The Role of Cryptococcal 3-Hydroxy fatty acids in Mediating *Cryptococcus*-amoebae Interactions

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DEDICATION

This dissertation is dedicated to my family: My lovely husband - Dr Chika Egenasi, my wonderful children - Chizaram and Chikamso Egenasi, my parents - Mr. and Mrs. Mmadubugwu, siblings and in-laws.

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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.

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Lynda Uju Madu

Candidate: M.Sc. degree

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DISSERTATION OVERVIEW

Microbial diversity is primarily driven by either genetic exchanges, even epigenetics and mutations which has led to the manifest of unique physiological qualities that are unique in some and absent in others. This has allowed some microbes to adapt and flourish in certain ecological niches while others may lack the genes that permit them to utilise some compounds. Toward this end, microbes may engage in relations wherein they can collaborate to breakdown and assimilate some compounds. As such, these microbes may co-exist in harmony. It is thus not surprising that communities that are more diverse and act in synergy tend to dominate and derive more benefit from the available resources than microbial communities that are less diverse.

However, more often than not, microbes may engage in direct competition in order to appropriate environmental advantage over other microbes. Microbes can be unforgiving and brutal when they compete and battle for survival and domination. An extreme form of competition is predation, wherein a predator extracts energy from the biomass of its killed prey. The predator also exerts territorial dominance by controlling its prey's population density. The predatory pressure can, at the same time, lead to prey developing counter mechanisms that enable them to survive in such harsh environments. This is the central question that is addressed herein in the dissertation by examining the relationship between amoeba (predator) and *Cryptococcus* (prey).

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.

CHAPTER 1

LITERATURE REVIEW

1.1 MOTIVATION

Fungi are a group of eukaryotic heterotrophs that can either be unicellular or multicellular microorganisms. They have paradoxical microbial factors that, in many respect, define human life. For example, in one instance these factors are essential for sustaining human life i.e. may be exploited to produce food or life-saving antibiotics. At the same time, some factors can terminate human life by potentiating patho-physiological processes that may result in a diseased-state and possibly death.

It thus follows that a lot of research has been conducted into understanding how microbial infection take hold and manifest a diseased-state, especially in susceptible hosts. Towards this end, many researchers have modelled these infections using animals in order to gain insights into cellular physiology and pathology. This has, in turn, revealed strategies for identifying potential drug targets. While such insight is pivotal, it is equally important to understand how a typical non-pathogenic fungus may evolve pathogenic factors. To this point, it becomes critical to study such microbes in their natural habitats and to characterise pressures (in these environments) that may select them to produce these factors – as this may also reveal novel targets for drug development. Of importance in this dissertation are the fungal species *Cryptococcus* (*C.*) *neoformans* and *Cryptococcus gattii*, which have emerged as major disease-causing microorganisms. Special attention is given to their interaction with amoebae, which is a natural predator of cryptococcal cells in the soil. The premise for studying this particular interaction is that it may shed light into how cryptococcal cells may potentially interact with macrophages,

which have been proposed to have evolved from free-living amoeba, during infections. Such information may be crucial in shedding light onto how pathogens, like *Cryptococcus neoformans*, may subvert the host's immunological response.

Thus, the purpose of this literature review is to discuss *Cryptococcus*, its natural habitat and interactions with amoebae in the soil. More to the point, how such interaction may have led to the origin of the capsule and 3-hydroxy fatty acids. The review will then conclude by examining what these lipid-based molecules are and what their biological role might be.

1.2 *CRYPTOCOCCUS NEOFORMANS*

1.2.1 Description

Kützing was the first person to use the term *Cryptococcus (C.)* when he described an organism in 1833 (Fonseca et al. 2011). This organism was subsequently shown to be an alga. The term (*Cryptococcus*) was later reserved to exclusively refer to a fungal genus by Vuillemin in 1901 (Fell and Statzell-Tallman, 1998). The first environmental isolation of *C. neoformans* was made in 1894 after its cells were isolated from fruit juice (Casadevall and Perfect, 1998). Interestingly, the same cells were separately isolated from a tibia lesion of a patient in 1894 by two German physicians namely, Busse and Buske (Casadevall and Perfect, 1998). It is because of the latter that *C. neoformans* was regarded as a pathogen.

Prior to the 1950s, cryptococcal infections were thought to be caused by one homogenous species. However, advances in serological techniques assessing antigenic differences, revealed that this “homogenous” species was rather a complex, made up of closely related species (Table 1) (Chen et al., 2010; Day, 2004). Moreover, advances in molecular techniques assessing genetic diversity, also led to the classification of this species into varieties and molecular types (Sidrim et al., 2010). While such information is critical to taxonomists; for clinicians (who are at the front line of managing cryptococcal infections) it is not meaningful. Therefore, in order to achieve nomenclatural stability that satisfies both clinicians and taxonomists, Kwon-Chung and Varma (2006) proposed that species within the complex be defined either as *C. neoformans* (serotype A, D and A-D) or *C. gattii* (serotype B and C) based on how these cells interact with immune cells, their ecological distribution and molecular properties.

Table 1. Classification of fungal species composing the *Cryptococcus neoformans* species complex (Chen et al., 2010; Day, 2004).

Species	Variety	Serotype		Ecological niche
		Non-hybrid	Hybrid	
C. neoformans	*grubii*	A	-	pigeon excreta
	gattii	B	-	eucalyptus trees
	gattii	C	-	eucalyptus trees
	neoformans	D	-	pigeon excreta
	grubii-neoformans	-	A-D	Unknown

C. neoformans cells are globose to ovoid in shape and between 2.5 µm to 10 µm in diameter Kwon-Chung (2011). In addition, cells are mainly found in a unicellular form i.e. yeast-state. However, at times, heterothallism is observed whereby compatible cells (a and α) may undergo sexual reproduction in order to rejuvenate and exchange genes leading to production of basidiospores. The conjugation of compatible cells occurs within the dikaryon, which is multicellular i.e. fungus-state (Figure 1) (Kwon-Chung, 1975, 1976).

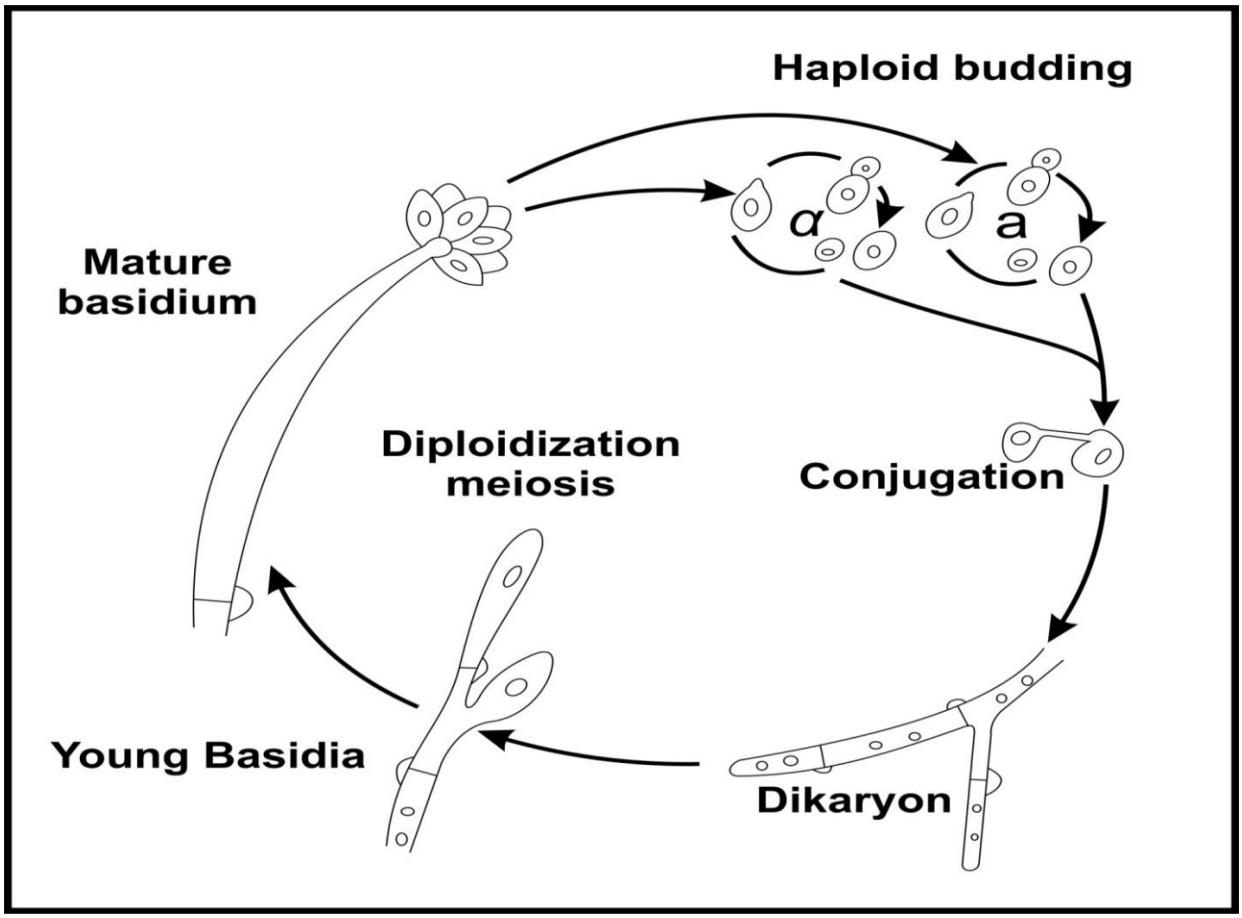


Figure 1. A pictogram illustrating two compatible mating types i.e. α and a, conjugating to form a typical basidiomycetous dikaryotic hyphae. This is then followed by the rejuvenation of genes and later, the restoration of the yeast phases. The pictogram is reproduced from Kurtzman et al. (2011).

Cryptococcal infections were not a common occurrence for most of the 20th century until 1970s. However, since the 1970s, there has an exponential increase in the number of cryptococcal infections. To a small degree, this increase in cryptococcal infections is due to an increase in the number of people undergoing modern medical interventions i.e. patients receiving cancer treatment or organ anti-rejection drugs. However, the single driver (and arguably the most important) of cryptococcal infections is HIV/AIDS (Chayakulkeeree and Perfect, 2008). According to the CDC, there are over a million cryptococcal cases reported each year and the majority of these infections occur in sub-Saharan Africa (Park et al., 2009). This region is also the epicentre of HIV infections (Levitz and Boekhout, 2006). In immunocompetent persons, cells are confined to the lungs following inhalation and cannot spread further (Hull and Heitman, 2002; Lin and Heitman, 2006). Thus, infections in healthy individuals are resolved and never cause problems. In persons with HIV (or defective immunity), infections are disseminated and somehow cells have a predilection for the brain (Casadevall and Perfect, 1998). There, the cells impair this organ's ability to reabsorb the cerebrospinal fluid. This in turn, leads to a pressure build-up within the skull (Figure 2). This resultant pressure then manifest in meningoencephalitis, which is a deadly inflammatory condition of the brain (Bose et al., 2003; Lin and Heitman, 2006).

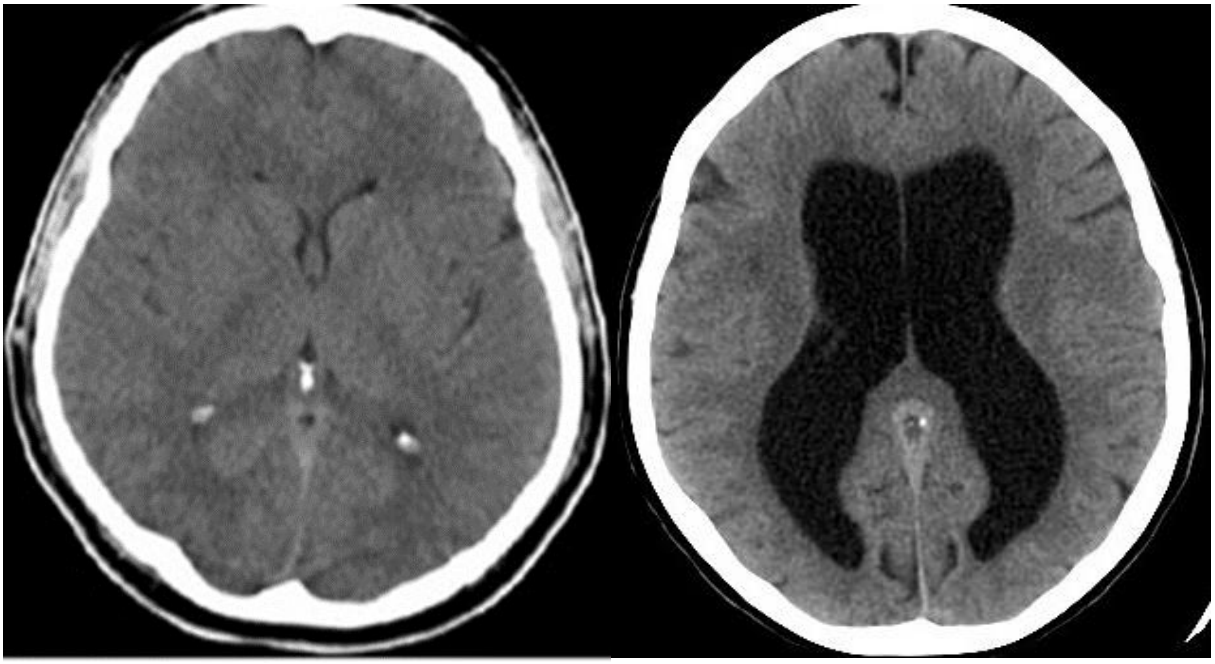


Figure 2. The pictogram depicts computerised tomography (CT) scans of two brains. The scan on the left, is that of a normal brain while that on the right is that of a diseased brain with black regions. These regions indicate the accumulation of cerebrospinal fluid, which could lead to death. Both the scans were obtained from Wikipedia and are freely available to the public. Normal brain was uploaded by Afiller - https://en.wikipedia.org/wiki/File:Brain_CT_scan.jpg and diseased brain was uploaded by Lucien Monfils - https://en.wikipedia.org/wiki/Hydrocephalus

1.3 INTERACTIONS BETWEEN *C. NEOFORMANS* AND AMOEBAE: POSSIBLE ORIGINS OF CRYPTOCOCCAL PATHOGENIC FACTORS

C. neoformans is a terrestrial fungus that is frequently found in the soil, especially in soil that is contaminated by bird droppings (Steenburgen and Casadevall, 2003). The soil, unlike air, is a physical space that is confined with boundaries. Thus, whatever nutrients

are available in that space are not boundless. Thus, to maximise the utilisation of such nutrients, microbes may work together to break down complex molecules in order to survive. Or, in some instances, they may enter into antagonistic relationships to appropriate environmental advantage over their competitors (Hunter, 2006; Comolli, 2014). An extreme form of this relationship is predation, wherein one microbe can feed on another. An example of a predator-prey relationship may involve amoebae (predator) and *C. neoformans* (prey). Amoebal species like *Acanthamoeba* are free living single-celled eukaryotes that are readily found in the soil and aquatic environments. Their size may range between 12 µm to 45 µm in cell diameter (Figure 3) (Marciano-Cabral and Cabral, 2003; Khan, 2014).

Amoebae species play a critical role in the ecosystem as they assist in controlling microbial population numbers and can also change the structure of microbial communities (Siddiqui and Khan, 2012). It has been noted that amoebae can decrease microbial population numbers by up to 60%. Importantly, this organism can recycle nutrients derived from the biomass of its prey back into the ecosystem (Siddiqui and Khan, 2012; Khan, 2014). To demonstrate this point, Sinclair and co-workers reported that soil containing amoebae and microbes are typically rich in minerals such as carbon, phosphorus and nitrogen compared to soil containing only bacteria (Sinclair et al. 1981).

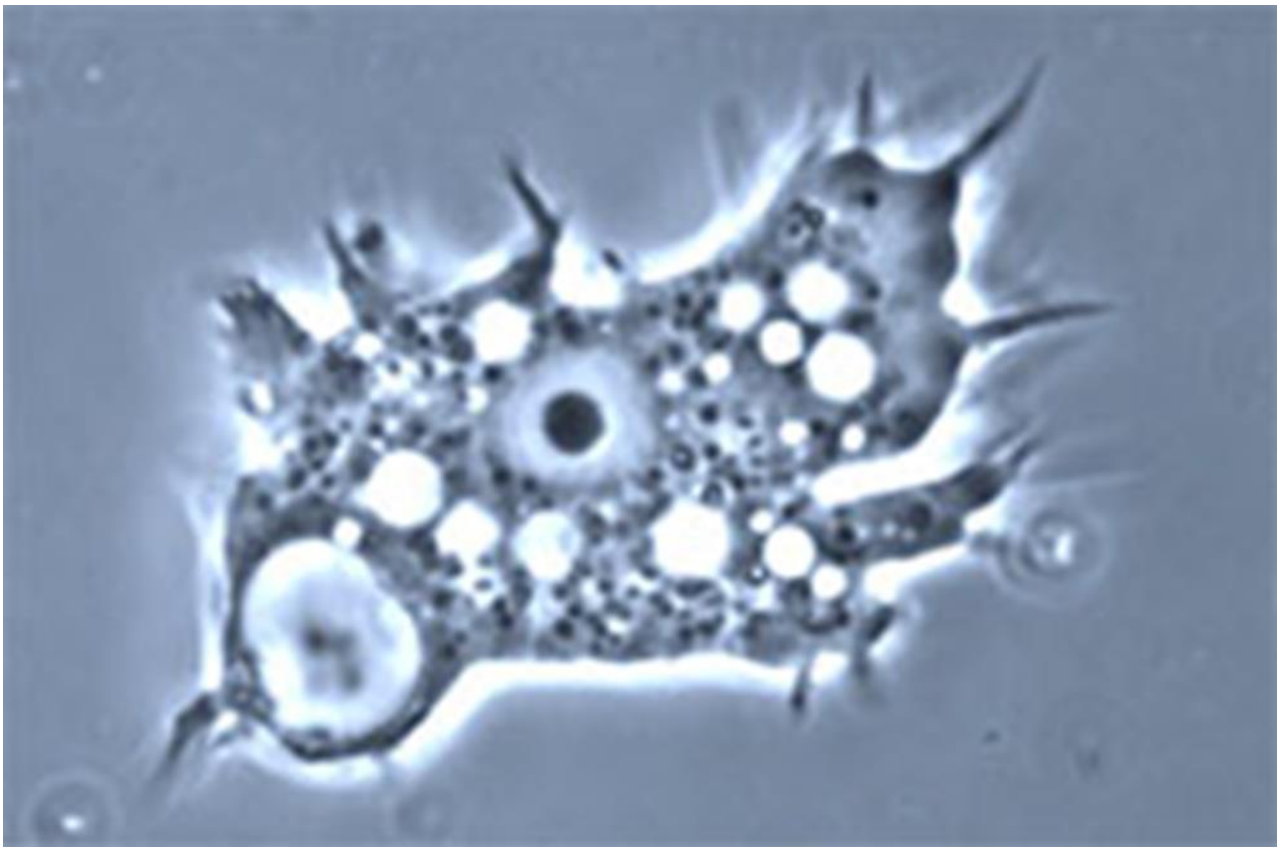


Figure 3. The morphology of *Acanthamoeba castellanii* trophozoite. The micrograph was downloaded from Carnt's page on ResearchGate and is available to the public. Credit to Carnt and Stapleton (2015).

