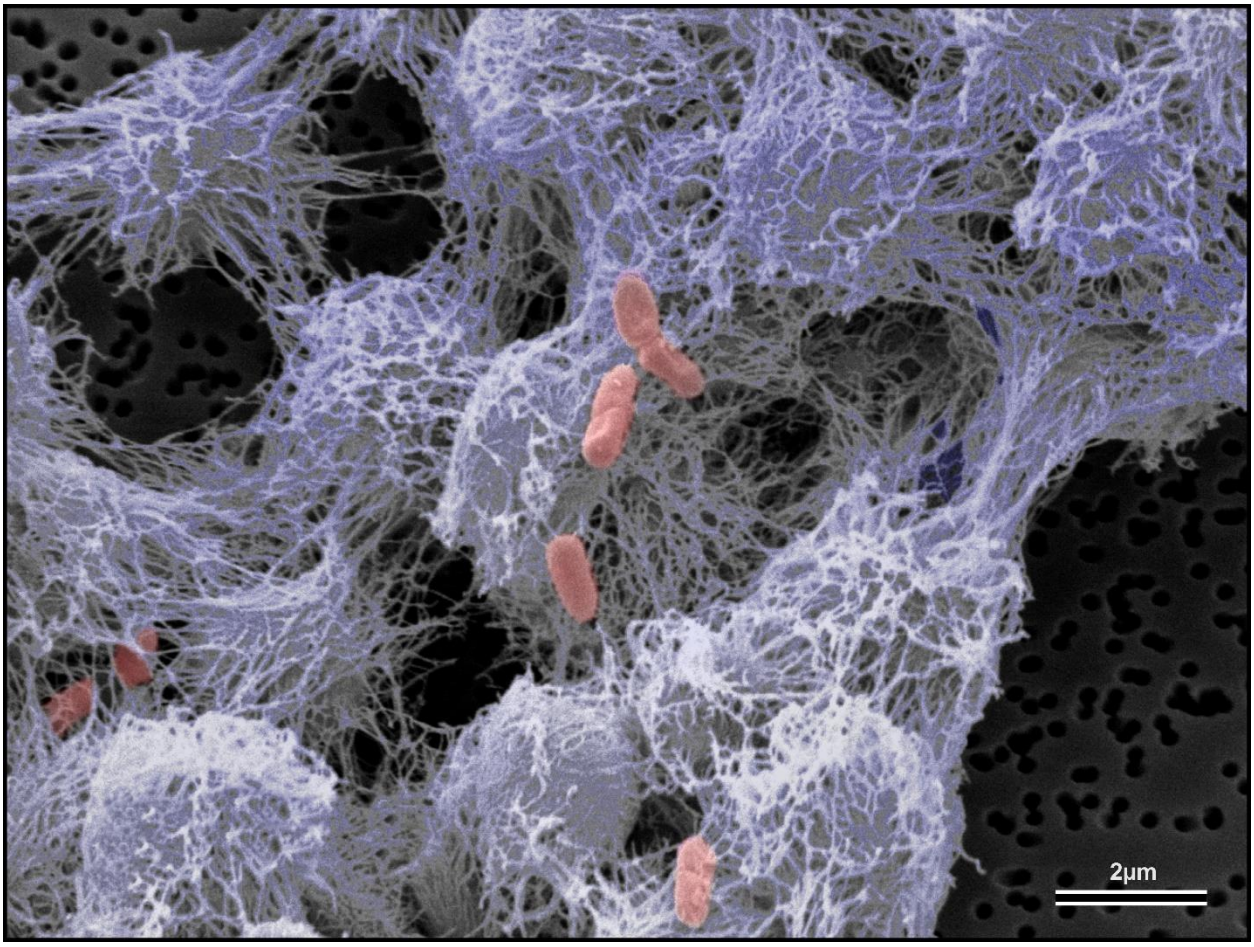

Figure 1. An SEM micrograph depicting interaction between *Cryptococcus* cells and *Pseudomonas* cells (orange). Co-culture cells were further distinguished using colour applied via CorelDRAW Graphics Suite X7. *Cryptococcus* cells = blue and *Pseudomonas* cells = orange.

At the same time, the noted reduction in *Pseudomonas* population numbers coincided with a decrease in the amount of pyocyanin that is secreted onto LB agar plates when directly comparing *Pseudomonas*-alone cell plates i.e. from cells not co-cultured with *Cryptococcus* cells to *Pseudomonas*-*Cryptococcus* co-cultured plates (Figure 2B). This further supports the argument that cryptococcal cells may be secreting a bioactive molecule (3-hydroxy fatty acids) that negatively affects *Pseudomonas* cells.

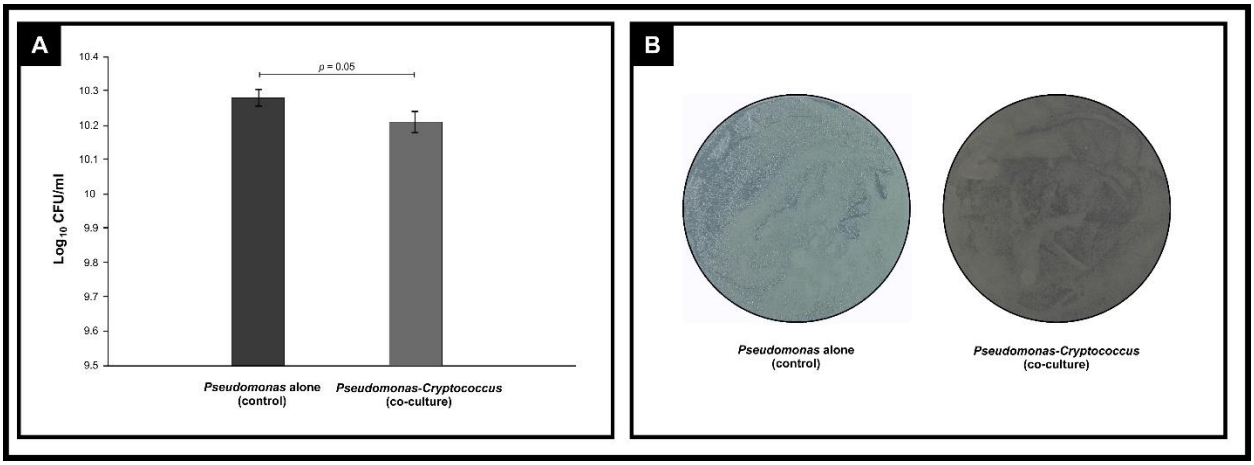

Figure 2. The effect of *Cryptococcus* cells on *Pseudomonas* cells when co-cultured. *Cryptococcus* cells exerted territorial dominance over *Pseudomonas* cells, which resulted in a significant reduction in *Pseudomonas* cell numbers after a 24-h period (A), and a reduction in pyocyanin production (B) when compared to *Pseudomonas* cells alone i.e. *Pseudomonas* cells not co-cultured with *Cryptococcus* cells. Pyocyanin production is depicted by a green pigmentation on LB agar plates. The LB agar results were similar for all repeats.

Next, the direct effect of 3-hydroxy fatty acids on *Pseudomonas* cells was determined. Here, *Pseudomonas* cells were inhibited in a dose-dependent manner by different concentrations of 3-hydroxy fatty acids i.e. 0.2 mM resulted in 12% growth reduction while 1 mM yielded 32% growth reduction when compared to non-treated cells after 24 h (Figure 3). Corollary, a dose-dependent reduction in pyocyanin production was observed when: 1) studying LB agar plates of *Pseudomonas* cells treated with different concentrations of 3-hydroxy fatty acids (Figure 4A), and 2) quantifying the amount secreted by cells (Figure 4B). More to the point, an 80% reduction in pyocyanin production at 0.2 mM and 92% reduction at 1 mM were calculated. At this point, the observed reduction in pyocyanin production may be a function of loss of *Pseudomonas* cells. However, other experiments are required to determine if 3-hydroxy fatty acids down regulate genes involved in pyocyanin production.

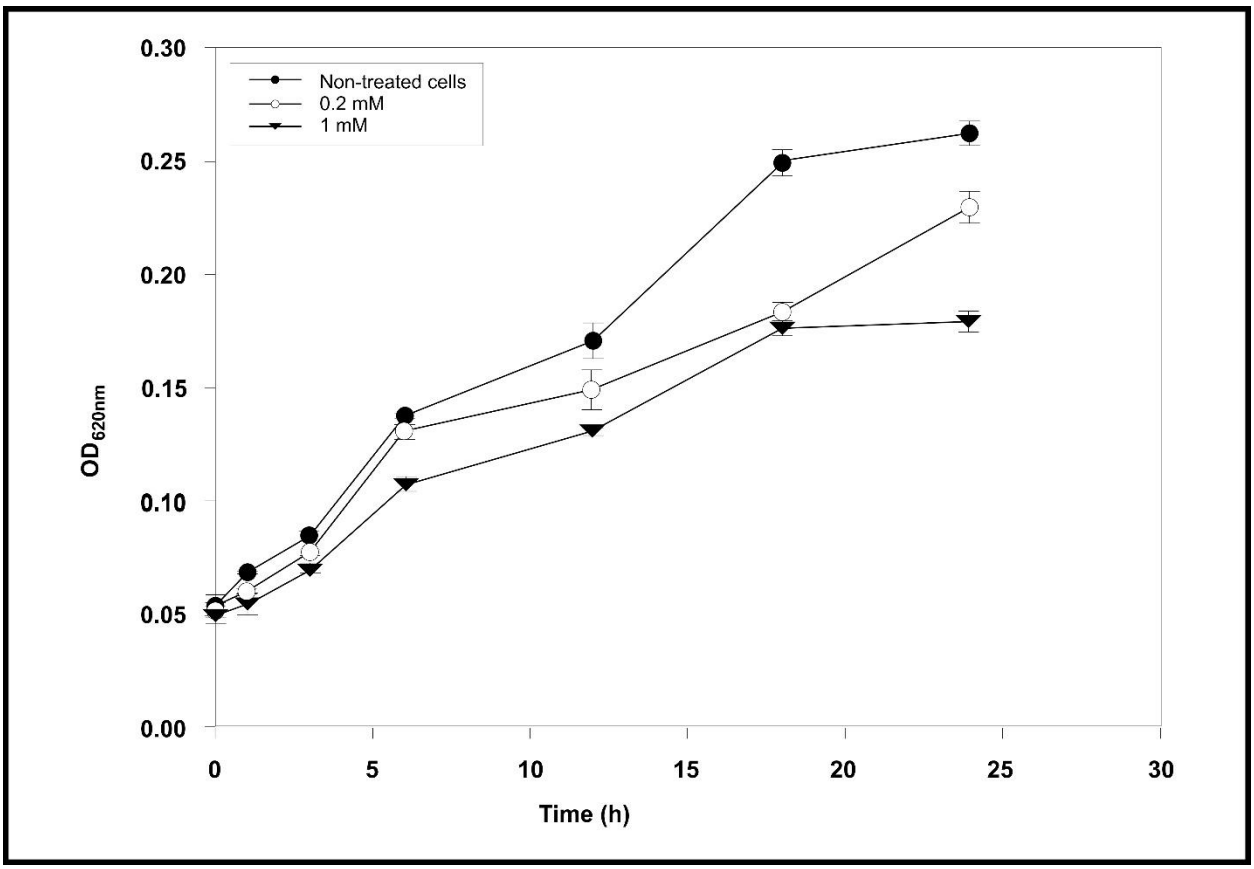

Figure 3. Treatment of *Pseudomonas* cells with 3-hydroxy fatty acids. 3-Hydroxy fatty acids inhibited *Pseudomonas* cells in a dose-dependent manner with the highest growth inhibition recorded for 1 mM after a 24-h incubation period.

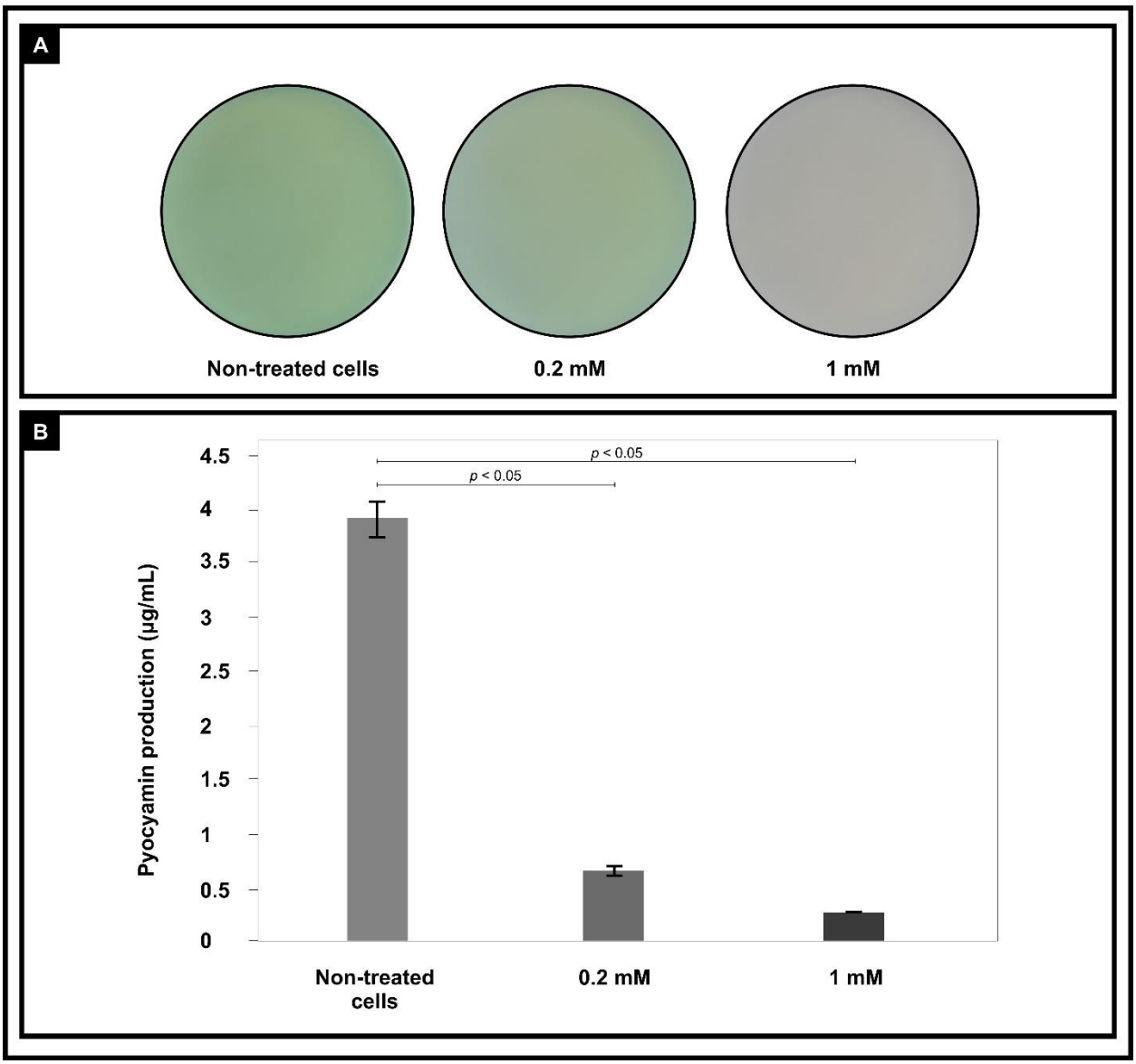

Figure 4. Treatment of *Pseudomonas* cells with 3-hydroxy fatty acids. (A) Represents a qualitative measurement of pyocyanin on LB plates based on visually inspecting the degree of pigmentation while (B) represents a quantitative measurement based on Essar *et al.*, (1990) calculation. In both cases, 3-hydroxy fatty acids led to a dose-dependent reduction in pyocyanin production. The LB agar results were similar for all repeats.

The treatment of *Pseudomonas* cells with 3-hydroxy fatty acids did not alter their outer ultrastructure i.e. causing cell walls to collapse or change their appearance (degree of smoothness

or roughness) as treated cells appeared similar to non-treated cells during SEM examination (Figure 5). Moreover, any significant size differences between treated cells and non-treated cells (Table 1) were not observed. When examining the inner ultrastructure of cells using Nano-SAM (Figure 6), we could differentiate treated cells from non-treated cells. To be specific, non-treated cells had indentations that were visible at the depth of 60 nm into the cell, after etching thin slices off at a sputter rate of 15 nm/min, that were not present in drug-treated cells. However, more cells have to be etched in order to draw concrete conclusions on the inner appearance of examined cells. At the moment, it cannot be deduced what these indentations represent. However, it is clear that treatment of cells revealed an altered internal organisation of cellular components – which may result in cells expressing a different physiological response.

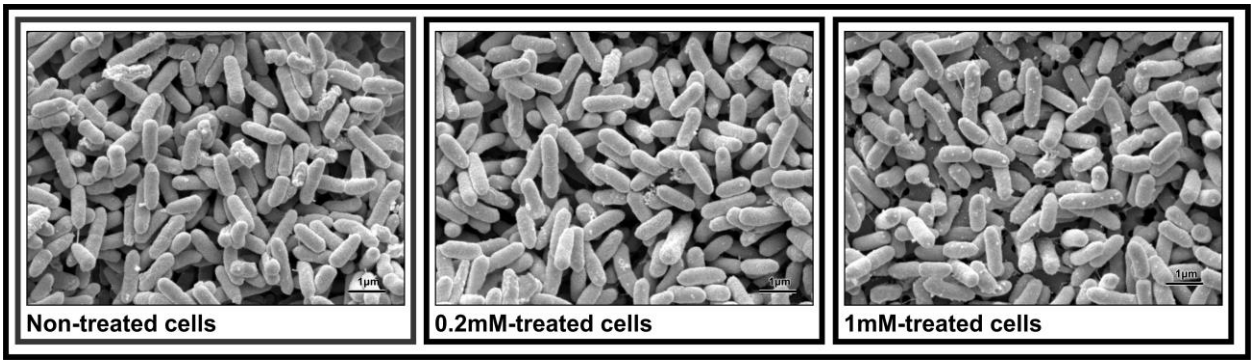

Figure 5. The effect of 3-hydroxy fatty acids on the outer ultrastructure of *Pseudomonas* cells. There were no significant differences between cells across the different experimental conditions i.e. no different in the degree of smoothness or roughness as well as cell size (length and width).

Table 1. The effect of 3-hydroxy fatty acids on *P. aeruginosa* cells. The figures represent averages of 100 measured cells per experimental condition. Cells were randomly selected from images taken from different positions on SEM stubs.

Experimental condition		**Cell size**	
Cell description	**Drug concentration**	**Length (µm)**	**Width (µm)**
Non-treated cells	0 mM	1.08 (+/- 0.0049)	0.37 (+/- 0.0005)
Treated cells	0.2 mM	1.10 (+/- 0.0055)	0.40 (+/- 0.0008)
Treated cells	1 mM	1.15 (+/- 0.0056)	0.39 (+/- 0.0008)

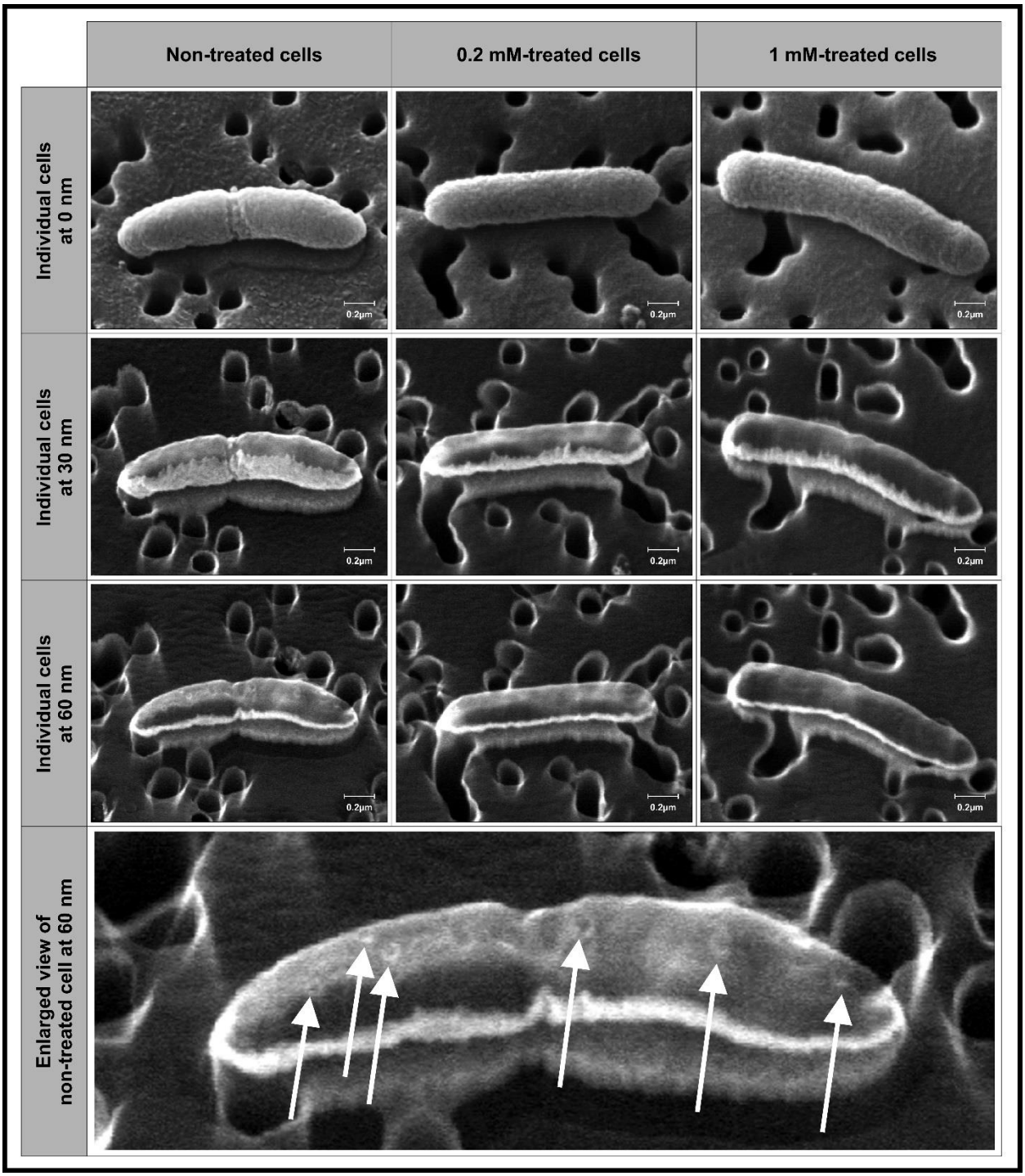

Figure 6. The effect of 3-hydroxy fatty acids on the inner ultrastructure of *Pseudomonas* cells. Non-treated cells revealed indentations inside cells at the depth of 60 nm following etching. At the moment, it is not possible to assign what these indentations represent. Nonetheless, these indentations are features that were absent in treated cells.