Amoebae predate on cells via a receptor-mediated process called phagocytosis. (Voelz et al., 2009; Freeman and Grinstein, 2014; Medina et al., 2014). The term refers to a process (-osis) wherein a targeted cell (kytos) is devoured (phagein) with a specialised cell compartment. This process was first documented by Elie Metchnikoff over a century ago when he observed amoeboid cells moving within a transparent starfish larva towards an inserted rose thorn (May and Machesky 2001; Tan and Dee, 2009). The process of engulfing foreign particles, is used by a number of organisms in nature i.e.

simple organisms like amoebae (as already discussed above) to complex organisms like animals that use the process to kill invading infectious agents. Interestingly, it has been theorised that phagocytic cells such as macrophages may have evolved from free-living amoebae (Siddiqui and Khan, 2012). In brief, the process entails the recognition of the targeted cell's pathogen-associated molecular patterns (found on the cell surface) by the phagocyte's pattern recognition receptors. This is then followed by actin polymerisation, which facilitates the movement/extension of pseudopods in order to capture the targeted cell. The targeted cell is then internalised and trapped within a compartment called a food vacuole (in amoebae) or phagosome (in macrophages). The harsh environment that prevails inside the compartment should, under normal physiological conditions, be sufficient to kill an internalised cell (Hurst, 2012; Winterbourn and Kettle 2013). The killing is facilitated by oxygen-independent and oxygen-dependent mechanisms. In the oxygen-independent mechanism, the lumen of the food vacuole is acidified and is flooded with antimicrobial peptides such as amoebapore and acanthaporin, among others (Medina et al., 2014; Leippe and Herbst, 2004). These peptides kill the cells by creating pores in the cell wall of targeted cells (Herbst et al., 2002). In the oxygen-dependent mechanism, the lumen is flooded with reactive oxygen species that target the macromolecules of the internalised cell. In the end, degraded cell is excreted as waste material that is rich in mineral nutrients (Chrisman et al., 2010).

The fact that amoebae can predate on other organisms in order to support their own growth comes with evolutionary consequences. To expound this point, with the exertion of sufficient predatory pressure, prey develops microbial factor(s) in order to

subdue the deleterious effects of the pressure. In the case of *Cryptococcus,* such a factor is the capsule, which is the principal virulence factor of this organism (Kozel and Gotschlich, 1982; Feldmesser et al., 2001; Steenburgen et al., 2001).

The cryptococcal capsule is a physical structure composed of a polysaccharide layer surrounding the cell wall. This cell wall structure (capsule) has been proven to be important to the survival of cryptococcal cells (Feldmesser et al., 2001; Zaragoza et al., 2008; Bojarczuk et al., 2016). The capsule forms a barrier to the extracellular space and also assists the cell to perceive its environment (Pommerville, 2010). In the main, this structure is composed of two large polysaccharide molecules viz. glucuronoxylomannan and galactoxylomannan, and to a smaller extend mannoproteins (Zaragoza et al., 2009). The cryptococcal capsule continues to remain a conundrum largely due to its incomprehensible qualities. This is because the capsule, which primarily evolved as a protective structure against hostile phagocytic amoebal cells in nature (Kozel and Gotschlich, 1982), can at the same time act as a protective anti-phagocytic layer when deployed against macrophages (Retini et al., 1996; Zaragoza et al., 2009). Studies as early as 1970s showed that acapsular strains were much more susceptible to phagocytosis than encapsulated strains (Zaragoza et al., 2008). This quality has allowed this terrestrial pathogen to transform itself into a very successful human pathogen, especially in susceptible hosts (Casadevall and Perfect, 1998; Buchanan and Murphy, 1998). As a result, there have been many studies focusing on this structure and its importance towards the survival of this organism both in nature and during infection

(Zaragoza et al., 2009). To illustrate the latter, capsule shedding and enlargement are briefly look at as examples.

The phenomenon of cells shedding their cell wall components, including capsules (Figure 4), when acted upon by phagocytic cells is well documented (Rietschel et al., 1994). The shed capsules can impair the functioning of pattern recognition receptors thus suppress internalisation of targeted cells or upon trapping a targeted cell within the lumen of the food vacuole, the shed capsule can alter the internal environment of a phagocytic cell (Bose et al., 2003).

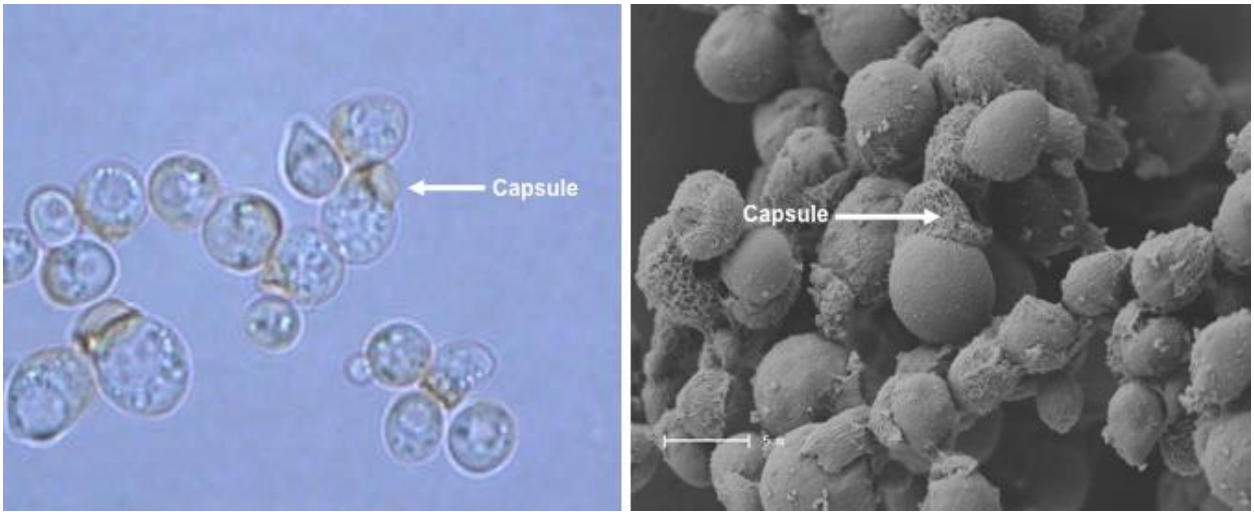


Figure 4. A light and scanning electron micrograph depicting cryptococcal capsules being shed, and arguably to deleterious effect. The above pictogram was obtained from Dr. Sebolai (unpublished data) and is used here with his permission.

Another interesting quality of cryptococcal cells is that they can undergo a morphological change that sees them transforming, via a mechanism not entirely

understood, from “normal-sized” cells of approximately 5 µm – 10 µm to “giant-sized” cells of 50 µm – 100 µm (Okagaki and Nielsen, 2012; Zaragoza and Nielsen, 2013) (Figure 5). It is believed that environmental conditions such as temperature, moisture content and nutrients may trigger the transformation that also involves capsule enlargement.

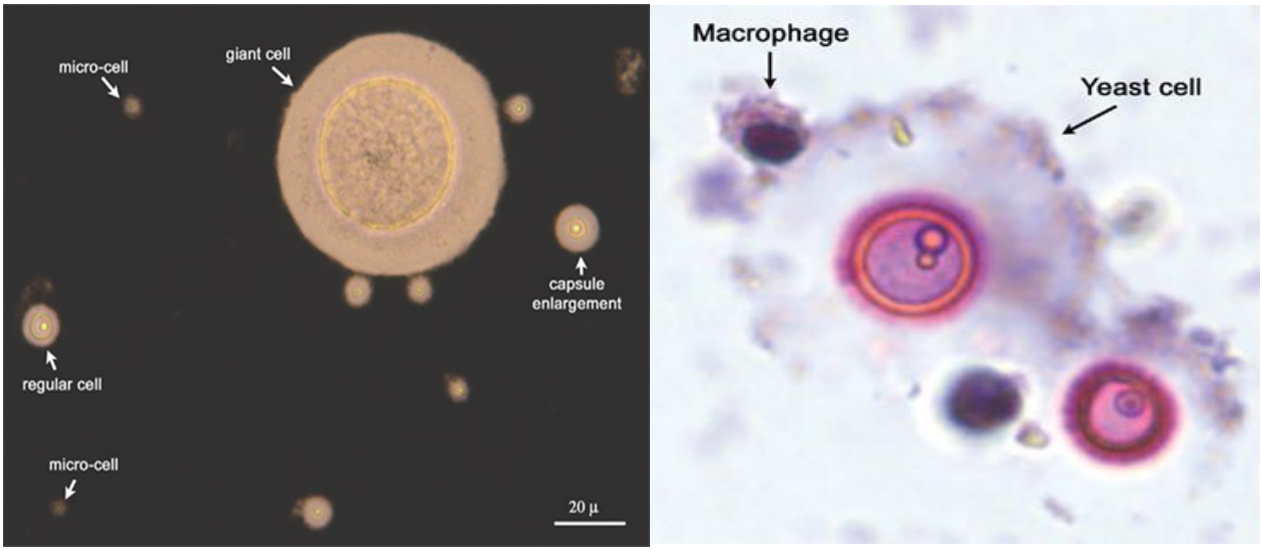


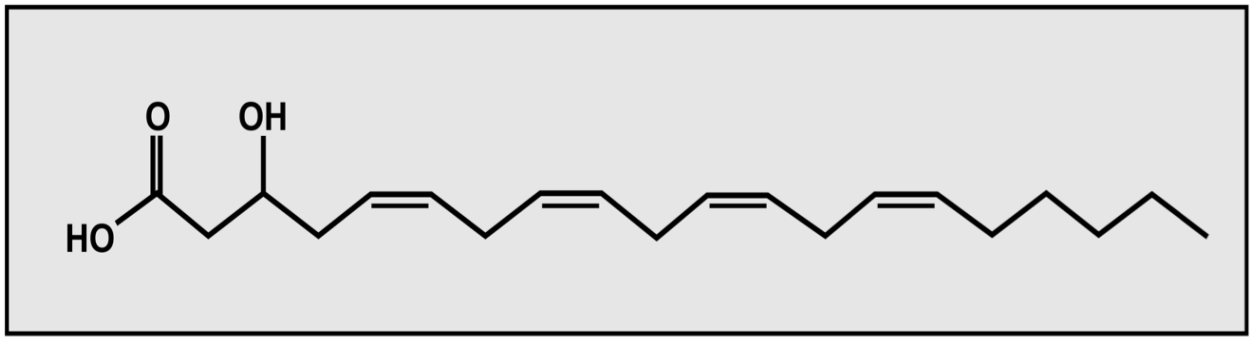
Figure 5. The pictures depicting enlarged cryptococcal cells. The picture on the left shows regular cells and a giant cell after capsule enlargement, whereas the picture on the right shows an enlarged cryptococcal cell that is impossible to be phagocytosed by a macrophage cell. The pictogram is reproduced from Kronstad et al., (2013).

Phagocytosis is a process that evolved a long time ago and is exquisitely effective in enabling phagocytes like amoeba to obtain their next meal. Toward this end, it is reasonable that other microbial factor(s) would act together with the capsule to overcome the workings of this process. Thus, it is not surprising that other anti-phagocytic factors,

like anti-phagocytic protein (app-1), have been identified (Luberto et al., 2003; Hull, 2011). Could one such additional factor be cryptococcal 3-hydroxy fatty acids? This molecule has been shown to be intimately associated with cryptococcal capsules of the strain *Cryptococcus neoformans* UOFS Y-1378 (Sebolai et al., 2007). However, the biological function of cryptococcal 3-hydroxy fatty acids is still not fully understood although it is reasonable to conclude (based on its proximity to the capsules) that it may play a role in pathogenesis. Similar to the capsule, this molecule may have also arisen as a result of predatory pressure emanating from their ecological niche.

3-Hydroxy fatty acids are oxygenated lipid-based molecules that are characterised by a hydroxyl group on the beta carbon of a fatty acid (Figure 6). These molecules are well distributed across the microbial kingdom i.e. from bacteria to fungi, including non-pathogenic ones (Kock et al., 2007). Here, these molecules were shown to be mainly found coating cell wall surfaces or associated with cell wall structures of many microbes (Takayama et al., 2005; Korf et al., 2005). These molecules are generally thought to be produced via an incomplete beta-oxidation process in the mitochondria, followed by extracellular secretion (Ciccoli et al., 2005; Sebolai et al., 2012). Sebolai and co-workers were able to provide cytological evidence to the latter (Sebolai et al., 2007, 2008). In their investigations, cryptococcal 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 and that 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. To definitely determine if 3-hydroxy fatty acids were present in the osmiophilic material, a polyclonal antibody that is specific for 3(*R*)-hydroxy fatty acids was reacted with cells during a TEM immuno-gold labelling assay. Here, it was shown 3-hydroxy fatty acids were present within the osmiophilic material found on capsules of cryptococcal cells. Moreover, they found that 3-hydroxy fatty acids were also present inside the characteristic spiky protuberances of *C. neoformans* UOFS Y-1378 capsules (Sebolai et al., 2008).



3-Hydroxy eicosatetraenoic acid

Figure 6. 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 be branched and saturated. The above pictogram was obtained from Dr. Sebolai and is used here with permission.

Currently, there is no information on the role of cryptococcal 3-hydroxy fatty acids specifically in the context of microbe-to-host interactions. In Gram negative bacteria, these molecules have been shown to influence the behaviour or subvert the functioning of host cells (Rietschel et al. 1994; Annane et al., 2005). To illustrate this point, it has been reported that the bacterial endotoxin, which has 3-hydroxy fatty acids as a constituent, can be shed intentionally by the bacteria into the host. Subsequently, this molecule can then initiate pathobiological processes that lead to the development of sepsis. Herein, the host's response to the toxin can lead to an increase in inflammation that in turn can cause widespread injury to the host's endothelium (Dinarello, 2000; Annane et al. 2005). Based on the above, it is reasonable to conclude that cryptcoccal 3-hydroxy fatty acids may also subvert host's function.

1.4 PURPOSE OF M.Sc. STUDY

The studies presented herein were grouped into two chapters, with each chapter addressing a specific research question. A brief description of each chapter is given below:

1. Chapter 2 focusses on the role of 3-hydroxy fatty acids in influencing interactions between cryptococcal cells and amoebae.
2. Chapter 3 evaluates the direct role of cryptococcal 3-hydroxy fatty acids in compromising the phagocytic machinery of amoebae.

It is hoped that information obtained from these studies may give an insight into how *Cryptococcus* may interact with macrophages which are also phagocytic in nature. This insight may reveal strategies to better manage cryptococcal infections.

1.5 REFERENCES

Annane, D., Bellissant, E. and Cavaillon, J. (2005). Septic shock. *Lancet.* 365, 63-78 DOI: 10.1016/S140-6736(04)17667-8

Bojarczuk, A., Miller, K. A., Hotham, R., Lewis, A., Ogryzko, N. V., Kamuyango, A. A., Frost, H., Gibson, R. H., Stillman, E., May, R. C., Renshaw, S. A. and Johnston, S. A. (2016). *Cryptococcus neoformans* intracellular proliferation and capsule size determines early macrophage control of infection. *Sci*. *Rep*. 6, 21489. DOI: 10.1038/SRep21489

Bose, I., Reese, A. J., Ory, J. J., Janbon, G. and Doering, T. L. (2003). A yeast undercover: the capsule of *Cryptococcus neoformans*. *Eukaryot*. *Cell* 2, 655-663. DOI: 10.1128/EC.2.4.655-663.2003

Buchanan, K. L. and Murphy, J. W. (1998). What makes *Cryptococcus neoformans* a pathogen? *Emerg. Infect. Dis.* 4, 71-78. DOI: 10.3201/eid0401.980109

Carnt, N. A. and Stapleton, F. (2015). Strategies for the prevention of contact lens-related *Acanthamoeba keratitis. Ophthalmic and Physiol. Opt.* 6, 77-92. DOI: 10.1111/opo.12271

Casadevall, A. and Perfect, J. R. (1998). *Cryptococcus neoformans*. Washington, US: American Society for Microbiology Press

Chayakulkeeree, M. D. and Perfect, J. R. (2008). Cryptococcosis, In *Infectious diseases: Diagnosis and treatment of human mycosis.* Eds. Hospenthal, D. R. and Rinaldi, M. G. (New Jersey, US: Hamana), 255-276

Chen, M., Li, X., Wu, S., Tang, X., Feng, B., Yao, Z., Pan, W., Liao, W. and Quan, Z. (2010). Molecular epidemiology of *Cryptococcus neoformans* species complex isolates from HIV-positive and HIV-negative patients in Southeast China. *Front*. *Med*. *China.* 4, 117-126. DOI: 10.1007/s11684-010-0011-z

Chrisman, C. J., Alvarez, M. and Casadevall, A. (2010). Phagocytosis of *Cryptococcus neoformans* by, and nonlytic exocytosis from, *Acanthamoeba castellanii*. *Appl. Environ. Microbiol*. 76, 6056–6062. DOI: 10.1128/AEM.00812-10

Ciccoli, R., Sahi, S., Singh, S., Prakash, H., Zafiriou, M. P., Ishdorj, G., Kock, J. L. F. and Nigam, S. (2005). Oxygenation by cyclooxygenase-2 (COX-2) of 3-hydroxyeicosatetraenoic acid (3-HETE), a fungal mimetic of arachidonic acid, produces a cascade of novel bioactive 3-hydroxy-eicosanoids. *Biochem. J.* 390, 737-747. DOI: 10.1042/BJ20041995

Comolli, L. R. (2014). Intra- and inter-species interactions in microbial communities. *Front*. *Microbiol*. 5, 629. DOI: 10.3389/fmicb.2014.00629

Day, J. N. (2004). Cryptococcal Meningitis. *Pract. Neurol.* 4, 274-285. DOI: 10.1111/j.1474-7766.2004.00254.x

Dinarello, C. A. (2000). Pro-inflammatory cytokines. *Chest*. 118, 503-508. DOI: 10.1378/chest.118.2.503

Feldmesser, M., Tucker, S. and Casadevall, A. (2001). *Cryptococcus neoformans*: Intracellular or extracellular? *Trends Microbiol*. 9, 418. DOI: 10.1016/S0966-842X(01)02035-2

Fell, J. W. and Statzell-Tallman, A. (1998). *Cryptococcus.* Vuillemin. In *The Yeasts, A Taxonomic Study, 4th edition.* Eds. Kurtzman, C. P. and Fell, J. W. (Amsterdam, NL: Elsevier B.V.), 742-767

Fonseca, A., Boekhout, T. and Fell, J. W. (2011). *Cryptococcus.* Vuillemin. In *The Yeasts, A Taxonomic Study, 5th edition*. Eds. Kurtzman, C. P., Fell, J. W. and Boekhout, T. (Amsterdam, NL: Elsevier B.V.), 1661-1737

Freeman, S. A. and Grinstein, S. (2014). Phagocytosis: receptors, signal integration, and the cytoskeleton. *Immunol*. *Rev*. 262, 193-215. DOI: 10.1111/imr.12212

Herbst, R., Ott, C., Jacobs, T., Marti, T., Marciano-Cabral, F. and Leippe, M. (2002). Pore-forming polypeptides of the pathogenic protozoon *Naegleria fowleri*. *J. Biol. Chem*. 277, 22353–22360. DOI: 10.1074/jbc.M201475200

Hull, C. M. (2011). Preventing phagocytosis takes more than a sweet disposition. *Cell. Host. Microbe*. 9, 174-175. DOI: 10.1016/j.chom.2011.03.006

Hull, C. M. and Heitman, J. (2002). Genetics of *Cryptococcus neoformans*. *Annu*. *Rev*. *Genet*. 36, 557-615. DOI: 10.1146/annurev.genet.36.052402.152652

Hunter, P. (2006). Entente cordiale: multiple symbiosis illustrates the intricate interconnectivity of nature. *EMBO Reports* 7, 861-864. DOI: 10.1038/sj.embor.7400785

Hurst, J. K. (2012). What really happens in the neutrophil phagosome? Free *Radic. Biol. Med*. 3, 508-520. DOI: 10.1016/j.freeradbiomed.2012.05.008

Khan, N. A. (2014). *Acanthamoeba*: Biology and Pathogenesis. Nottingham, UK: Caister Academic Press

Kock, J. L. F., Sebolai, O. M., Pohl, C. H., van Wyk P. W. J. and Lodolo, E. J. (2007). Oxylipin studies expose aspirin as antifungal. *FEMS Yeast Res.* 7, 1207-1217. DOI: 10.1111/j.1567-1364.2007.00273.x

Korf, J., Stoltz, A., Verschoor, J., De Baetselier, P. and Grooten, J. (2005). The *Mycobacterium tuberculosis* cell wall component mycolic acid elicits pathogen-associated host innate immune responses. *J. Immunol.* 35, 890-900. DOI: 10.1002/eji.200425332

Kozel, T. R. and Gotschlich, E. C. (1982). The capsule of *Cryptococcus neoformans* passively inhibits phagocytosis of the yeast by macrophages. *J. Immunol.* 129, 1675-1680. PMID: 7050244

Kronstad, J. W., Hu, G. and Jung, W. H. (2013). An encapsulation of iron homeostasis and virulence in *Cryptococcus neoformans*. *Trends Microbiol*. 21, 457-465. DOI: 10.1016/j.tim.2013.05.007

Kurtzman, C. P., Fell, J. W. and Boekhout, T. (2011). In *The Yeasts, A Taxonomic Study, 5th edition.* (Amsterdam, NL: Elsevier B. V.)

Kwon-Chung, K. J. (1975). A new genus, *Filobasidiella*, the perfect state of *Cryptococcus neoformans*. *Mycologia* 67:1197-1200. DOI: 10.2307/3758842

Kwon-Chung, K. J. (1976). Morphogenesis of *Filobasidiella neoformans*, the sexual state of *Cryptococcus neoformans*. *Mycologia* 68:821-833. DOI: 10.2307/3758800

Kwon-Chung, K. J. (2011). *Filobasidiella*. In *The Yeasts, A Taxonomic Study, 5th edition*. Eds. Kurtzman, C. P., Fell, J. W. and Boekhout, T. (Amsterdam, NL: Elsevier B. V.). 1443-1456

Kwon-Chung, K. J. and Varma, A. (2006). Do major species concepts support one, two or more species within *Cryptococcus neoformans*? *FEMS Yeast Res*. 6, 574-587. DOI: 10.1111/j.1567-1364.2006.00088.x

Leippe, M. and Herbst, R. (2004). Ancient weapons for attack and defense: the pore-forming polypeptides of pathogenic enteric and free-living amoeboid protozoa. *J. Eukaryot. Microbiol.* 51, 516-21. DOI: 10.1111/j.1550-7408.2004.tb00286.x

Levitz, S. M. and Boekhout, T. (2006). *Cryptococcus*: the once-sleeping giant is fully awake. *FEMS Yeast Res.* 6, 461–462. DOI: 10.1111/j.1567-1364.2006.00113

Lin, X. and Heitman, J. (2006). The biology of the *Cryptococcus neoformans* species complex. *Annu. Rev. Microbiol.* 60, 69-105. DOI: 10.1146/annurev.micro.60.080805.142102

Luberto, C., Martinez-Marino, B., Taraskiewicz, D., Bolanos, B., Chitano, P., Toffaletti, D. L., Cox, G. M., Perfect, J. R., Hannun, Y. A., Balish, E. and Del Poeta, M. (2003). Identification of App1 as a regulator of phagocytosis and virulence of *Cryptococcus neoformans. J. Clin. Invest.* 112, 1080–1094. DOI: 10.1172/JCI18309

Marciano-Cabral, F. and Cabral, G. (2003). *Acanthamoeba* spp. as Agents of Disease in Humans *Clin*. *Microbiol*. *Rev*. 2, 273-307. DOI: 10.1128/CMR.16.2.273-307.2003

Medina, G., Flores-Martin, S., Fonseca, B. B., Otth, C. and Fernandez, H. (2014). Mechanisms associated with phagocytosis of *Arcobacter butzleri* by *Acanthamoeba castellanii*. *Parasitol*. *Res*. 113, 1933-1942. DOI: 10.1007/s00436-014-3842-8

May, R. C. and Machesky, L. M. (2001). Phagocytosis and the actin cytoskeleton. *J. Cell Sci.* 114:1061–1077. PMID: 11228151

Okagaki, L. H. and Nielsen, K. (2012). Titan cells confer protection from phagocytosis in *Cryptococcus neoformans* infections. *Eukaryot. Cell* 11, 820–826. DOI: 10.1128/EC.00121-12

Park, B. J., Wannemuehler, K. A., Marston, B. J., Govender, N., Pappas P. G. and Chiller, T. M. (2009). Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS* 23, 525-530. DOI: 10.1097/QAD.0b013e328322ffac

Pommerville, J.C. (2010). Cell structure and function in the bacteria and archaea, In *Alcamo's fundamentals of microbiology: body systems,* Ed. Pommerville, J.C. (Sudbury, US: Jones and Bartlett Publishers), 98-128

Retini, C., Vecchiarelli, A., Monari, C., Tascini, C., Bistoni, F. and Kozel, T. R. (1996). Capsular polysaccharide of *Cryptococcus neoformans* induces proinflammatory cytokine release by human neutrophils. *Infect*. *Immun*. 64, 2897-2903. PMID: 8757810

Rietschel, E. T., Kirikae, T., Schade, F. U., Mamat, U., Schmidt, G., Loppnow, H., Ulmer, A. J., Zahringer, U., Seydel, U., Di Padova, F., Schereire, M. and Brade, H. (1994). Bacterial endotoxin: molecular relationships of structure to activity and function. *FASEB J.* 8, 217-225. PMID: 8119492

Sebolai, O. M., Pohl, C. H., Botes, P. J., Strauss, C. J., van Wyk P. W. J., Botha, A. and Kock, J. L. F. (2007). 3-Hydroxyl fatty acids found in capsules of *Cryptococcus neoformans. Can. J. Microbiol.* 53, 809-812. DOI: 10.1139/W07-045

Sebolai, O. M., Pohl, C. H., Botes, P. J., van Wyk P. W. J., Botha, A. and Kock, J. L. F. (2008). The influence of acetylsalicylic acid on oxylipin migration in *Cryptococcus neoformans* var. *neoformans* UOFS Y-1378. *Can. J. Microbiol.* 54, 91-96. DOI: 10.1139/w07-114

Sebolai, O. M., Pohl, C. H., Kock, L. J. F., Chaturvedi, V. and del Poeta, M. (2012). The presence of 3-hydroxy oxylipins in pathogenic microbes. *Prostaglandins Other Lipid Mediat.* 97, 17-21. DOI: 10.1016/j.prostaglandins.2011.11.001

Siddiqui, R. and Khan, N. A. (2012). *Acanthamoeba* is an evolutionary ancestor of macrophages: a myth or reality? *Exp*. *Parasitol.* 130, 95–97. DOI: 10.1016/j.exppara.2011.11.005

Sidrim, J. J. C., Costa, A. K. F., Cordeiro, R. A., Brilhante, R. S. N., Moura, F. E. A., Castelo-Branco, D. S., Neto, M. P. A. and Rocha, M. F. G. (2010). Molecular methods for the diagnosis and characterization of *Cryptococcus*: a review. *Can*. *J. Microbiol.* 56, 445-458. DOI: 10.1139/w10-030

Sinclair, J. L., McClellan, J. F. and Coleman, D. C. (1981). Nitrogen Mineralization by *Acanthamoeba* polyphaga in Grazed *Pseudomonas paucimobilis* Populations. *Appl. Environ. Microbiol.* 42, 667–671

Steenburgen, J. N. and Casadevall, A. (2003). The origin and maintenance of virulence of the human fungus *Cryptococcus neoformans*. *Microb. Infect.* 5, 667-675. DOI: 10.1016/S1286-4579(03)00092-3

Steenburgen, J. N., Shuman, H. A. and Casadevall, A. (2001). *Cryptococcus neoformans* interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. *Proc. Natl. Acad. Sci*. 98, 15245-15250. DOI: 10.1073/pnas.261418798

Takayama, K., Wang, C. and Besra, G. (2005). Pathway to synthesis and processing of mycolic acids in *Mycobacterium tuberculosis*. *Clin. Microbiol. Rev.* 18, 81-91. DOI: 10.1128/CMR.18.1.81-101.2005

Tan, S. Y. and Dee, M. K. (2009). Elie Metchnikoff (1845–1916): discoverer of phagocytosis. *Singapore Med. J.* 50, 456-457. PMID: 19495511

Voelz, K., Lammas, D. A. and May, R. C. (2009). Cytokine signalling regulates the outcome of intracellular macrophage parasitism by *Cryptococcus neoformans*. *Infect*. *Immun*. 77, 3420. DOI: 10.1128/IAI.00297-09

Winterbourn, C. C. and Kettle, A. J. (2013). Redox reactions and microbial killing in the neutrophil phagosome. *Antioxid. Redox. Signal.* 6. 642-60. DOI: 10.1089/ars.2012.4827

Zaragoza, O., Chrisman, C. J., Castelli, M. V., Frases, S., Cuenca-Estrella, M., Rodríguez-Tudela, J. L. and Casadevall, A. (2008). Capsule enlargement in *Cryptococcus neoformans* confers resistance to oxidative stress suggesting a mechanism for intracellular survival. *Cell Microbiol*. 10, 2043-2057. DOI: 10.1111/j.1462-5822.2008.01186.x

Zaragoza, O., Rodrigues, M. L., De Jesus, M., Frases, S., Dadachova, E. and Casadevall, A. (2009). The capsule of the fungal pathogen *Cryptococcus neoformans. Adv. Appl. Microbiol.* 68, 133-216. DOI: 10.1016/S0065-2164(09)01204-0

Zaragoza, O. and Nielsen, K. (2013). Titan cells in *Cryptococcus neoformans*: Cells with a giant impact. *Curr. Opin. Mirobiol.* 16, 409-413. DOI: 10.1016/j.mib.2013.03.006

CHAPTER 2

CRYPTOCOCCAL 3-HYDROXY FATTY ACIDS PROTECT CELLS AGAINST AMOEBAL PHAGOCYTOSIS

This study was performed by the candidate and has been published. Therefore, repetition of some information could not be avoided.

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Candidate's contribution: Co-designed experiments, performed the experiments and wrote the draft manuscript.

2.1 ABSTRACT

We previously reported on a 3-hydroxy fatty acid that is secreted via cryptococcal capsular protuberances - possibly to promote pathogenesis and survival. Thus, we investigated the role of this molecule in mediating the fate of *Cryptococcus (C.) neoformans* and the related species *C. gattii* when predated upon by amoebae. We show that this molecule protects cells against the phagocytic effects of amoebae. *C. neoformans* UOFS Y-1378 (which produces 3-hydroxy fatty acids) was less sensitive towards amoebae compared to *C. neoformans* LMPE 046 and *C. gattii* R265 (both do not produce 3-hydroxy fatty acids) and addition of 3-hydroxy fatty acids to *C. neoformans* LMPE 046 and *C. gattii* R265 culture media, causes these strains to become more resistant to amoebal predation. Conversely, addition of aspirin (a 3-hydroxy fatty acid inhibitor) to *C. neoformans* UOFS Y-1378 culture media made cells more susceptible to amoebae. Our data suggest that this molecule is secreted at a high enough concentration to effect intracellular signalling within amoeba, which in turn, promotes fungal survival.

Key words: 3-Hydroxy fatty acids, Amoeba, *Cryptococcus*, Phagocytosis, Protection.

2.2 INTRODUCTION

Cryptococcus neoformans lives primarily in the environment, wherein it is constantly isolated from soil contaminated with bird droppings (Lin and Heitman, 2006). In this ecological niche, cryptococcal cells interact with other organisms, often in a struggle to establish territorial dominance. To illustrate this point, cryptococcal cells have been reported to fall prey to foraging amoebae like *Acanthamoeba castellanii* (Steenburgen and Casadevall, 2003). Additionally, amoebae are said to have evolved efficient strategies to recognise, internalise and kill internalised microbes, which they use as a source of food (Bottone et al., 1994). And thus the constant struggle between this fungus and amoebae has, as a matter of natural course, selected this fungus to develop a protective structure i.e. the capsule, in order to evade predation (Kozel and Gotschlich, 1982; Feldmesser et al., 2001). Literature also suggests that cryptococcal cells when under attack from hostile phagocytic cells, including macrophages, express capsule production and enlargement as a defensive mechanism (Steenburgen and Casadevall, 2003; Fuchs and Mylonakis, 2006), because as pointed out by Feldmesser et al. (2001) "from the standpoint of *C*. *neoformans*; there might be little difference between a macrophage and amoeba"… It has been reported that the capsule can enlarge to up to 50 µm in size (Casadevall and Perfect, 1998). Unfortunately this defensive behaviour i.e. capsule production and enlargement, has also translated into this microbe establishing itself as a successful human pathogen, more so in susceptible hosts (Levitz and Boekhout, 2006). During infection, cryptococcal cells can be cleared or can take up residency inside macrophages while avoiding immuno-processing in order to disseminate (Casadevall and Perfect, 1998). Upon being internalised, cryptococcal cells (unlike some

pathogenic bacteria) do not prevent fusion of lysosomes to phagosomes (Horwitz, 1983; Voelz and May, 2010), and have adapted to proliferate inside macrophages regardless of the prevailing harsh environment (Levitz et al., 1999). While capsules may be critical in shielding cells (Casadevall and Perfect, 1998), much is still unknown about other metabolites and mechanisms that enable this pathogen to survive when internalised.