3-hydroxy fatty acids alter *Pseudomonas* membrane function

Membranes are important cellular components that regulate transportation in and out of the cell, and are also a site for recycling cofactors in respiring microbes like *Pseudomonas*. Given the chemistry of our test drug i.e. a saturated fatty acid molecule, it is possible that it will be incorporated into the membrane bilayer, and in turn, will lead to a more rigid bilayer, which is indicative of loss of membrane function. Under normal physiological conditions, unsaturated fatty acids give rise to a bilayer that is fluid and flexible, thus allowing for free flow of molecules and unimpaired functioning of integral proteins (Lee, 2004; Eyster, 2007). In this study, treated cells were unable to traffic significant amounts of adenylate kinase into the extracellular environment when compared to non-treated cells (Figure 7), and this may be an expression of a more rigid bilayer.

Furthermore, an increment in the dosage of the test drug also led to a dose-dependent accumulation of ROS (Figure 8). Thus accumulation of ROS may be as a result of a decrease in the freedom of movement of molecules involved in the cytochrome system (which are responsible for recycling cofactors and shuttling electrons to oxygen), in the resultant rigid bilayer (Horsfall and Cowling, 1979).

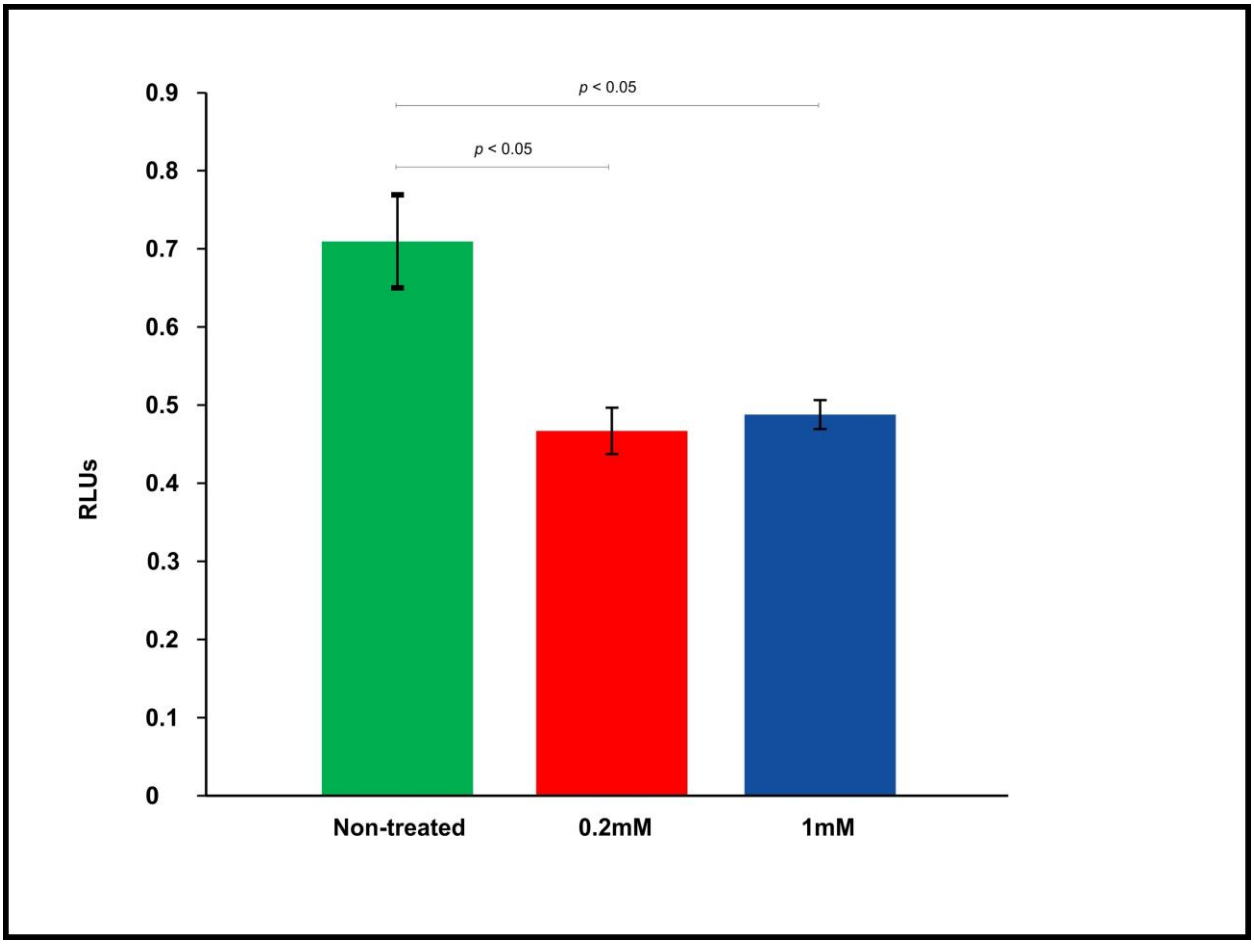

Figure 7. The effect of 3-hydroxy fatty acids on membrane function. Treatment of cells resulted in the inability of cells to traffic intracellular metabolites into the extracellular environment following a Toxilight® Bioassay.

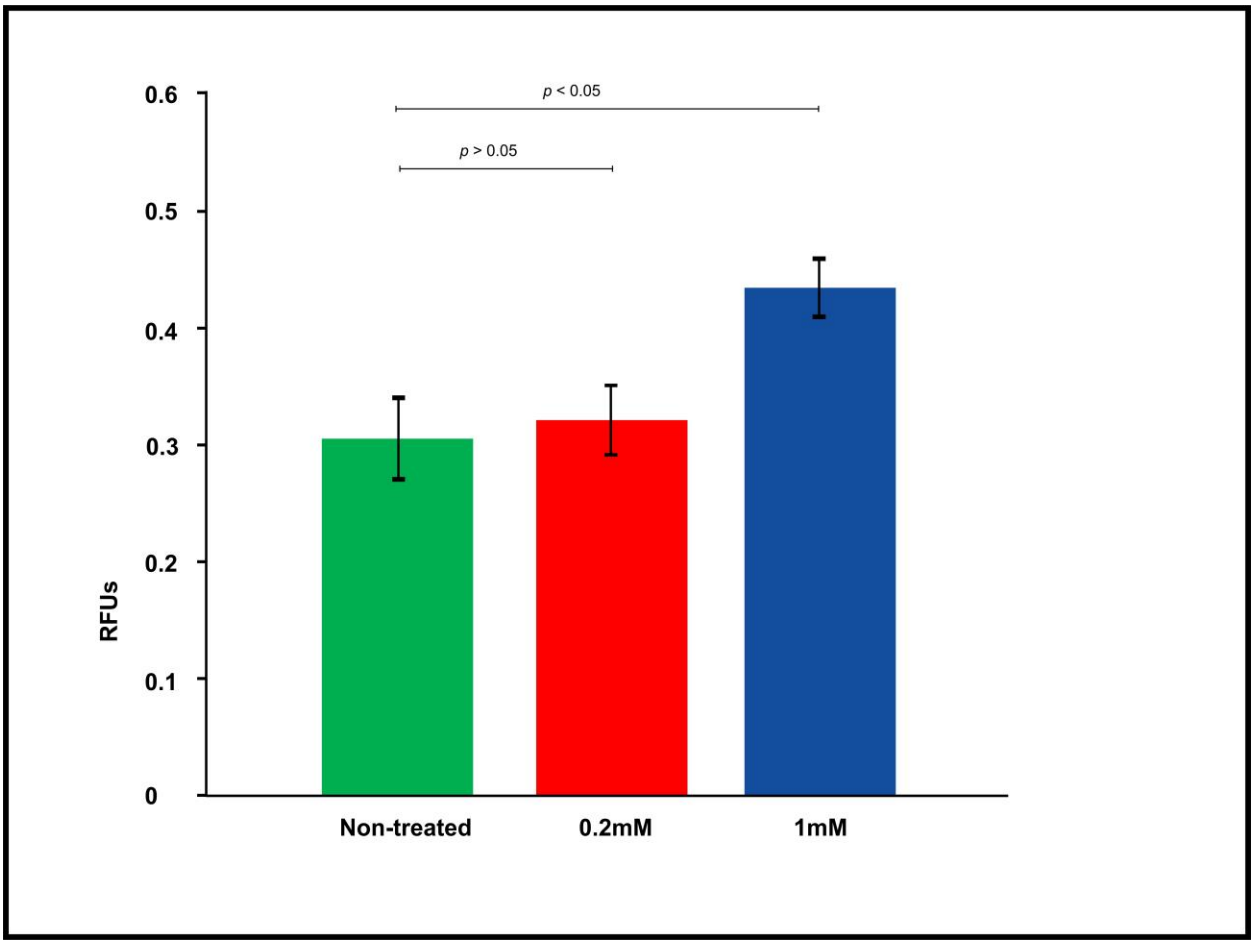

Figure 8. The effect of 3-hydroxy fatty acids on reactive oxygen species (ROS). An increment in the dosage of the test drug led to a dose-dependent accumulation of ROS in drug-treated cells. This dose-dependent accumulation may be a further indication of loss of membrane function.

In summary, microbes have evolved to colonise diverse niches ranging from the soil to the physical confined spaces of the human body. In these niches microbes rarely occur as homogenous communities, rather as mixed communities. Importantly, the growth of each community (i.e. ability to survive and flourish) is dependent upon the available space as well as materials in the environment it occupies. Thus, the latter sets up a stage for co-existing microbial communities to engage in antagonist interactions in order to appropriate territorial dominance

(Hunter, 2006; Comolli, 2014). In this regard, each microbial community secretes secondary metabolites such as anti-microbial agents, into the shared environment, to inhibit growth of competing communities. The outcome of such antagonist interactions is dynamic, and is never bias in favour of one community, as there may be other intermediaries involved. For example, during an infection, the body also responds to the collective behaviour of colonising cells, which may assist in determining the constitution of surviving microbial species (Comolli, 2014). Another factor is the quality of defensive armour one microbial population can deploy to protect itself i.e. a hard casing like a cryptococcal capsule. The capsule is reported to shield internalised cryptococcal cells against the chemical assault that is exerted by anti-microbial agents inside phagosomes (Voelz and May, 2010). The latter may, in part, explain how cryptococcal cells (as shown in this study) were able to withstand the chemical assault exerted by *Pseudomonas* cells, which have previously been reported to inhibit growth of cryptococcal cells (Rella *et al.,* 2012).

In the current study, it has been shown that cryptococcal 3-hydroxy fatty acids possess an anti-*Pseudomonas* quality, wherein further demonstrate that these molecules can inhibit *Pseudomonas* cells through impairing *Pseudomonas* membrane function. And for respiring cells like *Pseudomonas*, the membrane is critical to their survival. The idea that fatty acids and in particular cryptococcal 3-hydroxy fatty acids, may possess antimicrobial property is reasonable. Previously Sjogren *et al.,* (2003) documented that 3-hydroxy fatty acids i.e. 3-OH 10:0, 3-OH 11:0, 3-OH 12:0 and 3-OH 14:0, secreted by lactic acid bacteria displayed anti-microbial properties against a number of fungal species belonging to *Aspergillus, Penicillium, Kluyveromyces, Pichia* and *Rhodotorula*. Interestingly these authors argued that lactic acid bacteria secreted 3-hydroxy fatty acids to eliminate these contaminating fungal species, in the

bio-preservation of fermented products. Towards this end, it is conceivable that these molecules would also promote the survival of *C. neoformans* UOFS Y-1378 in nature.

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CHAPTER 3:

MICROBE-TO-HOST INTERACTIONS

This study has been independently performed by the candidate, and some parts of this study will be submitted to the journal "Journal of Infectious Diseases" for publication. As a result, repetition of some information could not be avoided.

3.1 ABSTRACT

The role of microbial 3-hydroxy fatty acids as mediators of the immune response is well documented, however not those of cryptococcal origin. Towards this end, we sought to determine if these molecules could subvert the course of immunological development following a macrophage-challenge study. A two-dimensional gel electrophoresis revealed that when macrophages were challenged with 0.2 mM of 3-hydroxy C9:0 (a 3-hydroxy fatty acid that is produced by *C. neoformans* UOFS Y-1378 cells), they yielded a protein spot that was absent on gels of untreated macrophages. Following in-gel digestion of the spot, this protein extract was identified using MALDI-ToF mass spectrometry. After matching three peptides (which resulted in a MOWSE score above 38), the protein extract was successfully identified as alpha-2-HS-glycoprotein. This protein is critical in countering the effects of opsonic pathogen-associated molecular patterns that lead to an innate pro-inflammatory response in order to maintain immunological homeostasis. This study is a first proteomics analysis of macrophage proteins that are activated by cryptococcal 3-hydroxy C9:0, wherein these molecules seem to play a role in diminishing the capability of the immune response to cryptococcal cells.

Key words: 3-Hydroxy fatty acids, 3-hydroxy C9:0, Alpha-2-HS-glycoprotein, *Cryptococcus*, Immune response.

3.2 INTRODUCTION

Cryptococcus (*C.*) *neoformans* has emerged as a major disease-causing fungal pathogen, and this is mainly due to a rise in the number of people with immunosuppressive conditions such as AIDS, and those that undergo modern medical interventions like organ transplantation (Levitz and Boekhout, 2006; Brown *et al.*, 2014a, b). More than one million cases of cryptococcal infections are reported annually (across the world), with the highest number of cases recorded in Sub-Saharan Africa (Park *et al.*, 2009). Cryptococcal infections usually begin with inhalation of airborne infectious fungal cells from the environment, which can then lodge within the alveolar space (Casadevall and Perfect, 1998). Here, the invading fungal cells are acted upon by macrophages (Voelz and May, 2010). Typically, a well-functioning immune system is capable of resolving the infecting cells. However, in immunocompromised persons, the fungal cells can subvert the course of immunological development (Johnston and May, 2013). Towards this end, the fungal cells can enter macrophages and use them for dissemination purposes in a so-called "Trojan-horse" model in order to reach the brain (Ma *et al.*, 2006). Upon reaching the brain, the cells can exit macrophages – importantly without invoking an immunological response (Voelz and May, 2010), and cause an often-deadly inflammatory condition of the brain (Dromer and Levitz, 2011). Under normal physiological conditions, T-helper 1 derived pro-inflammatory modulators such as interferon activate macrophages in order to clear infecting or invading cells (Boulware *et al*., 2010).

Conventionally, the capsule has been credited with protecting *C. neoformans* cells from being phagocytosed (Kozel and Gotschlich, 1982). Interestingly, when phagocytosed, the components of the capsule are shed, which can eventually manipulate the behaviour of immune

cells and associated immune modulators. More to the point, the capsule, mediates the evasion of immuno-processing by down-regulating inflammatory cytokines, depleting complement components, and reducing antigen-presenting mechanisms of macrophages, among other mechanisms (Retini *et al.,* 1996; Vecchiarelli, 2000; Vecchiarelli *et al.,* 2003). In some cases, the capsular components are not shed but rather directly trafficked to the extracellular environment of cryptococcal cells (Rodrigues *et al.,* 2007; Wolf *et al.,* 2014), where they can still exert deleterious effects on immune cells (Retini *et al.,* 1996; Vecchiarelli, 2000; Vecchiarelli *et al.,* 2003). An example of such trafficked compounds are 3-hydroxy fatty acids, which are closely associated with cryptococcal capsules (Sebolai *et al.,* 2007) and are trafficked to the extracellular environment of cells through spiky protuberances of shedding of capsules in an aspirin-sensitive release mechanism (Sebolai *et al.,* 2008).

3-Hydroxy fatty acids are regarded as secondary metabolites, which are thought to be produced through an enzymatic pattern that is similar to mitochondrial beta-oxidation, however incomplete (Venter *et al.,* 1997; Sebolai *et al.,* 2012). Under normal physiological conditions, these molecules rarely occur in significant quantities (Jones *et al.,* 2011). The latter suggests that cells produce these secondary metabolites for the purpose of promoting their own survival and/or pathogenesis. It is interesting to note that 3-hydroxy fatty acids have been shown to be critical to the pathogenesis of a number of medically important microbes. For example, 3-hydroxy fatty acids constitute part of Gram-negative bacteria's lipopolysaccharide (LPS), which is important in causing an upsurge in inflammatory cytokines during sepsis development, and mycolic acids (3-hydroxy fatty acids with long alpha alkyl branched chains) that can shield *Mycobacterium* cells against the chemical assault that is exerted by bactericidal agents inside phagosomes (Vander

Beken *et al.*, 2011). Additionally, in *Candida albicans*, 3-hydroxy fatty acids serve as a substrate for mammalian cyclooxygenases leading to production of a more potent class of prostaglandins during host infection (Ciccoli *et al.,* 2005). However, the immunological function of cryptococcal 3-hydroxy fatty acids has not been elucidated. Towards this end, the current study aimed to test the effect of this molecule, at estimated physiological concentrations (Madu *et al.*, 2015), on murine macrophages.

3.3 MATERIALS AND METHODS

Strain and cell line used, cultivation and standardisation

The fungal strain, *C. neoformans* UOFS Y-1378, was maintained on yeast-malt-extract (YM) agar (3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone, 10 g/l glucose, 16 g/l agar; Merck, South Africa) at 30ºC for 48 h. The murine macrophage cell line, RES234353453 (ScienCell Research Laboratories, Inqaba Biotec, South Africa), was grown on DMEM supplemented with 10% foetal bovine serum (FBS) (Biochrom AG, Germany), 2 mM of L-glutamine (Sigma-Aldrich, South Africa), streptomycin (20 µg/ml; Sigma-Aldrich) and penicillin (20 U/ml; Sigma-Aldrich) at 5% CO_2 and 37ºC. The macrophages were grown for 16 h before use according to the supplier's instructions. For cryptococcal cells, a loopful of cells was taken from a 48 h-old YM agar plate and grown in a 250 ml conical flask containing 100 ml of YNB broth (6.7 g/l; Difco Laboratories, United States) supplemented with 4% (w/v) glucose (Merck) at 30ºC for 48 h while agitating at 160 rpm. In light of anticipated co-culture experiments, cryptococcal cells were standardised to 1 x 10^6 cells in 10 ml of sterile phosphate buffered

solution (PBS; Oxoid, South Africa) while macrophages were standardised to 1 x 10^5 in 10 ml of fresh DMEM.

Visualisation of capsule shedding: Scanning electron microscopy (SEM)

In order to obtain ultrastructural detail of cells shedding their capsules, 1 ml suspension of 48 h-old cryptococcal cells in YNB broth (prepared as mentioned above) was used as material for scanning electron microscopy (SEM). The material was prepared according to the method of van Wyk and Wingfield, (1991). The material was chemically fixed using sodium-phosphate-buffered 3% glutaraldehyde (Merck) and similarly buffered osmium tetroxide (Merck) followed by dehydration in a graded ethanol (Merck, South Africa) series, critical-point dried (Bio-Rad Microscience Division, England), mounted on stubs, and sputter coated with gold using a SEM coating system (Biorad Microscience Division). Preparations were examined using a Shimadzu Superscan SSX 550 scanning electron microscope (Japan). Three replicate slides were prepared and examined.

Visualisation of *Cryptococcus*-macrophage interactions: Light microscopy and confocal laser scanning microscopy (CLSM)

To visualise interactive moments between cryptococcal cells (with shedding capsules) and macrophages, 100 µl of macrophages (1 x 10^5 cells per ml in DMEM) were seeded into wells of a chamber slide (Nunc® Lab-Tek® II Chamber Slide™ system; Sigma-Aldrich) and allowed to adhere for 2 h in the presence of 5% CO_2 at 37ºC. Next, 100 µl suspensions of cryptococcal cells (1 x 10^6 cells per ml in PBS) were added to the same wells. All cells were allowed to interact for additional 2 h (5% CO_2 at 37ºC) after which the wells were aspirated. Glutaraldehyde (Sigma-

Aldrich; 2.5% in PBS) was then added to wells to fix adhered cells for 1 h. The wells were again emptied and chambers dismantled before examining the slides with a Nikon TE 2000 light microscope (Japan).

To visualise macrophages with internalised cryptococcal cells, the phagocytosis stain, pHrodo^{TM} Green Zymosan A BioParticles (Life Technologies, United States) was used. The stain only fluoresces when excited at acidic pH, such as inside a phagosome. Cryptococcal cells were standardised to 1 x 10^6 cells per ml in PBS (which has a neutral pH) and stained (1 µl of stain : 999 µl of cells) in 1.5 ml plastic tubes for 2 h at room temperature while slowly agitating. At the same time, 100 µl suspensions of macrophages (1 x 10^5 cells per ml in DMEM) were transferred to wells on a chamber slide and allowed to adhere for 2 h in an incubator (CO_2 and 37^{o}C). Next, cryptococcal cells were washed with PBS, spun down and suspended in 1000 µl of sterile PBS. Next, suspensions of cryptococcal cells (100 µl) were transferred to the same wells. All cells were allowed to interact for 2 h. The interacting cells were fixed likewise with 2.5% glutaraldehyde for 1 h. The fixative was then aspirated. An antifade compound, 1,4-diazabicyclo[2.2.2]-octane (Sigma-Aldrich), was added to the slide (after dismantling its chambers) before viewing using a confocal laser-scanning microscope (CLSM; Nikon TE 2000; Tokyo, Japan). Three replicate slides were prepared and examined.