We previously reported on the presence and release of a 3-hydroxy fatty acid that is closely associated with capsules of *C. neoformans* UOFS Y-1378 (Sebolai et al., 2007, 2008). 3-Hydroxy fatty acids are regarded as secondary metabolites that have been implicated in the pathogenesis of other microbes (Deva et al., 2000; Ciccoli et al., 2005), but not in *C. neoformans*. Thus, in this study we sought to: 1) estimate the concentration of cryptococcal 3-hydroxy fatty acids being secreted, and 2) investigate the role of these molecules in mediating the fate of cryptococcal cells when acted upon by free-living hostile phagocytic cells.

2.3 MATERIALS AND METHODS

Strains, cultivation and standardization

The fungal strains, *C. neoformans* UOFS Y-1378 (held at the University of the Free State), *C. neoformans* LMPE 046 (held at the University of the Free State), and *C. gattii* R265 (a gift from R.C. May, University of Birmingham, UK), were 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 30^{o}C while amoeba, *Acanthamoeba castellanii* LMPE 187 (a gift from A. Idnurm, University of Missouri-Kansas City, USA), was grown on peptone-yeast extract glucose agar, PYG (ATCC medium 30234^{TM}) at 30^{o}C. 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 30^{o}C for 48 h while agitating at 160 rpm. For amoeba cells, cells were collected from a week old agar plate and cultivated in 50 ml centrifuge tubes (Becton-Dickinson Labware, United States) containing 25 ml of PYG broth at 30^{o}C for 48 h while shaking at 160 rpm. In light of anticipated co-culture experiments, cryptococcal cells were standardised to either 1 x 10^5 cells/ml, 1 x 10^6 cells/ml or 1 x 10^7 cells/ml in 10 ml of either fresh YNB broth or phosphate buffered solution (PBS; Oxoid, South Africa) while amoeba cells were standardised to 1 x 10^5 cells/ml in 10 ml of fresh PYG broth. All cells were placed on ice before use.

3-Hydroxy fatty acid extraction, analysis and relative quantification

3-Hydroxy fatty acids were extracted from 48 h cultures of *C. neoformans* UOFS Y-1378 [cell density after 48 h = 1.7 x 10^7 cells/ml (+/- 9.0 x 10^5)] and *C. gattii* R265 [cell density after 48 h = 2.3 x 10^7 cells/ml (+/-5.6 x 10^5)] using the modified Folch method. In brief, 2 ml of culture media (containing cells) were transferred to a 15 ml Falcon 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 in a fume hood. In a separate experiment, 1 ml of *C. gattii* R265 culture media (48 h) was spiked with 0.5 ml of the 3-hydroxy nonanoic acid 2 mM solution to yield a final concentration of 0.66 mM. Subsequently, 3-hydroxy fatty acids were extracted as detailed above. The analytical 3-hydroxy fatty acid standard viz. 3-OH C9:0, was obtained from Laradon Fine Chemicals (Sweden).

The 3-hydroxy fatty acid extracts (obtained from *C. neoformans* UOFS Y-1378 and *C. gattii* R265) were reconstituted in 50 µl of water, vortex mixed and centrifuged for 15 min at 10000 *g*. The supernatants were transferred to analytical vials with 200 µl fixed inserts and capped (Thermo-Fisher Ltd., United Kingdom). The samples were stored in the autosampler at 5°C and analysed within 72 h of reconstitution in negative electrospray ionisation (ESI) mode. Ultra High Performance Liquid Chromatography-Mass Spectrometry (UHPLC-MS) was performed according to the method reported in Gehmlich et al. (2015), applying a 5 µl sample injection volume (partial loop mode) on to a Hypersil Gold C18, (100 x 2.1mm, 1.9 µm particle size) UHPLC column (Thermo-Fisher Ltd.) with the Dionex U3000 UHPLC system coupled to a Thermo LTQ-FT-MS Ultra system (Thermo-Fisher Ltd.). Solvent A, HPLC grade water, and solvent B, HPLC grade methanol (J.T. Baker, United Kingdom) were acidified with 0.1% formic acid (Aristar grade, VWR Ltd., United Kingdom). The gradient programme was as follows: hold 100% A 0-1 min, 100% A - 100% B 1-3.5 min curve 3, hold 100% B 3.5-6 min, 100% B – 100%

A 6-7 min curve 3, hold 100% A 7-8 min. The LTQ-FT-MS Ultra system was operated under Xcalibur software (Thermo-Fisher Ltd.), in full scan mode (*m/z* 100-1000) at a mass resolution of 50,000 (FWHM defined at *m/z* 400). Prior to the analytical run, the LTQ and FT-MS were calibrated with the manufacturers recommended calibration mixture. The samples were analysed in a completely randomised order. A blank control sample was analysed at the start and end of the run, thus providing a measure of the sample background and also a measure of compound carry over. The relative peak areas of 3-hydroxy nonanoic acid (monoisotopic mass: 174.125595 Da, retention time (RT) 3.7 min) were obtained for each sample and the 3-hydroxy nonanoic acid analytical standard in the Qual Browser function of the Xcalibur software package (Thermo-Fisher Ltd.). The peak areas were calculated based upon the EIC for the major ESI negative mode base peak, 173.1182 *m/z* (the deprotonated parent ion M-H), which was detected at a retention time of 3.7 min. Peak areas were exported to Microsoft Excel, the standard deviation was calculated across the replicates within each of the defined experimental classes. Finally graphs were generated where error bar was representative of the standard deviation upon peak area.

Lipids were like-wise extracted from *C. neoformans* LMPE 046 [cell density = 1.9 x 10^7 cells/ml (+/- 3.0 x 10^5)] and *Acanthamoeba castellanii* LMPE 187 [cell density = 1.8 x 10^5 cells/ml (+/- 2.0 x 10^3)] and 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 method optimization 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 are 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 as supplementary data for comparison.

Visualisation of *Cryptococcus*-amoeba interactions: fluorescent microscopy and transmission electron microscopy

Fluorescent images were taken after cryptococcal cells (*C. neoformans* UOFS Y-1378) were stained with fluorescein isothiocyanate (Sigma-Aldrich, South Africa; 1 µl of stain : 999 µl of cells) for 2 h at room temperature, while at the same time amoeba cells (100 µl)

were allowed to adhere to wells on a chamber slide (Nunc® Lab-Tek® II Chamber Slide™ system; Sigma-Aldrich) at 30ºC. After this 2 h-period, cryptococcal cells were washed twice with PBS and added 100 µl to the chamber slide (for 2 h at 30ºC) in order to interact with amoeba cells (10 amoebae : 1 fungus). At the end of the interactive period, wells were washed twice with PBS to remove any cryptococcal cells not internalised. The slide was then fixed for 1 h with 2.5% glutaraldehyde (Sigma-Aldrich) following which, the fixative was aspirated. An antifade compound, 1,4-diazabicyclo[2.2.2]-octane (Sigma-Aldrich), was added to the slide before viewing using a confocal laser scanning microscope (CLSM; Nikon TE 2000; Tokyo, Japan). Material for transmission electron microscopy (TEM) was obtained from a 48 h co-culture (1 ml : 1 ml (v/v) of 10 amoebae : 1 fungus (*C. neoformans* UOFS Y-1378)) that was grown at 30ºC. The material was prepared for TEM viewing according to the method of van Wyk and Wingfield (1991). In brief, this co-cultured material was chemically fixed with 1.0 M (pH 7) sodium phosphate-buffered gluteraldehyde (3%) for 3 h and then for 1.5 h in similarly buffered osmium tetroxide. The material was next dehydrated in a graded acetone series. The TEM material was then embedded in epoxy resin and polymerized at 70ºC for 8 h. An LKB III Ultratome was used to cut 60-nm sections with glass knives. Uranyl acetate was used to stain sections for 10 min, followed by lead citrate for 10 min and the preparation viewed with a Philips EM 100 transmission electron microscope (van Wyk and Wingfield, 1991).

Cryptococcus phagocytosis assay

We assessed the ability of amoebae to internalise cryptococcal cells: 1) obtained from strains *C. neoformans* UOFS Y-1378, 2) *C. gattii* R265, 3) *C. neoformans* LMPE 046 in the absence or presence of 3-hydroxy C9:0 i.e. 0 mM, 0.2 mM and 1 mM, using the phagocytosis stain, pHrodo^{TM} Green Zymosan A BioParticles (Life Technologies, United States). The stain only fluoresces when excited at acidic pH, such as inside a food vacuole or phagosome. Cryptococcal cells were standardised to 1 x 10^6 cells/ml in PBS (which has a neutral pH) and stained (1 µl of stain: 999 µl of cells) for 1 h at room temperature while slowly agitating. Next, cryptococcal cells were washed with PBS, spun down and suspended in sterile 1000 µl of PBS. A 100-µl suspension of cells was then transferred to microtitre plate wells (Greiner Bio-One, Germany) and allowed to interact with amoebae (100 µl; 1 x 10^5 cells/ml) for 2 h or 6 h at 30^{o}C. The amoebae were standardised in fresh PYG broth (pH 7). At the end of the incubation period, the induced fluorescence was measured (492 nm; ex / 538 nm; em) using a Fluoroskan Ascent FL (Thermo-Scientific, United States) microplate reader, which converts logarithmic signals to relative fluorescence units. The fluorescence was also measured for fungal cells alone in order to normalise the readings.

Cryptococcus survival assay

The interactive outcome of amoeba cells and fungal cells was quantified by enumerating viable fungal cells by counting colony forming units (CFU). The above was based on a modified protocol previously detailed by Steenburgen et al. (2001). Here, cryptococcal

cells i.e. obtained from strains *C. neoformans* UOFS Y-1378, *C. gattii* R265 and *C. neoformans* LMPE 046, were added to amoeba cells in the absence or presence of 3-hydroxy C9:0 (0 mM and 0.2 mM) to yield a ratio of 10 (amoebae): 1 (fungus) i.e. 500 µl: 500 µl (v/v). These cultures were grown at 30^{o}C in 1.5 ml eppendorf tubes (Merck). After a 48 h interactive period, co-cultured cells were gently agitated and amoeba cells were lysed by forcibly pulling and pushing them through a needle (27 gauge x 20 mm; Novagen, South Africa) eight times (Steenburgen et al., 2001). For each tube, serial dilutions were made and plated out on YPD agar plates for 48 h at 30^{o}C. Additionally in a separate experiment, we determined the susceptibility levels of *C. neoformans* UOFS Y-1378 cells towards amoebae in the absence of 3-hydroxy fatty acids. To be specific, *C. neoformans* UOFS Y-1378 cells were initially treated with 1 mM aspirin, which is an inhibitor of 3-hydroxy fatty acids (Sebolai et al., 2008). Following a 48 h aspirin-treatment period, cells were then fed to amoebae at a ratio of 10 (amoebae): 1 (fungus). This co-culture was incubated as stated above and cryptococcal cells were enumerated in the same manner.

The effect of cryptococcal 3-hydroxy fatty acids on amoebae

A 100-µl suspension of amoeba cells concentrated to 10^5 cells/ml in PYG broth was added to wells of a sterile, disposable 96-well flat-bottom microtitre plates. Thereafter, aliquots of 100 µl of the test drug (3-hydroxy C9:0), at twice the desired final concentrations, were dispensed into wells. To the point, cells were tested at final concentrations of 0.2 mM and 1 mM of 3-hydroxy fatty acids. Amoeba cells were also tested against nonanoic acid (C9:0) at the same concentrations. The plates were then

incubated for 48 h at 30^{o}C. At the end of the incubation period, cells were reacted in the dark at 30^{o}C with 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT; Sigma-Aldrich) in the presence of menadione (Sigma-Aldrich) – in order to measure their metabolic activity (Polat et al., 2014). The optical density (OD) readings were measured, after 3 h of initiating the tetrazolium reaction, using a spectrophotometer (Biochrom EZ Read 800 Research, United Kingdom). Non-treated amoeba cells were included for reference.

Statistical note

All experiments, reported in this study, were performed in triplicate. And where appropriate, a student *t*-test was conducted to determine the statistical significance of data between the different experimental conditions.

2.4 RESULTS

Characterization of cryptococcal 3-hydroxy fatty acids

We previously assigned a structure of a 3-hydroxy fatty acid-based molecule (after detection with a 3*R*-hydroxy fatty acid-specific polyclonal antibody and initial GC-MS analysis) to a hydroxy fatty acid extracted from *C. neoformans* UOFS Y-1378 cultures (Sebolai et al., 2007). However, in order to determine the biological function(s) of these capsule-associated molecules, it is important to know their secreted concentration.

Therefore, in order to estimate concentrations produced by *C. neoformans* UOFS Y-1378, the extracted ion chromatograms (EICs) were compared between (1) the biological samples of *C. neoformans* UOFS Y-1378, (2) biological samples of *C. gattii* R265 that were spiked with 3-hydroxy nonanoic acid to a final concentration of 0.66 mM, and (3) a 0.1 mM solution of the 3-hydroxy nonanoic acid dissolved in water. Using a two-point calibration method, our analysis indicated that the biological sample concentration range of 3-hydroxy nonanoic acid was in the range 0.1 mM to 0.4 mM (Figure 1). Although these estimates were based upon comparisons to a single concentration level of the analytical standard rather than a full dilution series based calibration curve and matrix-matched standards – the extrapolated figures are sufficient for providing a concentration range that is suitable for conducting biological studies.

In light of our comparative studies, it was also important to establish if *C. gattii* R265 produced the same metabolite or not. Here, we analysed the authentic chemical standard for 3-hydroxy nonanoic acid and biological samples and matched the retention time (3.7 min and accurate *m/z* (173.1182; $[M-H]^-$) between standard and samples (Figure 2A-C). For *C. neoformans* UOFS Y-1378, as expected, we observed a similar MS/MS mass spectrum for the standard and samples showing the detection of a hydrogen-bound dimer ion ($[M+M-H]^-$), and a sodium-bridged dimer ion ($[M+Na+M-H]^-$) and in the same response ratios (Table 1; Figures 2A, B). With respect to *C. gattii* R265, we also noted elution of an unknown metabolite at a retention time that matched the analytical standard, although at very low levels approaching the limit of Fourier transform-ion cyclotron resonance-mass spectrometer detection. However, upon studying its mass spectrum, this

metabolite did not have the characteristic diagnostic MS/MS peaks of the chemical standard or the metabolite of interest (Table 1; Figure 2A, C). *C. neoformans* LMPE 046 and *Acanthamoeba castellanii* LMPE 187 were also shown to not produce any 3-hydroxy fatty acids (Supplementary Figure S1). Both their respective EICs did not show elution of our metabolite of interest after 2.05 min when referenced against the EIC of the analytical standard compound.

Visualisation of *Cryptococcus*-amoeba interaction

Cryptococcal cells often fall prey to foraging amoebae in nature. In order to reproduce a similar setting *in vitro*, *C. neoformans* UOFS Y-1378 was fed to *Acanthamoeba castellanii* LMPE 187, in order to view their interactions. Transmission electron micrographs revealed a moment when a cryptococcal cell was about to be captured by amoeba pseudopodia (Figure 3A, 3B) and after being trapped inside a food vacuole or phagosome (Figure 3C). The characteristic thick capsule, with the typical spiky protuberances of *C. neoformans* UOFS Y-1378, can clearly be seen in Figure 3D. We previously suggested these spiky protuberances may facilitate the release of 3-hydroxy fatty acids into the extracellular environment after detecting their presence inside protuberances following TEM immuno-gold labelling analysis (Sebolai et al., 2008). The fluorescent micrographs provide further pictorial evidence of a cryptococcal cell (in green) close to amoeba (in orange) (Figure 3E) and internalised cryptococcal cells (in green) (Figure 3F). It is reasonable to conclude that during such interactive moments, the source of 3-hydroxy

fatty acids can only be *C. neoformans* UOFS Y-1378 (Figure 2B) and not amoebae (Supplementary Figure S1) as per the LCMS results.

3-Hydroxy fatty acids protect cells from amoebal phagocytosis

In the absence of artificially added 3-hydroxy fatty acids, the test amoeba strain yielded significantly lower relative fluorescence units ($p < 0.05$) when co-cultured with *C. neoformans* strain UOFS Y-1378 compared to when co-cultured with *C. gattii* R265 and *C. neoformans* LMPE 046 at both 2 h (Figure 4A) and 6 h (Figure 4B). The latter implies that amoeba displayed less appetite to internalize *C. neoformans* UOFS Y-1378, which naturally produces 3-hydroxy fatty acids, when compared to *C. gattii* R256 and *C. neoformans* LMPE 046, which both do not. In order to investigate if 3-hydroxy fatty acids may be responsible for the displayed resistance expressed by *C. neoformans* UOFS Y-1378, we re-assessed the appetite of amoebae for *C. gattii* R256 cells and *C. neoformans* LMPE 046 cells when 3-hydroxy fatty acids were artificially added i.e. 0.2 mM and 1 mM, to their culture media. Here, addition of 3-hydroxy fatty acids made *C. gattii* R256 and *C. neoformans* LMPE 046 more resistant to amoebal internalization or less appetizing to be internalized at both 2 h and 6 h in a dose-dependent manner – as per the recorded lower relative fluorescence units when compared to higher readings obtained for *C. gattii* R256 and *C. neoformans* LMPE 046 in the absence of 3-hydroxy fatty acids (Figure 4). Additionally, *C. neoformans* UOFS Y-1378 displayed a dose-dependent resistance towards being internalised by amoebae when increasing amounts of 3-hydroxy fatty acids were artificially added. It is worthwhile to note that the number of *C. neoformans* UOFS

Y-1378 cells that were internalised (in the absence and presence of 3-hydroxy fatty acids) generally decreased over time. While on the other hand, the number of *C. gattii* R256 cells and *C. neoformans* 046 cells that were internalised (in the absence and presence of 3-hydroxy fatty acids) generally increased over time. It is also interesting to note that for each co-culture experiment, and specifically at 6 h, the number of internalised cryptococcal cells in the presence of 3-hydroxy fatty acids, were approximate to the number of internalised cryptococcal cells in the absence of 3-hydroxy fatty acids compared to at 2 h. This gap-narrowing or approximation suggest a level of adaptation by amoebae, over time, to the presence of 3-hydroxy fatty acids.