3-Hydroxy fatty acid extraction and analysis

As macrophages were going to be challenged with commercial 3-hydroxy fatty acids, it was prudent to determine if the test macrophages could be a source of 3-hydroxy fatty acids. Towards this end, lipids were extracted from 16 h macrophage cultures using the Folch method (Folch *et*

al., 1957). In brief, 2 ml of culture media (containing cells) was transferred to a 15 ml centrifuge tube (Becton-Dickinson Labware, United States) following which 2 ml of methanol-chloroform (HPLC-grade) solution (Merck, South Africa; 1:1, v/v) was added. The suspension was vortex mixed and allowed to stand for 20 min. Thereafter, distilled water (2 ml) was added to the above solution and allowed to stand for a further 20 min. The 3-hydroxy fatty acid fraction was collected from the chloroform layer following centrifugation (13000 *g* for 15 min), and was dried under a stream of nitrogen. The extracted lipids were then analysed on a ABSCIEX 3200 QTRAP hybrid triple quadrupole ion trap mass spectrometer (Toronto, Canada) with an Agilent 1200 SL HPLC stack as a front end. All data acquisition and processing was performed using Analyst 1.5 (ABSCIEX) software. Twenty microliter of each extracted sample was separated on a C18 (50mm x 4.6mm, XDB-C18, Agilent) column at a flow rate of 300 µl/min using an isocratic 90:10 [MeOH/0.1% formic acid: H_2O/0.1% formic acid (Merck, South Africa)] solvent composition for a total 3 min analysis time in positive mode. During initial optimization method, it was found that the analyte precursor ionizes in both positive and negative mode on this instrument but yielded better MRM transitions in positive only mode. Eluting analytes were ionised by electrospray in the TurboV ion source with a 400^{o}C heater temperature to evaporate excess solvent, 20 psi nebuliser gas, 20 psi heater gas and 20 psi curtain gas and the ion spray voltage was set at 5500 V. To analyse the samples, a targeted Multiple Reaction Monitoring (MRM) workflow was performed. The targeted analyses of 3-hydroxy nonanoic acid were performed using five MRM transitions [175.1>139.3 (quantifier); 175.1>97.2; 175.1>55.1; 175.1>69.1; 175.1>121.1 (qualifiers)]. The peak area on the chromatogram generated from the first and most sensitive transition was used as the quantifier while the other transitions were used as qualifiers. The qualifiers serve as an additional level of confirmation for the presence of the

analyte. The retention time for these two transitions needs to be the same. The EIC from the quantifier transition is shown in the results section and is compared to that of the analytical 3-hydroxy fatty acid standard viz. 3-hydroxy C9:0, which was obtained from Laradon Fine Chemicals (Sweden). This was done in triplicate. The analytical compound (3-hydroxy C9:0; obtained from Laradon Fine Chemicals (Sweden)), was prepared by complete dissolution in water (at ambient temperature and pressure) to yield a 2 mM stock solution.

Identification of protein(s) that influence the functioning of macrophages

Standardised 100 µl suspensions of macrophages (1 x 10^5 in 10 ml of DMEM) were seeded into microtitre plate wells (Greiner Bio-One, Germany) and then challenged with 100 µl of 3-hydroxy fatty acids i.e. 0.2 mM of 3-hydroxy C9:0 for 6 h while incubating at 5% CO_2 and 37ºC, in order to determine if these molecules influence the functioning of macrophages. In addition, macrophages were separately challenged with 100 µl of LPS (Sigma-Aldrich) standardised to 100 ng/ml for 6 h at 37ºC in the presence of 5% CO_2. The LPS was included for comparison purposes as it is a known pro-inflammatory modulator of macrophages (Aung *et al.,* 2006), and it has 3-hydroxy fatty acids (3-OH C14:0) as part of its structural make up (Rietschel *et al.,* 1994). Subsequently, macrophage lysates were obtained and subjected to two-dimensional (2D) SDS (sodium dodecylsulfate)-polyacrylamide gel electrophoresis (PAGE) coupled to a MALDI-ToF MS technique according to the method previously detailed by Ndimba *et al.,* (2003). In brief, after the 6 h incubation period, cells (from respective experimental conditions i.e. those treated with 3-hydroxy fatty acids and those with LPS) were transferred into 1.5 ml plastic tubes. Immediately after the cells were washed twice in PBS, harvested, and suspended in a 50 mM Tris-EDTA buffer solution, pH 7.4 (Sigma-Aldrich). The cells were subsequently ruptured using

the French press cell disrupter (Constant Cell Disruption Systems, United Kingdom) at a pressure of 36 kpsi. The proteins were precipitated and washed using cold 80% acetone. About 400 µl of the protein sample and 1600 µl of cold 80% acetone (1:5 ratios) were added into 2 ml Eppendorf tubes (Merck). The solutions were incubated overnight at -20ºC to precipitate proteins. Following protein precipitation, the samples were centrifuged at 13000 x *g* for 15 min and the supernatants were discarded. The pellets were then air dried at room temperature for 5 min and re-suspended in 100 µl of urea buffer [9 M urea, 2 M thiourea and 4% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)] followed by protein concentration determination using Bradford Assay (Bradford, 1976). Thus, equal amounts of the protein were then ready to be loaded onto the gel.

Two-dimensional gel electrophoresis of macrophage protein extracts was performed using 7 cm, ReadyStrip^{TM} immobilised pH gradient (IPG) strips of linear pH of range 3 to 10. The 2D gels were stained with Coomassie Brilliant Blue and scanned using a Pharos FX PLUS^{TM} (Bio-Rad). Furthermore, gel spots were de-stained with 200 µl of 50% acetonitrile (Sigma-Aldrich)/ 25 mM ammonium bicarbonate (Sigma-Aldrich) until clear. Samples were then dehydrated and desiccated with 100 µl acetonitrile (ACN; Sigma-Aldrich) followed by overnight trypsinisation at 37°C with 50 ng Trypsin solution (Promega). Peptides were then extracted with 10 µl 30% ACN; 0.1% trifluoroacetic acid (TFA; Sigma-Aldrich) for 30 min at room temperature with occasional vortex mixing. Mass spectrometry was used to identify selected protein spots that were well resolved and reproducible (across the two biological replicates). In brief, MALDI-TOF MS and LIFT MS/MS were performed using a UltrafleXtreme MALDI ToF/ToF system (Bruker Daltonics, Bremen, Germany) with instrument control through Flex

control 3.4. Approximately 0.5 µl of each digest was spot onto an 800 µm MALDI Anchor chip target plate for peptide mass fingerprinting. Peptides were ionized with a 337 nm laser and spectra acquired in reflector positive mode at 28 kV using 500 laser shots per spectrum with a scan range of *m/z* =700 to *m/z* = 4000. Spectra were internally calibrated using peptide calibration standard II (Bruker Daltonics, Bremen, Germany). This calibration method provided a mass accuracy of 50 ppm across the mass range 700 Da to 4000 Da. Peptide spectra of accumulated 3,000 shots were automatically processed using ProteinScape software (Bruker Daltonics, Bremen, Germany). Database interrogation was performed with the Mascot algorithm using the Swissprot database on a ProteinScape 3.0 workstation. One search parameter was performed i.e. Taxonomy-All entries, enzyme-trypsin; missed cleavages-1; fixed modification-carbamidomethyl(C); variable modification-oxidation (M); precursor tolerance - 50 ppm; fragment tolerance -0.7 Da. Candidate protein matches were considered as positive identification if: 1) their molecular weight search (MOWSE) score is greater than 38 and 2) more than one peptide was positively identified. Protein extracts from untreated macrophages (which were prepared likewise) were included for comparison reasons.

3.4 RESULTS AND DISCUSSION

Cryptococcal cells release 3-hydroxy C9:0 via the shedding of capsules

The cells of *C. neoformans* UOFS Y-1378 are characterised by shedding capsules as depicted in Figure 1. In addition, it was previously reported that the capsules of this particular strain have spiky protuberances, which are employed as “tunnels” for releasing 3-hydroxy C9:0 into the extracellular environment of cryptococcal cells (Sebolai *et al.*, 2008).

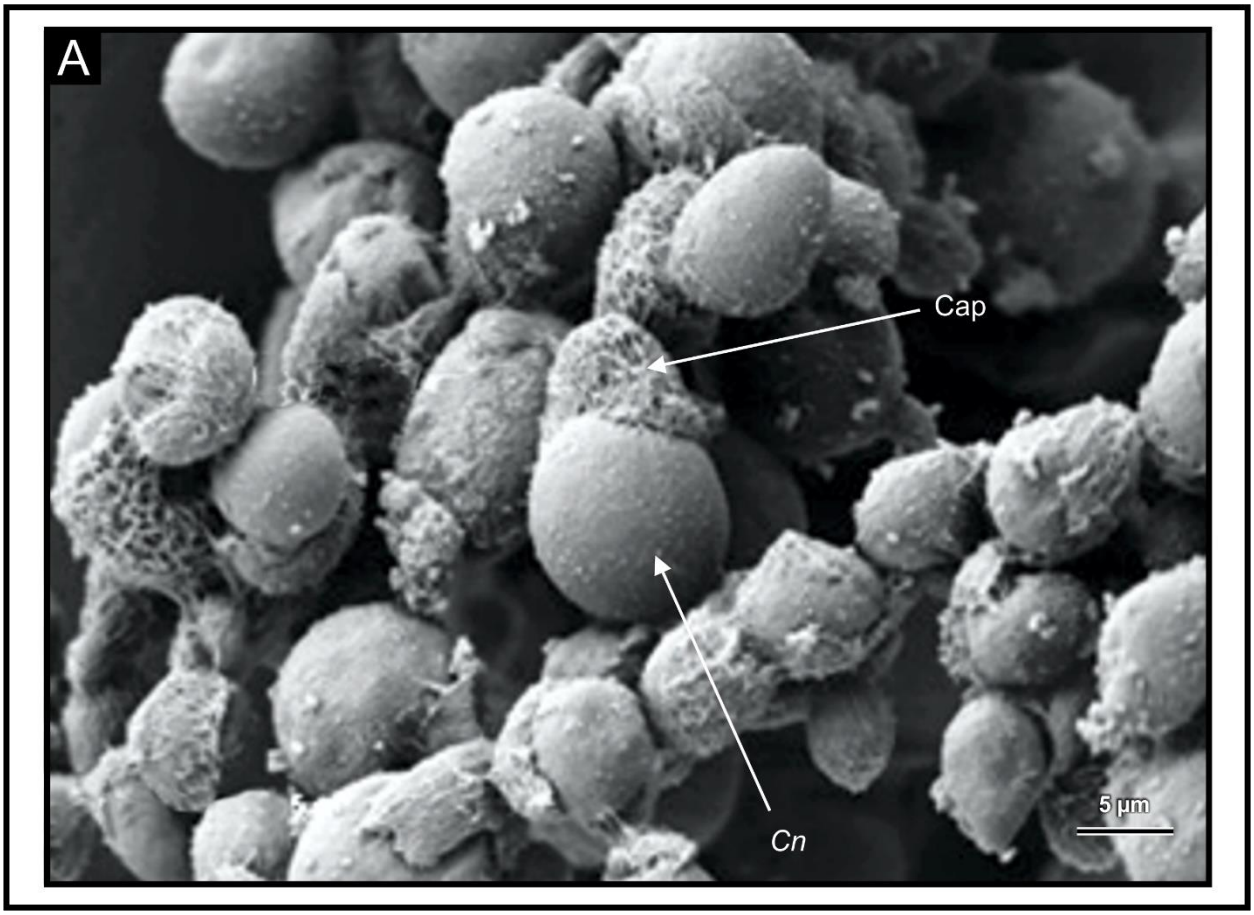

Figure 1. An SEM micrograph depicting *C. neoformans* UOFS Y-1378 cells shedding their capsules. Cap = capsule; *Cn* = vegetative body of *C. neoformans*.

Therefore, based on the above, it is reasonable to conclude that the shedding of the capsule as well as "capsular tunnels" or spiky protuberances are critical for the release of 3-hydroxy fatty acids in this cryptococcal strain. More importantly, it is hypothesised in the current study that upon release, these molecules would influence the behaviour of other cells, and in this case that of macrophages, sharing the same space as cryptococcal cells. It is clear as evidenced in Figure 2 that cryptococcal cells still maintain their capability to shed their capsules even when interacting with macrophages. It can be argued that the observed shedding facilitates the release of 3-hydroxy C9:0 and ensures that these molecules come into contact with macrophages. In

addition, the internalisation of cryptococcal cells as seen in Figure 3 is another method of introducing cryptococcal 3-hydroxy fatty acids into the internal space of macrophages. From there, these molecules can further influence the functioning of macrophages. Importantly, during such interactive moments i.e. between cryptococcal cells and macrophages, the only source of 3-hydroxy C9:0 is the fungus, and not macrophages.

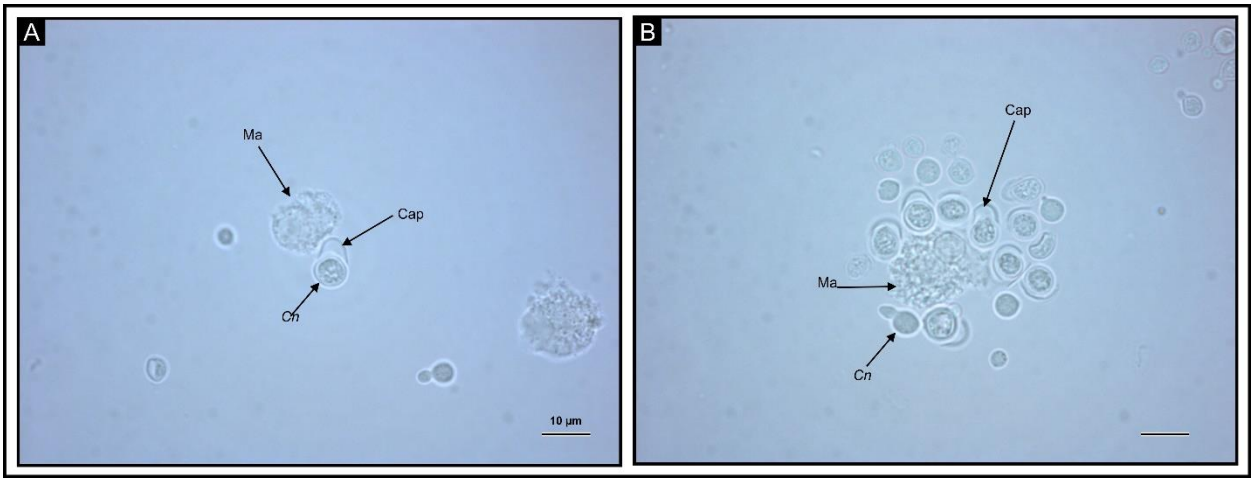

Figure 2. The visualisation of interactive moments between *C. neoformans* UOFS Y-1378 and test macrophages using a light microscope. The fungal cells are seen shedding their capsule, which in turn, facilitates the release of 3-hydroxy C9:0. Cap = capsule; *Cn* = *C. neoformans*, Ma = macrophage.

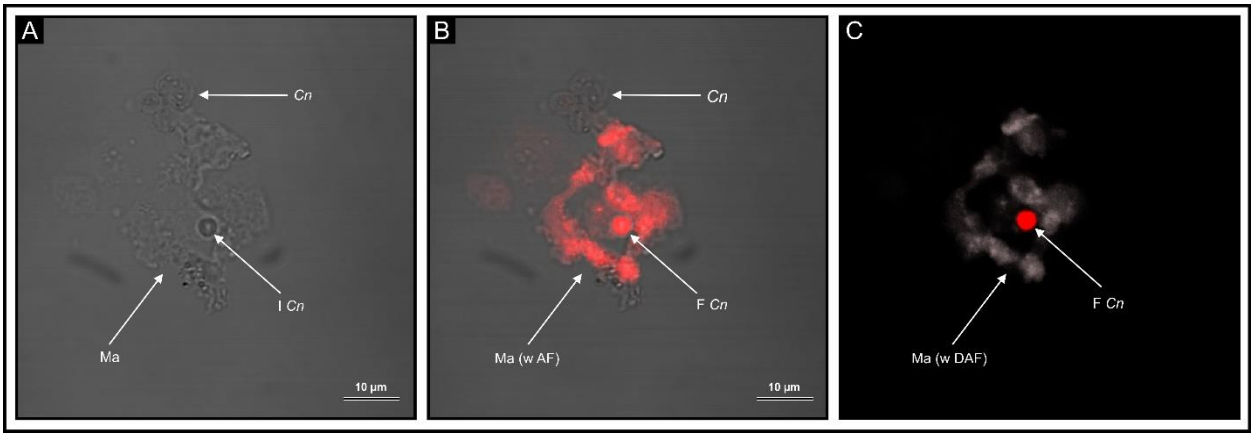

Figure 3. The visualisation of interactive moments between *C. neoformans* UOFS Y-1378 and test macrophages using a fluorescent microscope. The phagocytic stain, pHrodo^{TM} Green Zymosan A BioParticles, aided the visualisation of an internalised cryptococcal cell. In this paper, it is urged that internalisation is another method of introducing cryptococcal 3-hydroxy C9:0 into the internal space of macrophages. *Cn* = *C. neoformans*; F *Cn* = fluorescent *C. neoformans*; I *Cn* = internalised *C. neoformans*; Ma = macrophage; Ma (w AF) = macrophage with auto fluorescence; Ma (w DAF) = macrophage with dimmed auto fluorescence.

The latter is based on the inability of macrophages to yield a mass spectrum that shows the elution of our metabolite of interest (3-hydroxy C9:0) after 1.9 min when referenced against the mass spectrum of the commercial analytical standard compound i.e. 3-hydroxy C9:0 (Figure 4). This finding is important as it excludes the possibility that macrophages may produce 3-hydroxy C9:0 – because for purposes of this study, macrophages were artificially challenged with these molecules.

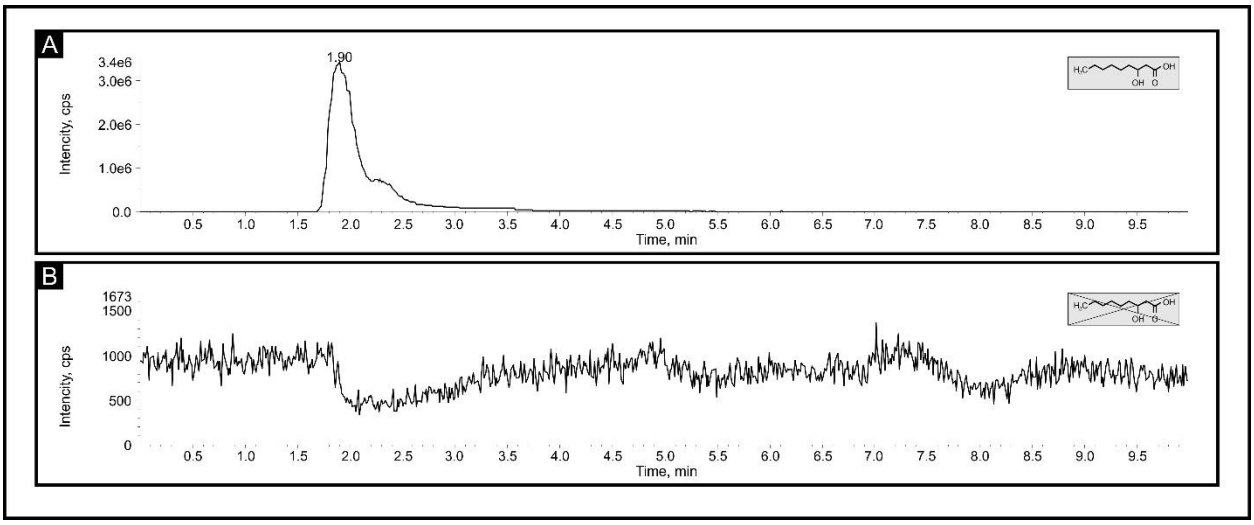

Figure 4. Determination of 3-hydroxy fatty acid production by cultures of macrophages. (A) = The EIC obtained for the analytical standard compound (3-hydroxy C9:0) showing elution of our metabolite of interest after 1.90 min. (B) = The EIC obtained for the test macrophage cell line showing no elution of 3-hydroxy C9:0 after 1.90 min, suggesting that macrophages do not produce 3-hydroxy C9:0.

3-Hydroxy C9:0 influences the functioning of macrophages

Macrophage protein extracts were separated by 2D gel electrophoresis and visualised following staining with Coomassie Brilliant Blue. A number of protein spots were observed on gels obtained from the different experimental conditions i.e. untreated macrophages, 0.2 mM 3-hydroxy C9:0-treated macrophages and 100 ng/ml-LPS-treated macrophages (Figure 5). The molecular weight of the protein spots ranged between 25 kDa and 116 kDa.