Next, we quantified the survival of fungal cells after being internalised. And as expected (in the absence and presence of 3-hydroxy fatty acids), *C. neoformans* UOFS Y-1378 was more resistant to amoebae i.e. few cells were successfully phagocytosed, compared to *C. gattii* R256 and *C. neoformans* LMPE 046 as per the number of recovered fungal colonies on agar plates (Figure 5). With respect to *C. neoformans* UOFS Y-1378, the observed phagocytosis outcome suggests that of the few cells that were successfully internalised (according to Figure 4 results); more of them were able to survive the phagocytic process and the opposite phenomenon is observed with respect to *C. gattii* R256 cells and *C. neoformans* LMPE 046 cells. This determination suggests that in addition to impairing cell internalisation, this molecule may also promotes intracellular survival. Once more, the addition of 3-hydroxy fatty acids to *C*. *neoformans* UOFS Y-1378, *C. gattii* R256 and *C. neoformans* LMPE 046 culture media significantly ($p < 0.05$) increased their level of resistance towards amoebae by promoting intracellular survival.

It is also striking to note that inhibition of 3-hydroxy fatty acid production using aspirin resulted in *C. neoformans* UOFS Y-1378 cells being more susceptible to amoebae compared to non-treated *C. neoformans* UOFS Y-1378 cells (Figure 6). In 2008, we showed that 3-hydroxy fatty acids are inhibited in a dose-dependent manner by increasing amounts of aspirin. Taken together, the above data points towards a possible protective quality that is exhibited by 3-hydroxy fatty acids.

3-Hydroxy fatty acids effect on amoebae

Exposure of amoeba cells to estimated physiological concentration of cryptococcal 3-hydroxy fatty acids i.e. 0.2 mM, yielded XTT reduction values that were highly comparable ($p = 0.11$; not-significant) to readings obtained for non-treated amoeba cells. However, at 1 mM concentration, the XTT reduction values were significantly ($p < 0.05$) lower compared to that of non-treated amoeba cells (Figure 7A). Next, these XTT reduction values were translated into percentage reduction in metabolic activity (% RMA) in order to determine the measure of cytotoxicity exerted by 3-hydroxy fatty acids on amoebae. Towards this end, 0.2 mM yielded 12% RMA while 1 mM yielded 43% RMA (Figure 7B). These findings suggest that 3-hydroxy fatty acids do not negatively affect the growth of amoebae, more so at estimated physiological concentrations; and this is crucial as cryptococcal cells require a viable host for intracellular survival. Moreover, the former also suggests that amoebae do not metabolise 3-hydroxy fatty acids to support their growth, as addition of 3-hydroxy C9:0 to the culture media did not increase their metabolic activity. This further supports the argument that 3-hydroxy fatty acids may be particularly secreted

to impair the phagocytic machinery of amoeba. When considering the effect of nonanoic acid (C9:0; which is structurally close to 3-hydroxy C9:0) on amoeba's metabolic activity, this molecule was observed to exert a greater negative effect on the metabolic activity of amoeba cells at 0.2 mM ($p < 0.05$) and 1 mM ($p < 0.05$) compared to 3-hydroxy fatty acids (Figure 7 C). The latter also translated into significantly higher %RMA at 0.2 mM (42%) and 1 mM (73%) compared to 3-hydroxy C9:0 (Figure 7D).

2.5 DISCUSSION

Cryptococcus species are reported to act as parasites of phagocytic cells wherein they can successfully establish an intracellular lifestyle (Levitz et al., 1999, Heitman et al., 2011) and in some instances, use phagocytic cells to disseminate to other organs (Voelz and May, 2010). To date, the capsule has been credited with shielding cells against the effects of phagocytic cells. This study shows that in addition to capsules, 3-hydroxy fatty acids also have a role to play possibly in concert with the capsule. To the point, our findings suggest that 3-hydroxy fatty acids are secreted at a high enough concentration to display a protective quality that prevents internalisation and possibly promote intracellular survival. Importantly, at this concentration, the molecule does not kill or negatively affect its host cell and this is paramount to any parasite's quest to successfully establishing an intracellular lifestyle.

Phagocytosis 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 (Freeman and Grinstein, 2014). To be internalised, a cell must first bind to the surface of amoeba. However, as Bottone et al. pointed out, not all cells that are bound are internalised and eventually phagocytosed by amoebae (Bottone et al., 1994). The latter may be driven, for example, by antagonistic microbial secretomes that inhibit microbial recognition. Towards this end, 3-hydroxy fatty acids could impair the intracellular signalling mechanism that is required to initiate phagocytosis. To ascertain this, further studies are however required in order to understand the molecular mechanism(s) that confer the protective quality of cryptococcal 3-hydroxy fatty acids. The idea of lipids being trafficked into the extracellular environment of microbes including *C. neoformans*, has previously been reported on by Rodrigues et al. (2007). In their paper, these authors point out that upon release – the secreted molecules can mediate host-pathogen interactions in favour of the pathogen leading to a diseased-state. Importantly, they highlight lipids such as phosphotidylserine that may decrease microbial killing – thus promoting pathogenesis (Rodrigues et al., 2007). It is also not surprising that our 3-hydroxy fatty acid may impair the phagocytic machinery of amoebae as the role of fatty acids in preventing phagocytosis is well documented. In one study, short chained fatty acids were reported to prevent the release of lysozymes (Eftimiadi et al., 1987), and in other another, fatty acids reduced hydrogen peroxide production thus impairing phagocytosis (Bellinati-Pires et al., 1993).

It was encouraging to also observe that inhibition of cryptococcal 3-hydroxy fatty acids made cells more susceptible to amoebal phagocytosis. Aspirin inhibits of 3-hydroxy fatty acid production by outcompeting the product of 3-hydroxyacyl-CoA dehydrogenase activity in the mitochondria as a result of structural similarities between aspirin's active metabolite, salicylate, and the product (Glasgow et al., 1999). It will be interesting to determine if treatment of cryptococcal cells with aspirin will also chemosensitise macrophages to successfully phagocytose internalised cells as we have shown. However, when using aspirin, caution should be taken to realise the desired outcome to the exclusion of adverse effects. In this study, we tested aspirin at a concentration, 1 mM, which is considered as optimum for safe and effective therapy in the blood (Levy, 1976). The latter thus points to the possible application of aspirin as a candidate drug that can prevent cryptococcal cells from establishing an intracellular lifestyle.

2.6 REFERENCES

Bellinati-Pires, R., Waitzberg, D. L., Salgado, M. M. and Carneiro-Sampaio, M. M. (1993). Functional alterations of human neutrophils by medium-chain triglyceride emulsions: evaluation of phagocytosis, bacterial killing, and oxidative activity. *J. Leukoc. Biol.* 53*,* 404-410

Bottone, E. J., Perez, A. A., Gordon, R. E. and Qureshi, M. N. (1994). Differential binding capacity and internalisation of bacterial substrates as factors in growth rate of *Acanthamoeba* spp. *Med. Microbiol.* 40, 148-154. DOI: 10.1099/0022261540-2-148

Casadevall, A. and Perfect, J. R. (1998). *Cryptococcus neoformans*. Washington, DC: American Society for Microbiology Press

Ciccoli, R., Sahi, S., Singh, S., Prakash, H., Zafiriou, M. P., Ishdorj, G., Kock, J. L. F. and Nigam, S. (2005). Oxygenation by cyclooxygenase-2 (COX-2) of 3-hydroxyeicosatetraenoic acid (3-HETE), a fungal mimetic of arachidonic acid produces a cascade of novel bioactive 3-hydroxy-eicosanoids. *Biochem. J.* 390*,* 737-747. DOI: 10.1042/BJ20041995

Deva, R., Ciccoli, R., Schewe, T., Kock, J. L. F. and Nigam, S. (2000). Arachidonic acid stimulates cell growth and forms a novel oxygenated metabolite in *Candida albicans*. *Biochim. Biophys. Acta.* 1486, 299-311. DOI: 10.1016/S1388-1981(00)00073-1

Eftimiadi, C., Buzzi, E., Tonetti, M., Buffa, P., Buffa, D., van Steenburgen, M.T., de Graaff, J. and Botta, G.A. (1987). Short-chain fatty acids produced by anaerobic bacteria alter the physiological responses of human neutrophils to chemotactic peptide. *J. Infect.* 14, 43-53. DOI: 10.1016/S0163-4453(87)90808-5

Feldmesser, M., Tucker, S. and Casadevall, A. (2001). *Cryptococcus neoformans*: Intracellular or extracellular? *Trends Microbiol.* 9, 418. DOI: 10.1016/S0966-842X(01)02035-2

Freeman, S. A. and Grinstein, S. (2014). Phagocytosis: receptors, signal integration, and the cytoskeleton. *Immunol. Rev.* 262, 193-215. DOI: 10.1111/imr.12212

Fuchs, B. B. and Mylonakis, E. (2006). Using non-mammalian hosts to study fungal virulence and host defense. *Curr. Opin. Microbiol.* 9, 346-351. DOI: 10.1016/j.mib.2006.06.004

Gehmlich, K., Dodd, M. S., Allwood, J. W., Kelly, M., Bellahcene, M., Lad, H. V., Stockenhuber, A. Hooper, C., Ashrafian, H., Redwood, C. S., Carrier, L. and Dunn, W. B. (2015). Changes in the cardiac metabolite caused by perhexiline treatment in a mouse model of hypertrophic cardiomyopathy. *Mol. BioSyst.* 11, 564-573. DOI: 10.1039/c4mb00594e

Glasgow, J. F. T., Middleton, B., Moore, R., Gray, A. and Hill, J. (1999). The mechanism of inhibition of β-oxidation by aspirin metabolites in skin fibroblasts from Reye's syndrome patients and controls. *Biochim. Biophys. Acta.* 1454, 115-125. DOI: 10.1016/S0925-4439(99)00025-3

Heitman, J., Kozel, T. R., Kwon-Chung, K. J., Perfect, J. R. and Casadevall, A. (2011). *Cryptococcus* from Human Pathogen to Model Yeast. Washington, DC: American Society for Microbiology Press

Horwitz, M. A. (1983). The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. *J. Exp. Med.* 158, 2108-2126. DOI: 10.1084/jem.158.4.1319

Kozel, T. R. and Gotschlich, E. C. (1982). The capsule of *Cryptococcus neoformans* passively inhibits phagocytosis of the yeast by macrophages. *J. Immunol.* 129, 1672-1680. DOI: 10.1111/j.1567-1364.2006.00113.x

Levitz, S. M. and Boekhout, T. (2006). *Cryptococcus*: The once-sleeping giant is fully awake. *FEMS Yeast Res.* 6, 461-462. DOI: 10.1111/j.1567-1364.2006.00113.x

Levitz, S. M., Nong, S. H., Seetoo, K. F., Harrison, T. S., Speizer, R. A. and Simon, E. R. (1999). *Cryptococcus neoformans* resides in an acidic phagolysosome of human macrophages. *Infect. Immun.* 67*,* 885-890. PMID: 9916104

Levy, G. (1976). Pharmacokinetics of aspirin in man. *J. Investig. Dermatol.* 67*,* 667-668. DOI: 10.1111/1523-1747.ep12544495

Lin, X. and Heitman, J. (2006). The biology of the *Cryptococcus neoformans* species complex. *Annu*. *Rev*. *Microbiol*. 60, 69-105. DOI:10.1146/annurev.micro.142102

Polat, Z. A., Walochnik, J., Obwaller, A., Vural, A., Dursun, A. and Arici, M. K. (2014). Miltefosine and polyhexamethylene bigua- nide: a new drug combination for the treatment of *Acanthamoeba* keratitis. *Clin. Exp. Ophthalmol.* 42, 151-158. DOI: 10.1111/ceo.12120

Rodrigues, M. L., Nimrichter, L., Oliveira, D. L., Franses, S., Miranda, K., Zaragoza, O., Alvarez, M., Nakouzi, A., Feldmesser, M. and Casadevall, A. (2007). Vesicular polysaccharide export in *Cryptococcus neoformans* is a eukaryotic solution to the problem of fungal trans-cell wall transport. *Eukaryot. Cell.* 6, 48-59. DOI: 10.1128/EC.00318-06

Sebolai, O. M., Pohl, C. H., Botes, P. J., Strauss, C. J., van Wyk, P. W. J., Botha, A. and Kock, J. L. F. (2007). 3-Hydroxy fatty acids found in capsules of *Cryptococcus neoformans*. *Can. J. Microbiol.* 53*,* 809-812. DOI: 10.1139/W07-045

Sebolai, O. M., Pohl, C. H., Botes, P. J., van Wyk, P. W. J. and Kock, J. L. F. (2008). The influence of acetylsalicylic acid on oxylipin migration in *Cryptococcus neoformans var. neoformans* UOFS Y-1378. *Can. J. Microbiol.* 54*,* 91-96. DOI: 10.1139/w07-114

Steenburgen, J. N., Shuman, H. A. and Casadevall, A. (2001). *Cryptococcus neoformans* interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. *Proc. Natl. Acad. Sci.* 98, 15245-15250. DOI: 10.1073/pnas.261418798

Steenburgen J. N. and Casadevall, A. (2003). The origin and maintenance of virulence for the human pathogenic fungus *Cryptococcus neoformans*. *Microbes Infect.* 5, 667-675. DOI: 10.1016/S1286-4579(03)00092-3

van Wyk, P. W. J. and Wingfield, M. J. (1991). Ascospores ultrastructure and development in *Ophiostoma cucullatum*. *Mycologia* 83, 698-707. DOI: 10.2307/3760427

Voelz, K. and May, R. C. (2010). Cryptococcal interactions with the host immune system. *Eukaryot. Cell* 9, 835-846. DOI: 10.1128/EC.00039-10

Table 1. Molecule annotation of the cryptococcal 3-hydroxy fatty acid molecule based on the comparison of retention time (RT) and accurate *m/z* determinants.

Sample details		**Characterization of 3-hydroxy fatty acid extracts**				
Experimental class	**Replicates**	**RT (min)**	***Diagnostic MS/MS peaks**			**†Analyte structure**
			$[M-H]^-$	**$[M+M-H]^-$**	**$[M+Na+M-H]^-$**	
Analytical standard	*n* = 5	3.70	173.118	347.244	369.225	3-Hydroxy C9:0
C. neoformans	*n* = 5	3.72	173.118	347.244	369.225	3-Hydroxy C9:0
C. gattii	*n* = 5	3.72	-	-	-	Unknown

* $[M-H]^-$ = Deprotonated molecular ion; $[M+M-H]^-$ = Hydrogen-bound dimer ion; and $[M+Na+M-H]^-$ = Sodium-bridged dimer ion.

† 3-Hydroxy C9:0 = 3-Hydroxy nonanoic acid.

2.7 FIGURES

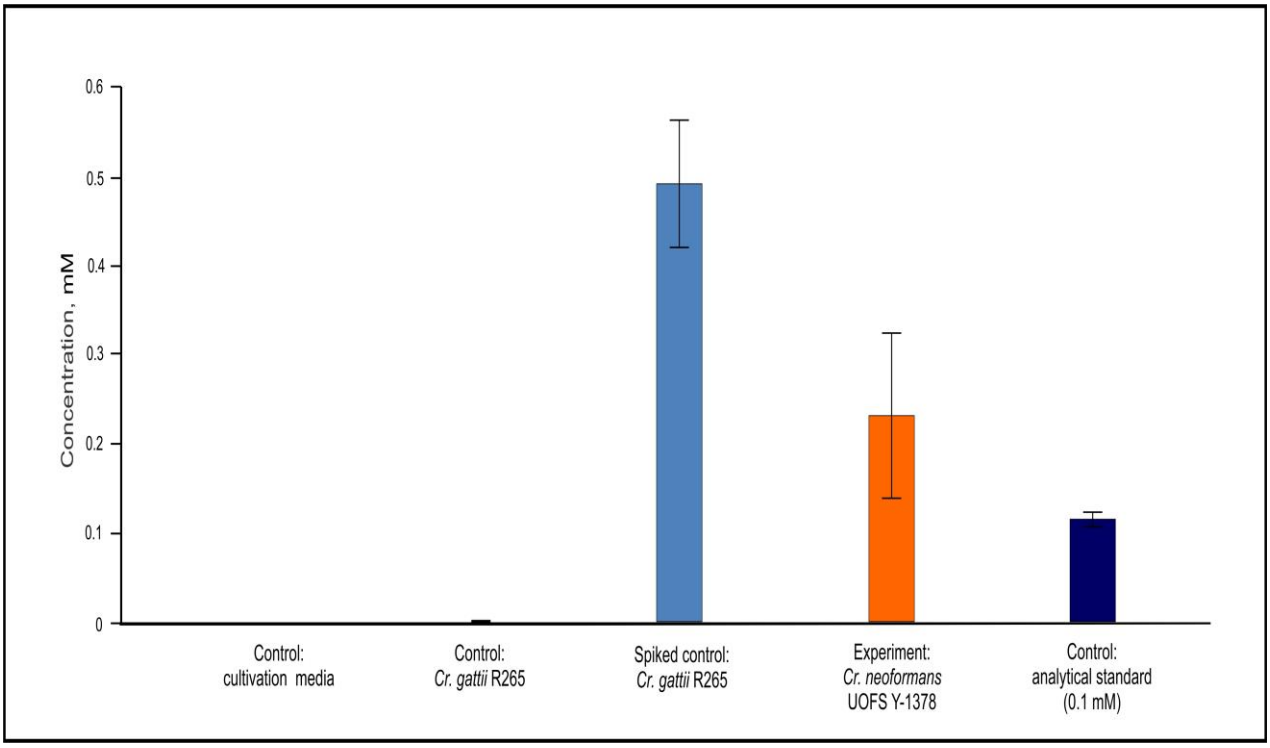

Figure 1. Estimation of the physiological concentrations of 3-hydroxy nonanoic acid produced by *C. neoformans* UOFS Y-1378. Using a two-point calibration method, we extrapolated the secreted concentration to be in the range: 0.1 mM to 0.4 mM. For each sample class, five biological replicates were analysed and the values are the mean with standard deviation indicated by error bars. *C. gattii* was spiked with the analytical standard solution at a final concentration of 0.66 mM.

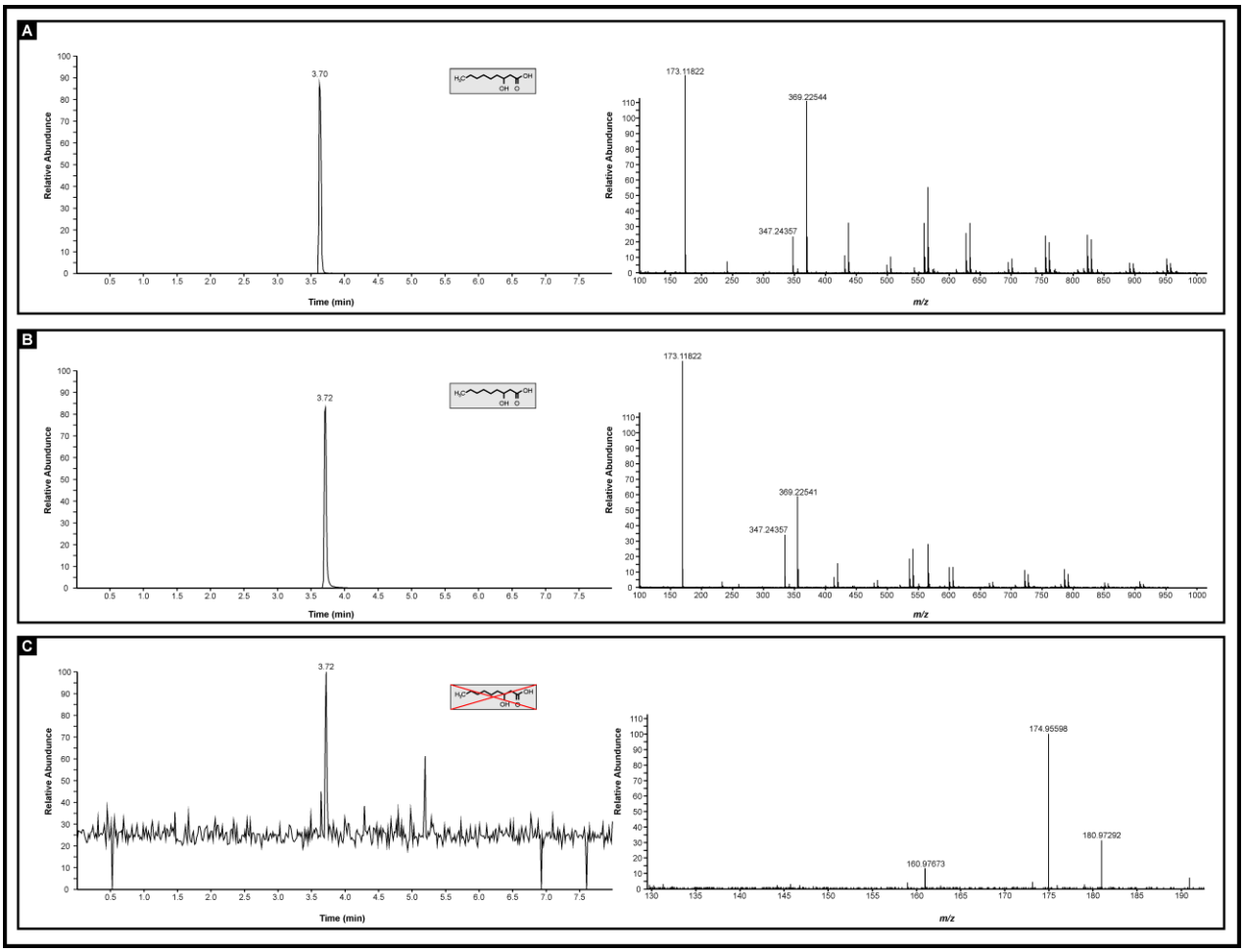

Figure 2. Characterisation of 3-hydroxy fatty acids in *C. neoformans* UOFS Y-1378 and *C. gattii* R265. (A) = The EIC (top left graphic) obtained for the analytical standard compound (3-hydroxy nonanoic acid) and its corresponding mass spectrum (top right graphic). (B) = The EIC (middle left graphic) obtained for *C. neoformans* analyte and its corresponding mass spectrum (middle right graphic). *C. neoformans* had a similar profile (i.r.o. retention time and diagnostic peaks) suggesting this fungus produces 3-hydroxy nonanoic acid. (C) = The EIC (bottom left graphic) obtained for *C. gattii* and its corresponding mass spectrum (bottom right graphic). *C. gattii* produced an unknown metabolite, which had a similar retention time to the analytical standard compound**.**

However, we could not detect diagnostic peaks ($[M-H]^-$, $[M+Na+M-H]^-$ and $[M+M-H]^-$) that are characteristic of our metabolite of interest (3-hydroxy nonanoic acid).

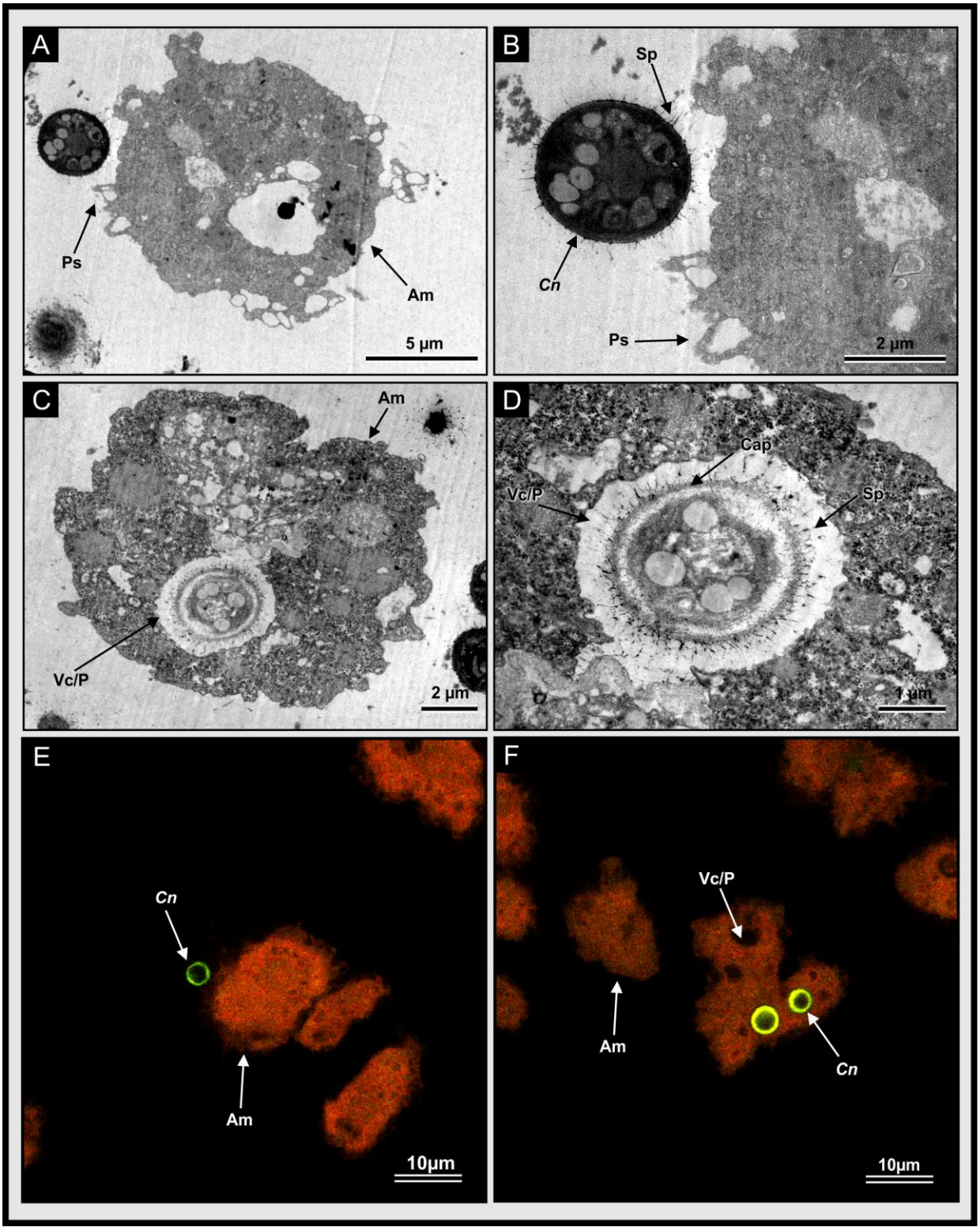

Figure 3. The visualisation of interactive moments between *C. neoformans* UOFS Y-1378 cells and amoebae, *Acanthamoeba castellanii* LMPE 187. (A) Shows a cryptococcal cell

in close proximity to an amoeba cell. (B) Is an enlargement of (A) showing pseudopodia about to catch a cryptococcal cell. (C) Shows a cryptococcal cell, with characteristic capsular protuberances, that has been internalised while (D) is an enlargement of an internalised cell inside the food vacuole. (E) And (F) are fluorescent micrographs, which show internalised *Cryptococcus* cells (depicted in a green-colour) inside an autofluorescing amoebae (depicted in an orange colour). Am = amoeba, C = *Cryptococcus*, Cap = capsule, Fv/p = food vacuole or phagosome, Ps = pseudopodia. Sp = spiky protuberances.

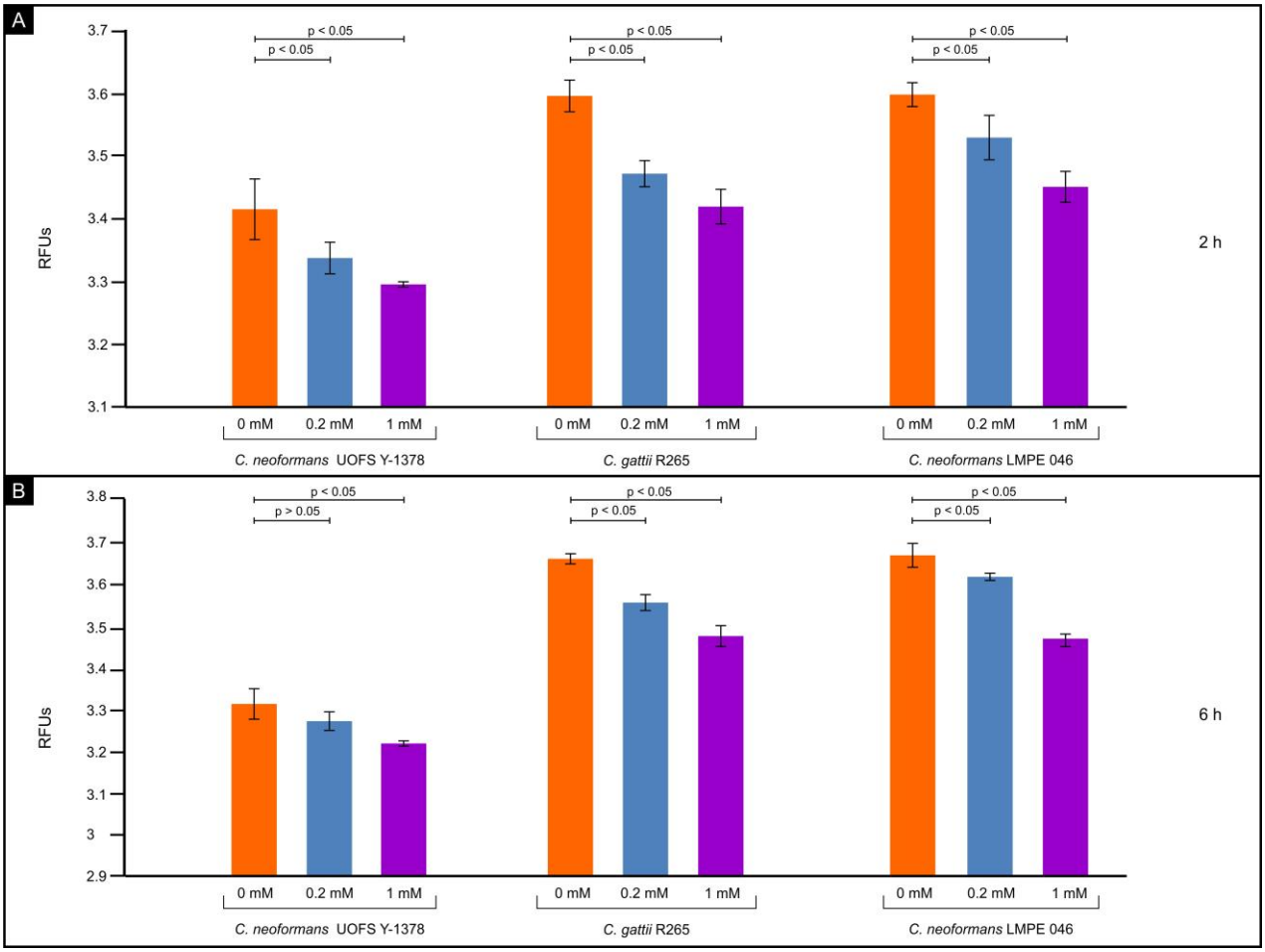

Figure 4. The results of the phagocytosis assay of cryptococcal cells co-cultured with amoeba. Through using the phagocytosis stain, pHrodo™ Green Zymosan A BioParticles, the appetite of amoebae to internalise *C. neoformans* UOFS Y-1378, *C. neoformans* LMPE 046 and *C. gattii* R265, in the presence and absence of 3-hydroxy fatty acids, was measured after 2 h (A) and 6 h (B) of co-culturing. At both time intervals, *C. neoformans* UOFS Y-1378 cells were more resistant ($p < 0.05$) to amoebae compared to *C. neoformans* LMPE 046 cells and *C. gattii* R265 cells based on the recorded relative fluorescence units. Addition of 3-hydroxy fatty acids to *C. neoformans* UOFS Y-1378, *C. neoformans* LMPE 046 and *C. gattii* R265 cells, at both 2 h and 6 h, seem to have significantly ($p < 0.05$) decreased their vulnerability towards amoebae when compared to

C. *neoformans* UOFS Y-1378, *C. neoformans* LMPE 046 and *C. gattii* R265 cells in the absence of 3-hydroxy fatty acids.

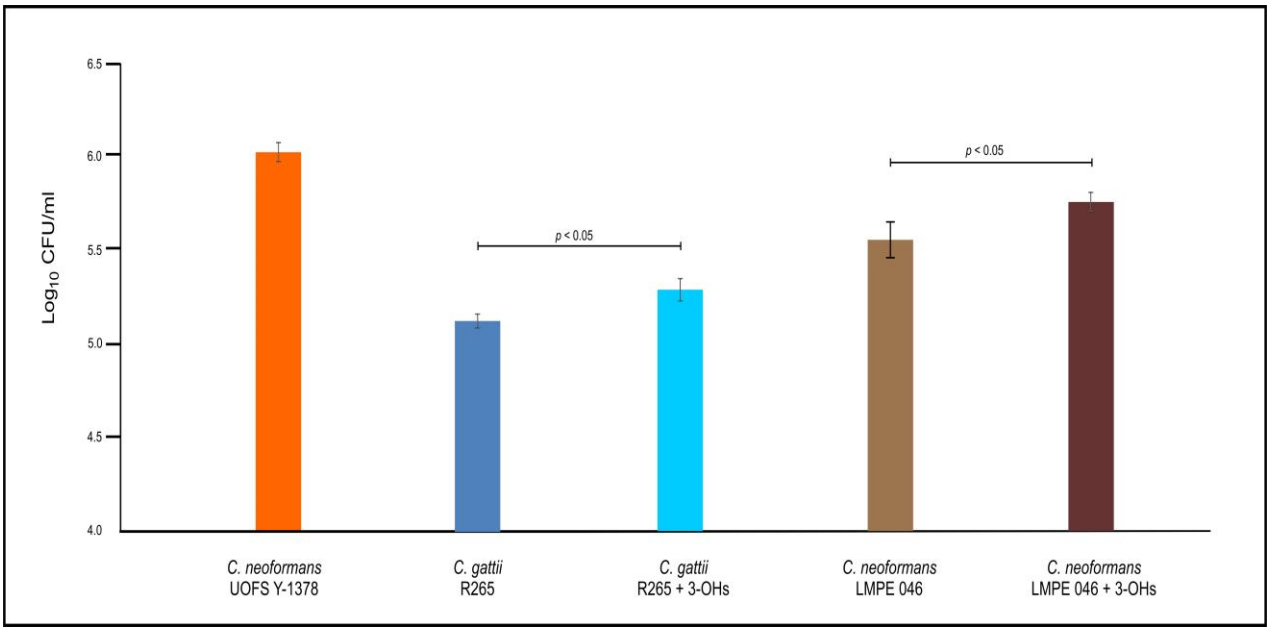

Figure 5. The results of the survival assay of cryptococcal cells co-cultured with amoeba. The results obtained corroborate the phagocytosis assay outcome. As expected, *C. neoformans* UOFS Y-1378 was naturally resistant to amoebae and thus more cells ($p < 0.05$) were recovered on agar plates compared to recovered cells of *C. neoformans* LMPE 046 and *C. gattii* R265. The addition of 3-hydroxy fatty acids to cultures of *C. neoformans* UOFS Y-1378, *C. neoformans* LMPE 046 and *C. gattii* R265 made these cells also resistant to amoebae.