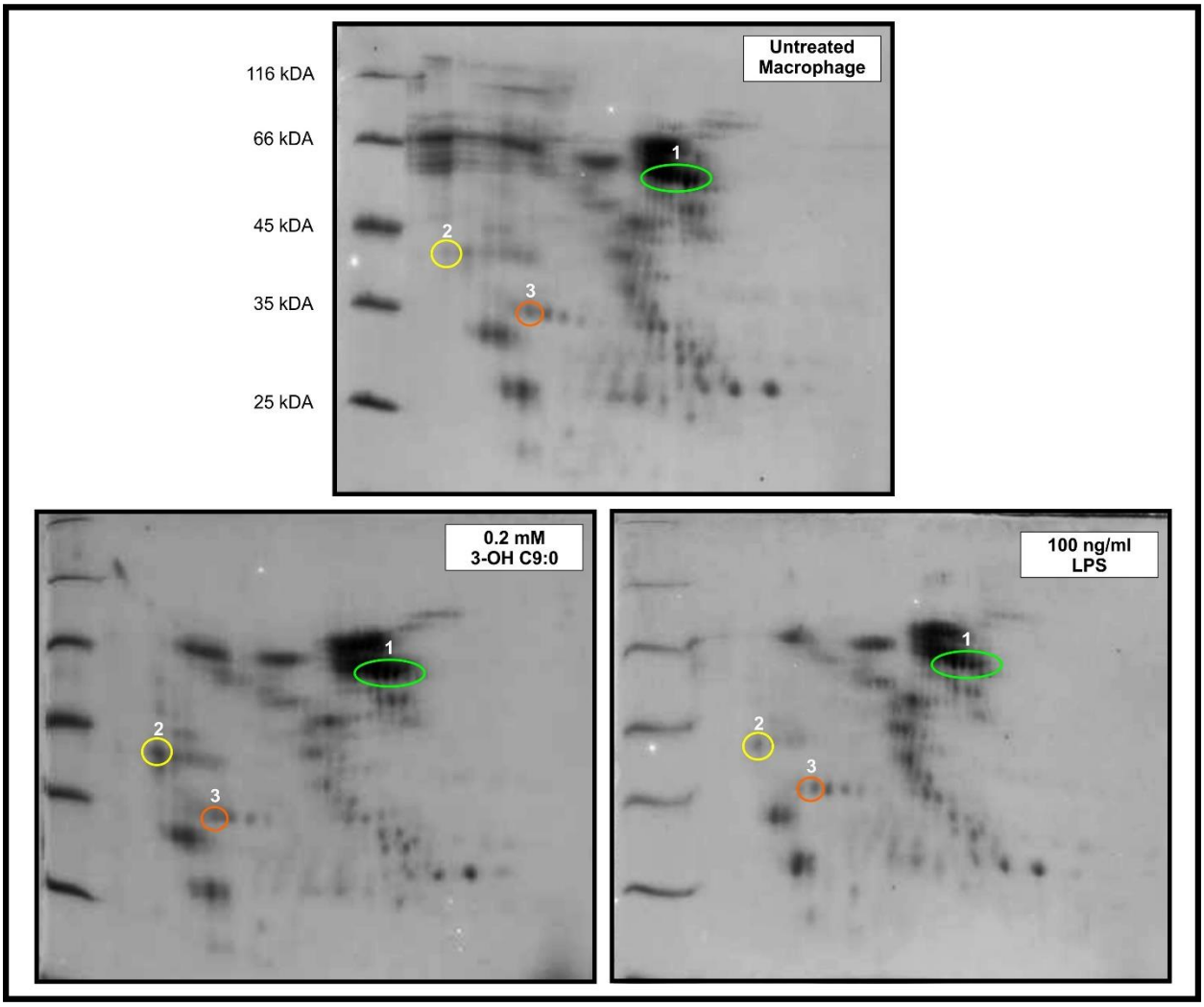

Figure 5. Two-dimensional SDS PAGE (pH 3 - 10) showing the three selected gel spots trypsinised, which were obtained from the test experimental conditions i.e. untreated macrophages, macrophages treated with 0.2 mM 3-hydroxy C9:0 or macrophages treated with 100 ng/ml LPS. These spots were subsequently subjected to MALDI-ToF mass spectrometer for identification.

Three spots were selected for identification with MALDI-ToF on the basis that: 1) spot 1 showed varying degrees of abundance on all gels, 2) spot 2 was absent on the untreated macrophage gel and showed varying degrees of abundance on treated macrophage gels, and 3) spot 3 showed varying degrees of abundance on all gels. Furthermore, spot 1 and spot 2 were acidic in nature and thus had isoelectric points that ranged between 5 and 6, while spot 3 had a pI of 9.89 (Table 1). Importantly, only one spot (i.e. spot 2) was positively identified based on satisfying the two conditions of the criteria provided in the materials and method section i.e. this spot: 1) yielded a MOWSE score greater than 38, and 2) more than one peptide, from the protein sequence, could be positively matched (Table 1). The other two spots (spot 1 and spot 3) did not satisfy the set criteria. Nevertheless, spot 1 and spot 3 were identified as serum albumin and lysophosphoslipid acyltransferase respectively (Table 1).

On the basis of the above, spot 2 was positively identified as alpha-2-HS-glycoprotein as per the obtained MALDI-ToF MS spectrum (Figure 6). Close examination of this protein's spot on the gels (obtained from the different experimental conditions) revealed that this protein has varying degrees of abundance between different experiments. The spot was more abundant when macrophages were challenged with 3-hydroxy C9:0 compared to when challenged with the LPS. However, caution should be taken when interpreting such qualitative results and a more appropriate quantitative method (such as qPCR or ELISA) should be used to assay for the abundance of alpha-2-HS-glycoprotein before concrete conclusions can be drawn. Given that this protein is crucial to the functioning of macrophages in response to infection or cellular injury (Wang *et al.*, 1998; Li *et al.*, 2011; Wang and Sama, 2012), the discussion henceforth will be limited to alpha-2-HS-glycoprotein.

Table 1. The identification of macrophage culture spot proteins using MALDI-ToF mass spectrometry.

Spot[a]	Best Match	Accession[b]	MOWSE score	pI	Matching peptides	Peptide sequence
1	Serum albumin	ALBU_BOVIN	85.97	5.78	1	K.DAFLGSFLYEYSR.R
2	Alpha-2-HS-glycoprotein	FETUA_BOVIN	193.76	5.78	3	R.GYKHTLNQIDSVK.V K.HTLNQIDSVKVWPR.R K.QDGQFSVLFTK.C
3	Lysophospholipid acyltransferase	MBOA7_HUMAN	33.80	9.89	1	R.YWNMTVQWWLAQYIYK.S

a = spot number as shown on 2D gel (Figure 5); b = accessions were obtained from SwissProt database: www.uniprot.org/

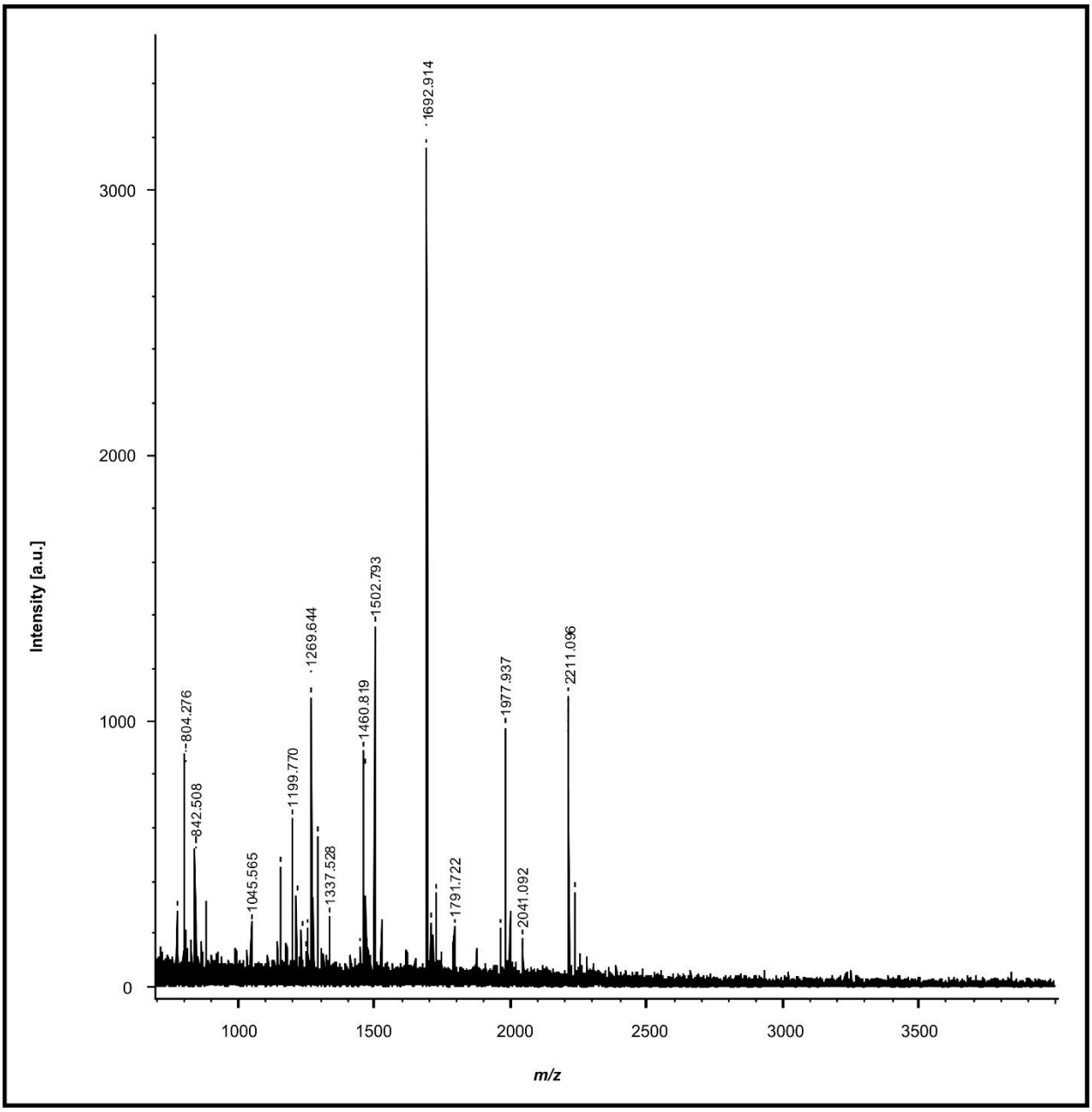

Figure 6. MALDI-ToF mass spectrum showing peptide signal from gel spot 3 (see Figure 5). This spot was successfully identified to be alpha-2-HS-glycoprotein, which is a protein that is critical in maintaining immunological homeostasis.

Recently, Wang and Sama (2012) put forward an argument that "infection and injury are two seemingly unrelated processes that often converge on common innate inflammatory responses mediated by pathogen- or damage-associated molecular patterns. If dysregulated, an excessive inflammation manifested by over production and release of pro-inflammatory mediators (e.g., TNF, IFN-γ, and HMGB1) may adversely lead to many pathogenic consequences. As a counter-regulatory mechanism, the liver strategically reprioritises the synthesis and systemic release of acute phase proteins (APP) including the fetuin-A (also termed alpha-2-HS-glycoprotein for the human homologue)". Although alpha-2-HS-glycoprotein was first discovered in liver cells, this protein has since been discovered in other cell lines including macrophages (Wang *et al.,* 1998). Taken together, the above statement clearly establishes this protein as an anti-inflammatory modulator that is critical in maintaining immunological homeostasis. To this end, it is not surprising that pathogens would deliberately secrete molecules such as 3-hydroxy C9:0 or LPS, which could alter the expression levels (either up- or down-regulate) of this protein – as per the observed varying degrees of this protein's abundance on the gels (Figure 5). It seems that 3-hydroxy C9:0 may be up-regulating this protein's expression level, which in turn, would translate into diminished capability of macrophages to resolve or clear the infecting cryptococcal cells through phagocytosis. The latter assertion is reasonable to draw when considering the recent findings documented Madu *et al.* (2015). These authors demonstrated that amoebae displayed diminished capability to phagocytose *C. neoformans* UOFS Y-1378 (a strain that naturally produces 3-hydroxy C9:0), when compared to *C. gattii* R256 and *C. neoformans* 046 (which both do not produce 3-hydroxy C9:0). In order to investigate if 3-hydroxy C9:0 may be responsible for the diminished phagocytic capability observed towards *C. neoformans* UOFS Y-1378 cells, they re-assessed the capability of amoebae

to phagocytose *C. gattii* R256 cells and *C. neoformans* LMPE 046 cells when 3-hydroxy C9:0 was artificially added (0.2 mM and 1 mM) to their culture media. Strikingly, they observed that addition of 3-hydroxy C9:0 made *C. gattii* R256 cells and *C. neoformans* LMPE 046 cells more resistant to amoebal phagocytosis in a dose-dependent manner (Madu *et al.*, 2015). Considering the above, it is clear that production of 3-hydroxy C9:0 is a trait that is maintained by *C. neoformans* UOFS Y-1378, and importantly mediates the fate of this cryptococcal strain when it is acted upon by phagocytic cells i.e. either amoeba (in the soil) or macrophages (in the body).