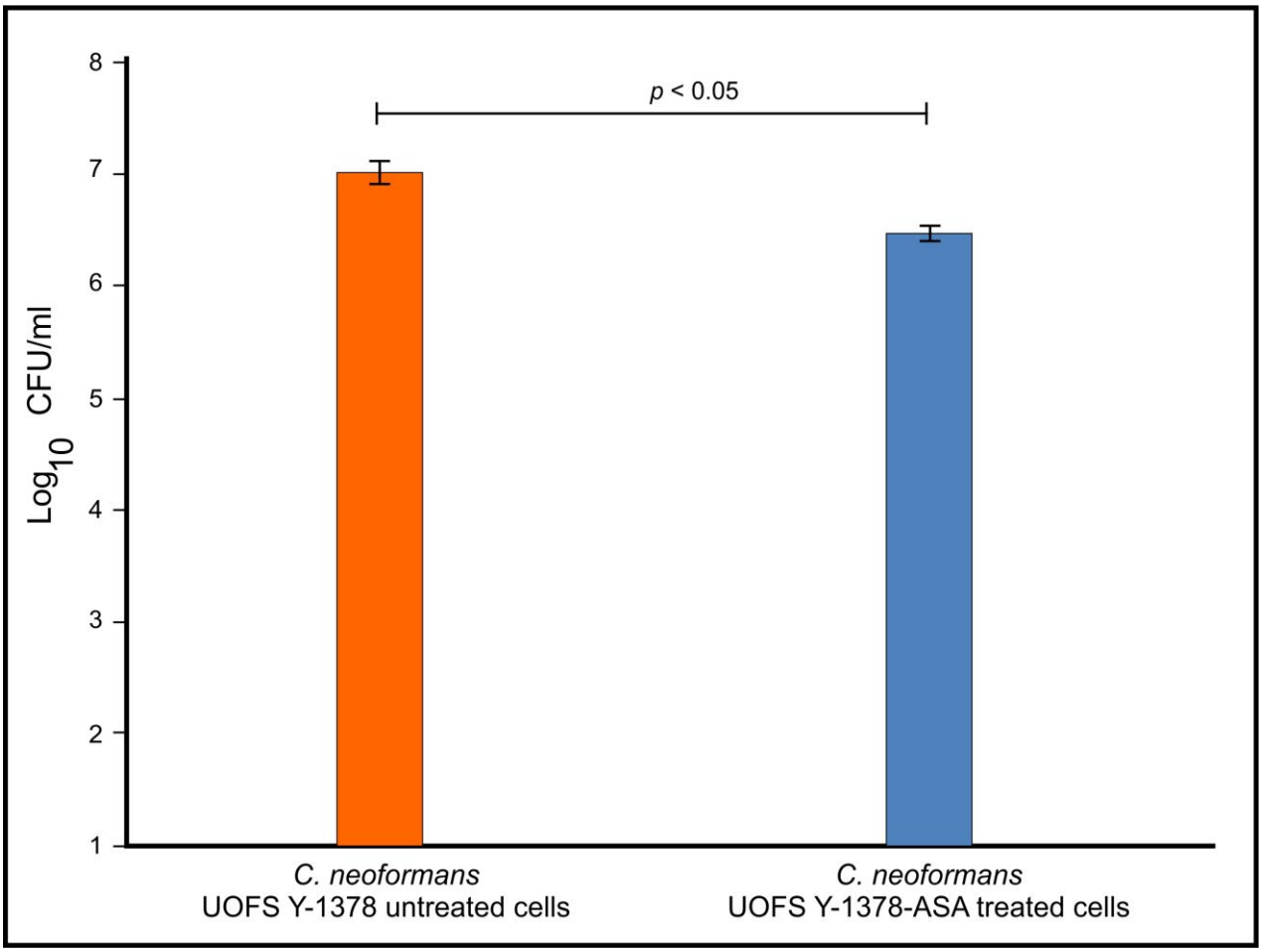

Figure 6. The results of the survival of aspirin-treated *C. neoformans* UOFS Y-1378 cells and non-treated *C. neoformans* UOFS Y-1378 cells co-cultured with amoeba. The inhibition of 3-hydroxy fatty acids by aspirin made *C. neoformans* more susceptible ($p < 0.05$) to amoebae as less cells were recovered on agar plates when compared to non-treated cells.

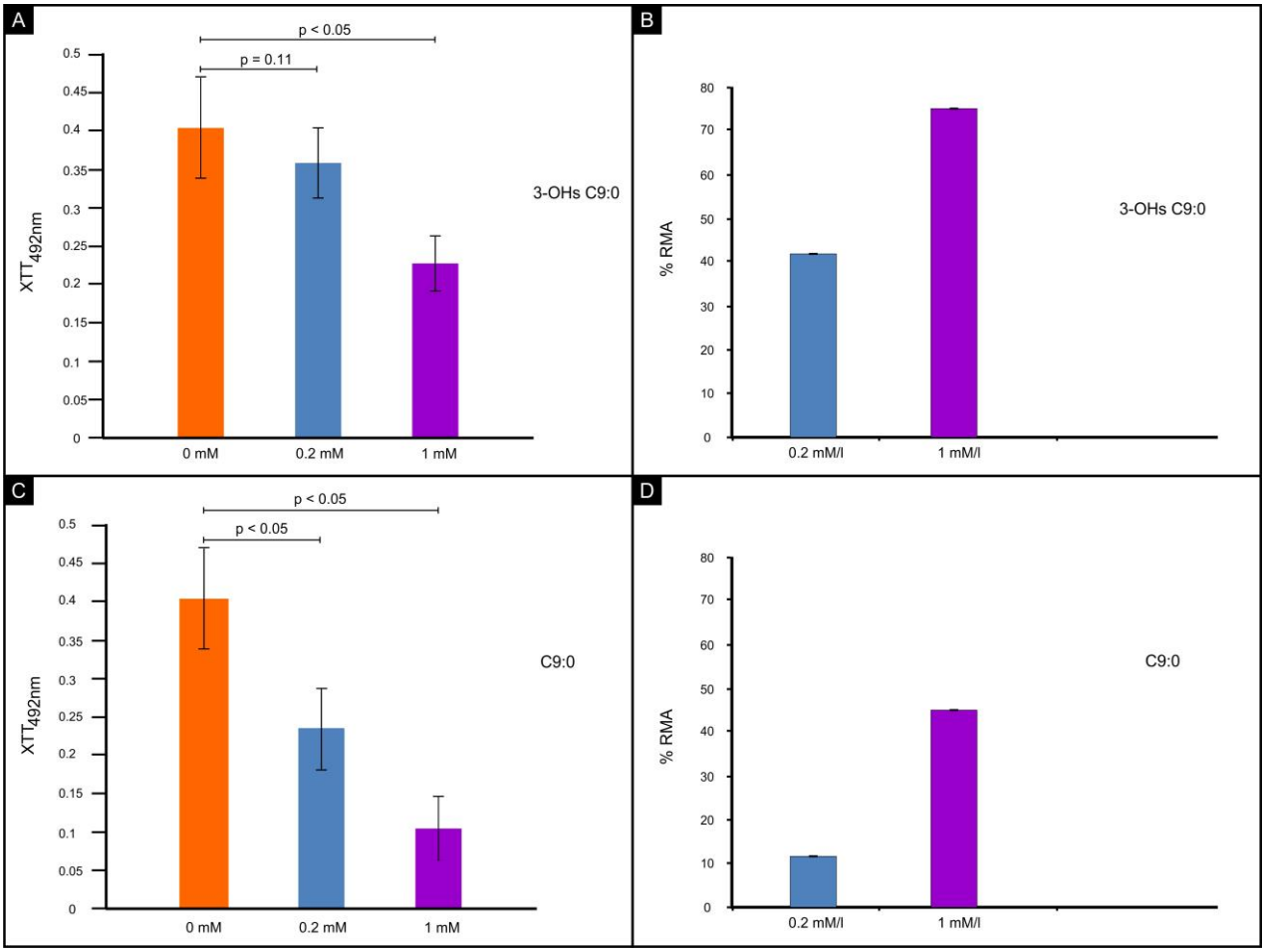

Figure 7. The cytotoxicity effect of 3-hydroxy fatty acids on amoebae. 3-Hydroxy nonanoic acid (3-OHs C9:0) was shown to be less effective as an anti-amoebae agent (A, B) when compared to nonanoic acid (C9:0) (C), which yielded a higher percentage reduction in metabolic activity (% RMA) (D). At the estimated physiological concentration of 0.2 mM, 3-hydroxy nonanoic acid yielded XTT reduction readings that were highly comparable (p = 0.11) to readings obtained for non-treated amoebae after a 48 h period. The latter suggest that this molecules does not negative affect the growth of amoebae.

2.8 SUPPLEMENTARY FIGURE

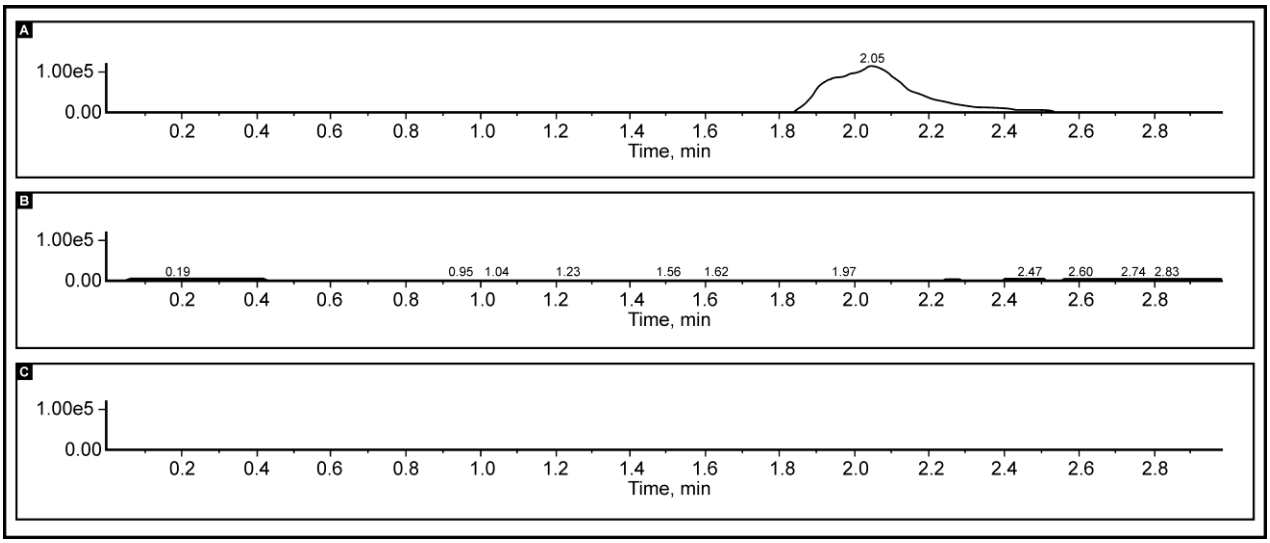

Figure S1. Determination of 3-hydroxy fatty acids production by cultures of *C. neoformans* LMPE 046 and *Acanthamoeba castellanii* LMPE 187. (A) = The EIC obtained for the analytical standard compound (3-hydroxy nonanoic acid) showing elution of our metabolite of interest after 2.05 min. (B) = The EICs obtained for *C. neoformans* LMPE 046 and (C) *Acanthamoeba castellanii* LMPE 187 showing no elution of 3-hydroxy nonanoic acid after 2.05 min, suggesting these species do not produce 3-hydroxy nonanoic acid.

CHAPTER 3

ELUCIDATION OF THE ROLE OF 3-HYDROXY FATTY ACIDS IN *CRYPTOCOCCUS*-AMOEBA INTERACTIONS

This study was performed by the candidate and has been published. As a result, repetition of some information could not be avoided.

Madu, U. L., Ogundeji, A. O., Pohl, C. H., Albertyn, J. and Sebolai, O. M. (2017). Elucidation of the role of 3-hydroxy fatty acids in *Cryptococcus*-amoeba interactions. *Front*. *Microbiol*. 8, 765. DOI: 10.3389/fmicb.2017.00765

Candidate's contribution: Co-designed experiments, performed the experiments and wrote the draft manuscript.

3.1 ABSTRACT

We previously reported that 3-hydroxy fatty acids promoted the survival of cryptococcal cells when acted upon by amoebae. To expand on this, the current study sought to explain how these molecules may protect cells. Our data suggest that 3-hydroxy fatty acids may subvert the internalisation of cryptococcal cells via suppression of the levels of a fetuin A-like amoebal protein, which may be important for enhancing phagocytosis. Additionally, we show that an acapsular strain (that is devoid of 3-hydroxy fatty acids) was protected against the effects of hydrogen peroxide when exogenous 3-hydroxy fatty acids were present, but not in the absence of 3-hydroxy fatty acids. A similar response profile was noted when a strain with a capsule was challenged with hydrogen peroxide. We also show that cryptococcal cells that naturally produce 3-hydroxy fatty acids were more resistant to the effects of amoebapore (an amoeba-specific hydrolytic enzyme), compared to cells that do not produce these molecules. Taken together, our findings suggest that 3-hydroxy fatty acids possess an anti-phagocytic activity that may be expressed when cells interact with macrophages. This may allow the yeast cells to evade immuno-processing.

Keywords: 3-Hydroxy fatty acids (3-hydroxy C9:0), Amoeba, *Cryptococcus*, Phagocytosis, Shield.

3.2 INTRODUCTION

3-Hydroxy fatty acids are lipid-based molecules that are oxygenated and whose amphiphilic quality is evident when immersed in water (Tsitsigiannis and Keller, 2007; Sebolai et al., 2012). The chemical structure of these molecules is characterised by a hydroxyl group on the beta carbon from the carboxylic group – and their hydrocarbon chain can be linear or branched, saturated or unsaturated and may be linked to other macromolecules (Kock et al., 2003). It has been reported that these molecules are produced via an incomplete enzymatic pathway similar to mitochondrial beta oxidation (Kock et al., 2007; Sebolai et al., 2012). Herein, 3-hydroxy fatty acids are intermediate products that are poorly catalysed by 3-hydroxyacyl-CoA dehydrogenase and consequently are secreted (Sebolai et al., 2008). This escape from the mitochondria suggests these molecules have no apparent function in the primary catabolism of the concerned organism.

It is thus not surprising that 3-hydroxy fatty acids are regarded as secondary metabolites that have, for example, been successfully implicated in microbial pathogenesis (Sebolai et al., 2012). To illustrate this point, Ciccoli and co-workers (2005) reported that *Candida albicans* can scavenge arachidonic acid from a host's infected cells and convert it into a 3-hydroxy fatty acid (3-hydroxy eicosatetraenoic acid) via incomplete beta oxidation. In turn, the produced 3-hydroxy fatty acid can act as a substrate for the host's cyclooxygenase-2 enzyme, leading to the production of 3-hydroxy prostaglandins, which are more potent pro-inflammatory mediators compared to non-hydroxylated prostaglandins.

The presence of 3-hydroxy fatty acids has also been documented in *Cryptococcus* (*C*.) *neoformans* (Sebolai et al., 2007). In an attempt to elucidate the function(s) of these molecules, Madu and co-workers (2015) studied how they may influence *Cryptococcus*-amoeba interactions These researchers concluded that, at a physiological concentration, 3-hydroxy fatty acids protected cryptococcal cells by impairing the ability of amoeba to internalise and phagocytose cells. However, the mechanisms underlying this protective ability to 3-hydroxy fatty acids have not been elucidated yet. Therefore, the current study aims to determine how these molecules may impair the phagocytic process and/or protect internalised cells. This study may offer insight into how these molecules could assist cells to evade immuno-processing when acted upon by macrophages, which are said to have evolved from free-living amoebae (Siddiqui and Khan, 2012).

3.3 MATERIALS AND METHODS

Strains, cultivation and standardisation

The fungal strains, *C. gattii* R265 (does not produce 3-hydroxy C9:0), *C. neoformans* LMPE 046 (does not produce 3-hydroxy C9:0), *C. neoformans* UOFS Y-1378 (produces 3-hydroxy C9:0) and *C. neoformans* LMPE 101 (acapsular strain that does not produce 3-hydroxy C9:0), were used in the study. These strains were grown 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) plates at 30^{o}C for 48 h. Cells (representing the respective strains) were separately standardised using a haemocytometer (Marienfield, Germany) to a final

concentration of 1 x 10^6 cells/ml in 10 ml of distilled water before use. For amoebapore (BioWorld, United States) sensitivity testing, five colonies (representing either R265, LMPE 046, UOFS Y-1378 or LMPE 101) were scraped off and suspended in 10 ml of distilled water. At the end, the cells were standardised to prepare final inocula of between 0.5 x 10^5 and 2.5 x 10^5 CFU/ml in RPMI 1640 medium (Sigma-Aldrich, South Africa) according to EUCAST guidelines (Arendrup et al., 2015).

The amoeba (*Acanthamoeba castellanii*) strain LMPE 187, used in the study, was grown on peptone-yeast extract glucose broth, PYG (ATCC medium 30234^{TM}) at 30^{o}C for a week. The cells (in trophozoite state) were then standardised using a haemocytometer to a final concentration of 1 x 10^5 cells/ml in 10 ml of sterile PYG broth and the viability of cells was determined to be 80% using trypan blue.

3-Hydroxy fatty acids

The 3-hydroxy fatty acid standard (3-hydroxy C9:0), was purchased from Larodan (Sweden). This compound was tested at a final concentration of 0.2 mM, which is the estimated physiological concentration secreted by *C. neoformans* UOFS Y-1378 (Madu et al., 2015).