In summary, this study is a first proteomics analysis of macrophage proteins activated by cryptococcal 3-hydroxy C9:0. The identified protein, alpha-2-HS-glycoprotein, is a known anti-inflammatory modulator of macrophages (Wang and Sama, 2012) that is critical in determining the outcome of an infection or cellular injury in the body. The findings of this study are a further step in the process of understanding the role of this molecule in promoting the survival and/or pathogenesis of cryptococcal cells when interacting with other cell types. Towards this end, there are on-going studies in our research group that examines how these molecules may also influence the expression levels of pro-inflammatory modulators.

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DISSERTATION SUMMARY

Cryptococcus neoformans is today recognised as an important human pathogen that has arisen from a non-pathogenic terrestrial fungus. Its interactions (specifically antagonistic interactions with other microbes (such as amoeba) in its natural habitat, soil, has led to cryptococcal cells evolving elegant offensive and defensive strategies to survive in nature. Importantly, these qualities seem to be maintained and activated whenever cryptococcal cells engage in antagonistic interactions with other cell types i.e. when in competition with *Pseudomonas* cells, which is the subject of chapter two, as well as when engaging in parasitism with macrophages, which is the subject of chapter three. Towards this end, in this dissertation, special attention was given to the role of the secondary metabolite, cryptococcal 3-hydroxy C9:0, in mediating the fate of *C. neoformans* when in contact with these other cell types.

In chapter two, it was sought to explore the interactive outcome between cryptoccocal cells and *Pseudomonas* cells. Both cell types can, in their individual capacity, lead to the development of pneumonia. While the host body has optimal conditions (temperature, ambient air, etc.) to promote the growth of these two pathogens, their growth may be limited by the available nutrients within the confined space of the host lung. It is therefore reasonable to conclude that these microbes would engage in competition in order to exert territorial dominance over the other. To demonstrate this point *in vitro*, *Pseudomonas* cells were co-cultured with cryptococcal cells over a period of time. It was subsequently determined that cryptococcal cells dominated *Pseudomonas* cells. This domination manifested in a significant reduction ($p = 0.05$) in the number of *Pseudomonas* cells over a 24-h period. In order to determine if the observed domination could be as a result of the cryptococcal 3-hydroxy fatty acid, 3-OH-C9:0, negatively affecting the growth of *Pseudomonas* cells, *Pseudomonas* cells were (in a separate experiment) directly challenged with this molecule. And here it was

shown that the growth of *Pseudomonas* cells was inhibited in a dose-dependent manner by increasing concentrations of this molecule. Importantly, 3-OH-C9:0 inhibited growth of *Pseudomonas* cells, possibly leading to their death, via altering their membrane function following the incorporation of this saturated molecule into the bilayer leading to a more rigid membrane. Taken together, these findings suggested cryptococcal 3-OH-C9:0 possesses antimicrobial properties, that when secreted into the extracellular environment, negatively affect the fate of surrounding microbes.

In chapter three the role of 3-OH-C9:0 in mediating the function of macrophages was investigated. Macrophages are critical in clearing infecting or invading microbial cells through phagocytosis. However, phagocytosis is a receptor-mediated process that is governed by a balance between pro-signal molecules that promote phagocytosis and anti-signal molecules that inhibit it. Thus, it is not surprising that microbes would counter the action of macrophages by producing anti-signal molecules that would subvert the capability of macrophages to clear infecting cells. Critically, the designed macrophage-challenge study showed that 3-OH-C9:0 induced macrophages to produce the protein alpha-2-HS-glycoprotein. This protein is an anti-inflammatory modulator that is important in maintaining immunological homeostasis. This implies this protein counters the effects of pro-inflammatory modulators, which drive the T-helper 1 response in order to clear microbial infections. Furthermore, it was established that macrophages do not produce 3-hydroxy C9:0, and this highlighted that macrophages would not deliberately produce these anti-signal molecules in order to undermine their responsibility of clearing infections.

Based on the findings reported in this dissertation, a picture emerges that clearly points that 3-hydroxy fatty acids are crucial to the survival and pathogenesis of cryptococcal

cells. According to the Centers of Disease Control and Prevention, cases of disseminated cryptococcal infections remain a major cause of morbidity and mortality. Thus there are considered efforts to manage cryptococcal infections. The studies presented in the dissertation highlight 3-hydroxy fatty acid biosynthetic route as a potential target for controlling the pathogenesis of this medically important fungus. Towards this end, the usage of animals in modelling disseminated cryptococcal infections should be considered in order to clearly establish the therapeutic benefits of drugs such as aspirin, which has previously been shown to inhibit production of these molecules in a dose-dependent manner.

Key words: Anti-microbial, Anti-inflammatory modulator, 3-Hydroxy C9:0, *Cryptococcus*, Competition, Immunity, Macrophages, Parasitism, *Pseudomonas*, Survival.

VERHANDELING OPSOMMING

Cryptococcus neoformans word tans erken as ‘n belnagrike menslike patogeen wat onstaan het vanaf ‘n nie-patogeniese terrestriële fungus. Sy interaksies (veral antagonistiese interaksies) met ander mikrobe (soos amoeba) in sy natuurlike habitat, grond, het daartoe gelei dat *Cryptococcus* selle elegante aanvals- en verdedigingstrategieë ontwikkel het om in die natuur te oorleef. Dit is van belang dat hierdie eienskaape blyk behou en geaktiveer te word wanneer *Cryptococcus* selle deelneem aan antagonistiese interaksies met ander tipes selle d.i. wanneer hulle kompeteer met *Pseudomonas* selle, die onderwerp van hoofstuk twee, asook wanneer hulle deelneem aan parasitisme met makrofage, die onderwerp van hoofstuk drie. Ter nastrewe van hierdie doel, word in hierdie verhandeling veral aandag gegee aan die rol van die sekondêre metaboliet, 3-hidroksie C9:0 (3-OH-C9:0) vanaf *Cryptococcus*, in bepaling van die lot van *C. neoformans* wanner dit in kontak is met hierdie ander seltipes.

In hoofstuk twee is gepoog om die interaktiewe uitkoms tussen *Cryptococcus* selle en *Pseudomonas* selle te ondersoek. Beide seltipes kan individueel lei tot ontstaan van longontsteking. Alhoewel die gasheerliggaam optimale toestande (temperatuur, lug ens.) bevat om die groei van hierdie twee patogene te promoveer, mag hulle groei beperk word deur die beskikbare voedingstowwe binne die beperkte spasie van die gasheerlong. Dit kan dus redelikerwys afgelei word dat hierdie mikrobes sal deelneem aan kompetisie om territorial domanansie oor die ander uit te oefen. Om hierdie punt *in vitro* te demonstreer is *Pseudomonas* selle saam met *Cryptococcus* selle gekweek oor ‘n seker periode. Gevolglik is gevind dat *Cryptococcus* selle *Pseudomonas* selle domineer. Hierdie domineering het gemanifesteer as ‘n beduidende afname (p = 0.05) in die getal *Pseudomonas* selle oor ‘n tydperk van 24-h. Ten einde te bepaal of die waargeneemde dominering kan wees as gevolg van negatiewe invloed van die *Cryptococcus* 3-hidroksie vetsuur, 3-OH-C9: 0, op die groei van *Pseudomonas* selle, is die *Pseudomonas* selle (in 'n aparte eksperiment) direk blootgestel

aan hierdie verbinding. Hier is aangetoon dat die groei van *Pseudomonas* selle in 'n dosis-afhanklike wyse geïnhibeer word deur toenemende konsentrasies van hierdie verbinding. Wat belangrik is, 3-OH-C9:0 het groei van *Pseudomonas* selle geïnhibeer, wat moontlik kon lei tot hulle dood, via die wysiging van hul membraanfunksie na die invoeging van hierdie versadigde molekule in die dubbellaag, wat lei tot 'n meer rigiede membraan. Hierdie bevindinge dui saam daarop dat *Cryptococcus* 3OH-C9:0 antimikrobiese eienskappe besit, wat wanneer dit afgeskei word in die ekstrasellulêre omgewing, ’n negatiewe invloed op die lot van omliggende mikrobes gehad het.

In hoofstuk drie word die rol van 3-OH-C9:0 in die bevordering van die funksie van makrofage ondersoek. Makrofage is van kritieke belang in die opklaar van infeksie of indringer mikrobiese selle deur fagositose. Maar fagositose is 'n reseptor bemiddelde proses wat deur 'n balans tussen pro-sein molekules wat fagositose promoveer en anti-sein molekules wat dit inhibeer, gereguleer word. Dus is dit nie verbasend dat mikrobes die werking van makrofage sou teen werk deur die vervaardiging van anti-sein molekules wat die vermoë van die makrofage sou ondermyn om infekterende selle te verwyder nie. Die ontwerpte makrofaag-blootstellingstudie het getoon dat 3-OH-C9:0 makrofage geïnduseer het om die proteïen, alpha-2-HS-glikoproteïen, te produseer. Hierdie proteïen is 'n anti-inflammatoriese modulator wat belangrik is in die handhawing van immunologiese homeostase. Dit impliseer hierdie proteïen die effek van pro-inflammatoriese modulators, wat die T-helper 1 reaksie om mikrobiese infeksies op te klaar dryf, teenwerk. Verder is vasgestel dat makrofage nie 3-hidroksie C9:0 produseer nie, en dit beklemtoon dat makrofage nie doelbewus hierdie anti-sein molekules sou produseer om sodoende hul verantwoordelikheid vir die opklaar van infeksies te vermy nie. Gebaseer op die bevindinge in hierdie verhandeling, verskyn ’n beeld wat duidelik wys dat die 3-hidroksie vetsure noodsaaklik is vir

die oorlewing en patogenese van *Cryptococcus* selle. Volgens die Centers van Disease Control & Prevention, bly gevalle van verspreide *Cryptococcus* infeksies 'n groot oorsaak van morbiditeit en mortaliteit. Dus word ernstige pogings aangewernd om *Cryptococcus* infeksies te bestuur. Die studies voorgedra in hierdie verhandeling, beklemtoon die 3-hidroksie vetsuur biosintetiese weg as 'n potensiële teiken vir die beheer van die patogenese van hierdie medies belangrike fungus. Dit ten doel, moet die gebruik van diere in modellering van verspreide *Cryptococcus* infeksies oorweeg word om die terapeutiese voordele van middels soos aspirien, wat voorheen reeds aangetoon is om die produksie van hierdie molekules in 'n dosis-afhanklike wyse te inhibeer, duidelik te vestig.

Sleutelwoorde: Anti-mikrobiese, Anti-inflammatoriese modulator, 3-hidroksie C9:0, *Cryptococcus*, Kompetisie, Immuniteit, Makrofage, Parasitisme, *Pseudomonas*, Oorlewing.