Glucuronoxylomannan (GXM) isolation

Crude GXM was isolated in anticipation of experiments wherein it was used for comparison purposes. The isolation was done according to a protocol previously detailed by Zaragoza et al. (2008). In short, a loopful of scraped *C. neoformans* UOFS Y-1378 (grown for 48 h on YM plates) colonies was used to inoculate a 500 ml conical flask containing 250 ml of Difco-yeast nitrogen base (YNB) broth (6.7 g/l YNB and 40 g/l glucose; Becton, Dickinson and Company, United States) that was supplemented with 2% glucose (Merck, South Africa). The flask was placed on a rotary shaker (160 rpm/min) and cultivated for 48 h at 30°C. The cells were washed five times with distilled water and finally suspended in 40 ml of distilled water. The cells were then heat-killed at 55°C for 30 min before being irradiated (dosage of approximately 200 grays) with a ^{137}Cs gamma-irradiator (kept at HEPRO Cape, South Africa) in order to make cells shed their capsule (GXM). After irradiation, the cells were centrifuged at 3500 x *g* to mobilise the shed GXM into the supernatant.

Next, the lipids were removed from the collected supernatant via using a modified Folch lipid extraction protocol. In brief, the supernatant (10 ml) was transferred to a 50 ml Falcon tube (Becton-Dickinson Labware, United States). Following which, 10 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 (10 ml) was added to the above solution and allowed to stand for a further 20 min. The chloroform fraction that contained 3-hydroxy fatty acids was disposed and the water

fraction containing the GXM was kept. The isolated GXM was then lyophilised, weighed and reconstituted in sterile water to make a stock solution of 10 mg/ml.

To confirm the isolation, 100 µl of the delipidated stock solution was subjected to an agglutination (Meridian Bioscience, Inc., United States) assay and ELISA (IMMY, United States) assay. Both assays were performed according to manufacturer's protocol. The optical density (OD) readings were measured at 450 nm using a spectrophotometer (Biochrom EZ Read 800 Research, United Kingdom).

Fetuin A and NOX-1 (NADPH oxidase-1) ELISA assays

A 100 µl suspension (in YPG broth) of standardised amoebae was seeded into designated wells of a sterile microtitre plate (Greiner Bio-One, Germany). Next, the cells were challenged with 3-hydroxy C9:0 (1:1, v/v) at a final concentration of 0.2 mM or 100 µl of isolated GXM to yield a final concentration of 2.5 mg/ml. Non-treated amoeba cells were included as a control. The plate was then incubated at 30^{o}C for a 6 h period. At the end, the supernatant was aspirated and accordingly transferred to a sterile microtitre plate specific for the human-based fetuin A (Abcam, United Kingdom) while the cells were kept to harvest the lysate according to a protocol and materials provided in the NADPH oxidase-1 (human-based) kit (LifeSpan BioSciences, United States). The lysate was then transferred to a sterile microtitre plate specific for NADPH oxidase-1. The supernatant and lysate plates were respectively treated according to the manufacturer's protocols. Each ELISA plate was then read at 450 nm using a Biochrom EZ spectrophotometer.

The effect of hydrogen peroxide on cryptococcal cells

Standardised cells (1 x 10^6 cells/ml), in distilled water, of the strain LMPE 101 were used in this test. A 75 µl suspension of cells was separately transferred to wells of a sterile microtitre plate. These cells were then treated with 75 µl of hydrogen peroxide (60 µM). In other wells, cells (75 µl) were treated with 75 µl of hydrogen peroxide (240 µM) in the presence of either GXM (150 µl of 10 mg/ml) or 3-hydroxy C9:0 (150 µl of 0.8 µM). The plate was incubated for 3 h at 37^{o}C. After incubation, the plate was gently agitated and the contents aspirated. Of this, 100 µl was used to make a 1:10 dilution in distilled water. Thereafter, 100 µl was used to create a uniform lawn of cells on a YM agar plate. The plates were incubated for 48 h at 30^{o}C before colony forming units (CFU) were counted. The considered concentration of hydrogen peroxide was similar to that reported to accumulate inside phagosomes (Dupré-Crochet et al., 2013).

In addition, the levels of hydrogen peroxide in the medium containing hydrogen peroxide-treated cells and hydrogen peroxide-treated in the presence of GXM or 3-hydroxy C9:0 was measured as previously detailed by Noble and Gibson (1970). In brief, a microtitre plate was prepared as above-mentioned and at a specific time interval i.e. 3-h, 6-h and 24-h, an absorbance reading was taken at 360 nm. This reading was then multiplied by a molar extinction coefficient value of 43.6, in order to estimate the levels of hydrogen peroxide.

The effect of amoebapore on cryptococcal cells

The effect of amoebapore (amoebal antimicrobial peptide) was independently assessed on biological duplicates of the strains R265, LMPE 046, UOFS Y-1378 and LMPE 101 at final concentrations (prepared in RPMI 1640 medium) of 3.25 µM or 7.5 µM. In short, a 100 µl of the standardised inoculum (0.5×10^5 and 2.5×10^5 CFU/ml) was aliquoted into designated wells of a microtitre plate. The cells, in the wells, were then treated with 100 µl of amoebapore (at twice the stated desired final concentrations). The plate was incubated for 48 h at 37^{o}C. After 48 h, the optical density (OD) of each well was measured at 562 nm using a Biochrom EZ spectrophotometer. The resultant effect of amoebapore on the growth of treated cells was compared to that of non-treated cells.

In order to assess if amoebapore killed cells by creating pores on cell walls, cells (i.e. UOFS Y-1378 and LMPE 101) treated with 7.5 µM, were prepared on a separate microtitre plate as stated above (for drug sensitivity testing) before being viewed by transmission electron microscopy (TEM). The cells were prepared for TEM viewing according to the method of van Wyk and Wingfield (1991). In short, the cells were chemically fixed with 1.0 M (pH 7) sodium phosphate-buffered gluteraldehyde (3%) for 3 h and then for 1.5 h in similarly buffered osmium tetroxide. The cells were next dehydrated in a graded acetone series, embedded in epoxy resin and polymerized at 70^{o}C for 8 h. An LKB III Ultratome was used to cut 60-nm sections with glass knives. Uranyl acetate was used to stain sections for 10 min, followed by lead citrate for 10 min and the preparation viewed with a Philips EM 100 transmission electron microscope.

Statistical note

All experiments, unless otherwise stated, were performed in triplicate. Where appropriate, a student t-test was used to determine the statistical significance of data between the control and different experimental conditions.

3.4 RESULTS

3-Hydroxy fatty acids alter the phagocytic behaviour of amoebae

In this study, fetuin A was assayed based on prior unpublished work in our group, which revealed that macrophages challenged with 3-hydroxy fatty acids produced this protein. This protein is a major globulin component of serum that has been shown to regulate the function of macrophages (Wang et al., 1998). More to the point, this glycoprotein has been reported to mediate the uptake of particulate material such the uptake of some bacteria (*Escherichia coli* and *Staphylococcus aureus*; both are not known to produce 3-hydroxy fatty acids) including apoptotic cells, by phagocytes (van Oss et al. 1974; Jersmann et al., 2003).

The GXM, which was used for comparison purposes in this experiment and others, was successfully isolated (Figure S1). Our results suggest that amoebae also produce a fetuin A-like protein (Figure 1), which may have a similar function as that expressed in macrophages. This assertion is reasonable as macrophages have been theorised to have

evolved from amoebae (Siddiqui and Khan, 2012) and in particular, this protein may have evolved from cystatin by gene duplication. Hence, it is conceivable that amoeba may have this protein. When considering the results, it was clear that there was a significant reduction ($p < 0.01$) in the levels of fetuin A when cells were challenged with either GXM (74% reduction) or 3-hydroxy C9:0 (68% reduction) compared to non-treated amoebae. Given the function of this protein, which (among others) is to promote macrophage phagocytosis (Jersmann, et al., 2003), the GXM results are conceivable primarily because this molecule is said to be anti-phagocytic (Zaragoza et al., 2009; Del Poeta, 2004). Therefore, by extension, the data suggests that like GXM, 3-hydroxy C9:0 (which is intimately associated with cryptococcal capsules (Sebolai et al., 2007)) may also be anti-phagocytic. This finding may, in part, explain the findings of Madu et al., wherein they reported that the addition of 3-hydroxy C9:0 to the co-culture media resulted in reduced ability of amoebae to internalise cryptococcal cells compared to the absence of this molecule (Madu et al., 2015). Taken together, this points to impairment of the phagocytic process.

3-Hydroxy fatty acids protect cells against oxidative damage

It was first sought to determine if 3-hydroxy C9:0 affected the functioning of NADPH oxidase-1, which could in turn alter the amount of oxidative radicals produced inside the amoebal food vacuoles (Figure 2). Here, no meaningful deductions could be made as there was no statistical significance ($p > 0.05$) between the non-treated amoebae and experimental conditions i.e. 3-hydroxy C9:0-treated amoebae or GXM-treated amoebae.

Therefore to compliment this experiment, it was then sought to determine if 3-hydroxy C9:0 could protect cells against the oxidative effects of hydrogen peroxide (when cells were directly challenged) (Figure 3). Previously, Zaragoza and co-workers (2008) showed that GXM protected cells against hydrogen peroxide. Our results confirm this finding. When comparing LMPE 101's non-treated cells (an acapsular strain that also does not produce 3-hydroxy C9:0 (data not shown)) data to the corresponding hydrogen peroxide-treated cells', GXM-treated cells' and 3-hydroxy C9:0-treated cells' data; significantly fewer CFUs were obtained under the treatment conditions. More importantly, this strain yielded significantly more CFUs ($p < 0.05$) when challenged with hydrogen peroxide in the presence of GXM or 3-hydroxy C9:0 compared to when this strain was challenged with hydrogen peroxide in the absence of the two test molecules. A similar response pattern was observed when examining the results obtained for the strain UOFS Y-1378, which has a capsule (Figure S2). Also of interest, the levels of hydrogen peroxide in the medium did not dissipate over a 24-h period and remained more or less the same (Figure S3). This suggests that the reported results specifically for hydrogen peroxide-treated cells and hydrogen peroxide-treated in the presence of GXM or 3-hydroxy C9:0, are not as a result of diminished levels of hydrogen peroxide in the test medium but rather speak to the protective quality of GXM and 3-hydroxy C9:0.

3-Hydroxy fatty acids protect cells against amoebapore

Amoebapore is an amoeba-specific digest peptide that creates pores in cell walls of targeted cells (Leippe et al., 1994). To test the effectiveness of amoebapore in killing

cryptococcal cells, we considered its activity against four strains: 1) one that has a capsule and produces 3-hydroxy C9:0 (UOFS Y-1378), 2) two that have capsules but no 3-hydroxy C9:0 (R265 and LMPE 046), and 3) one without a capsule and 3-hydroxy C9:0 (LMPE 101) (Figure 4). Our data show that the strain UOFS Y-1378 was resistant to amoebapore at both test concentrations, and was rather stimulated by this protein. The same phenomenon was observed for the other two capsular strains (R265 and LMPE 046), although the level of growth stimulation was less than for UOFS Y-1378. However, the strain LMPE 101 (which lacks a capsule and 3-hydroxy fatty acids) was sensitive to amoebapore. Based on these results, it is reasonable to conclude that 3-hydroxy C9:0 in UOFS Y-1378 may have acted in concert with the capsule to shield cells hence resulted in the greatest level of resistance that were recorded. On the other hand, the absence of 3-hydroxy fatty acids (in applicable strains) led to a decrease in the levels of resistance. Logically, the strain without 3-hydroxy fatty acids and the capsule was the most susceptible. Nonetheless, an independent study is required to determine if addition of 3-hydroxy fatty acids would lead to strains showing resistance towards amoebapore.

TEM was subsequently performed on UOFS Y-1378 and LMPE 101 in order to assess the effect of this amoebapore on the ultrastructure of cells. The cell wall of UOFS Y-1378 was without pores while that of LMPE 101 showed a nick in the cell wall (Figure 5). The latter could explain the resistance and susceptibility that was respectively expressed by UOFS Y-1378 and LMPE 101.

3.5 DISCUSSION

C. neoformans has evolved to emerge as an important disease-causing microorganism (Casadevall and Perfect, 1998; Levitz and Boekhout, 2006). In part, this is attributed to the ability of cells to subvert the functioning of macrophages. Of concern is how cells are able to take up residency in macrophages, without evoking an immunological response, and subsequently disseminate (Voelz and May, 2010). Some researchers have reasoned that species such amoeba, which predate on microbes like *Cryptococcus*, serve as a pivotal training ground wherein a prey is selected to produce an arsenal of microbial survival factors (Molmeret et al., 2005; Casadevall, 2008).

Traditionally, the capsule has been credited with protecting cells against phagocytic cells (Bose et al., 2003; Del Poeta, 2004; Zaragoza et al., 2009). However, it has emerged that there are other molecules, which may also promote the survival of cryptococcal cells when acted upon phagocytic cells, such as the anti-phagocytic protein-1 (Luberto et al., 2003). Thus, the current study is an extension to the uncovering of other protective molecules, and importantly positions 3-hydroxy fatty acids as being anti-phagocytic in nature. In particular, we showed that these molecules altered the phagocytic behaviour of amoebae by affecting fetuin A and protected cells against oxidative damage and amoebapore. These results offer insight into how these molecules may impair the intracellular signalling mechanism, mediated by the fetuin-A-like protein, which is required to initiate internalisation. Interactions between fetuin (which is an acute phase protein) and fatty acids are known (Pal et al., 2012). Importantly, the interactions of this protein

with mediators that inhibit or promote inflammation or those that inhibit or promote phagocytosis as is the case in the current paper, define its unique properties. Toward this end, it was not surprising to note 3-hydroxy C9:0 decreased the levels of this protein. The effect of 3-hydroxy C9:0 may not be long term and may be abrogated – as it is possible that during an infection, the manifestation of a subclinical inflammation in response to the presence of invading cells may increase its levels. Thus, this may explain how cells may escape phagocytosis (arguably maybe only initially) even though they have been recognised and destined for internalisation.

In addition, we have shown how some cells that were unable to escape internalisation, could survive the harsh internal environment (hydrogen peroxide) found inside the food vacuole of amoebae or phagosomes of macrophages. The role of lipids in preventing oxidative damage is not clear cut. Typically, a radical like hydrogen peroxide (has a predilection of targeting double bonds of unsaturated fatty acids) can enter a cell by simple diffusion and gain access to its membranes. Based on the results we recorded in the current study, it is possible that 3-hydroxy C9:0 may have been incorporated into the membranes and in turn, influenced the fluidity. In his dissertation, Mochochoko showed that treatment of *Pseudomonas* cells with 3-hydroxy C9:0 impaired membrane fluidity as cells were unable to traffic molecules in and out (unpublished data). In such a scenario, hydrogen peroxide (which can easily diffuse into a cell) may have been prevented from entering the cells hence its ineffectiveness to reduce CFU counts in the presence of 3-hydroxy C9:0.

There are also lipid-soluble non-enzymatic antioxidants such as tocopherol, which can neutralise the effect of hydrogen peroxide. To the point, this molecule can be targeted and oxidised by reactive oxygen species like hydrogen peroxide, and in turn prevent lipid peroxidation (Latifi et al., 2009). Based on the latter, it is also possible that after incorporation of 3-hydroxy C9:0 into membranes, these molecules may like-wise be targeted and oxidised – hence the effect of hydrogen peroxide was abrogated. With respect to observed amoebapore results, it is possible that 3-hydroxy C9:0 may form a complex with this antimicrobial peptide to neutralise it. Lipids have previously been shown to block the activity of many antimicrobial peptides that exhibit membrane disrupting properties (Malanovic and Lohner, 2016). In their paper, these authors further make the point that trapping of antimicrobial peptides sufficiently reduces the total concentration of these peptides on the cytoplasmic membrane.

Taken together, these findings may contribute to our understanding of how cryptococcal cells may survive amoebal phagocytosis as well as provide insight into survival mechanisms inside phagosomes of macrophages during dissemination. This is supported by the idea that cryptococcal cell would recognise both amoebae and macrophages as the same predatory cell (Steenburgen et al., 2001; Steenburgen and Casadevall, 2003), and thus this fungus will accordingly display the same defensive behaviour in order to escape phagocytic processing.

In conclusion, the presented results highlight the importance of 3-hydroxy fatty acids to the pathogenesis of *C. neoformans*. It will be interesting to see if similar results could be observed in macrophages as well as in laboratory animals, and additionally, if creation of a 3-hydroxy fatty acid-deficient mutant (via deletion of beta-oxidation gene(s)) will result in a less virulent strain that is susceptible to amoeba or macrophage action.

3.6 REFERENCES

Arendrup, M. C., Guinea, J., Cuenca-Estrella, M., Meletiadis, J., Mouton, J. W., Lagrou, K., et al. (2015). European Committee for Antimicrobial Susceptibility Testing (EUCAST) definitive document E.Def 7.3: method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts. EUCAST, Denmark.

Bose, I., Reese, A. J., Ory, J. J., Janbon, G. and Doering, T. L. (2003). A yeast undercover: the capsule of *Cryptococcus neoformans. Eukaryot. Cell* 2: 655-663. DOI: 10.1128/EC.2.4.655-663.2003

Casadevall, A. (2008). Evolution of intracellular pathogens. *Ann. Rev. Microbiol*. 62: 19-33. DOI: 10.1146/annurev.micro.61.080706.093305

Casadevall, A. and Perfect, J. R. (1998). *Cryptococcus neoformans*. Washington, USA: ASM Press.

Ciccoli, R., Sahi, S., Singh, S., Prakash, H., Zafiriou, M. P., Ishdorj, G., Kock, J. L. F. and Nigam, S. (2005). Oxygenation by cyclooxygenase-2 (COX-2) of 3-hydroxyeicosatetraenoic acid (3-HETE), a fungal mimetic of arachidonic acid produces a cascade of novel bioactive 3-hydroxy-eicosanoids. *Biochem. J.* 390: 737-747. DOI: 10.1042/BJ20041995

Del Poeta, M. (2004). Role of phagocytosis in the virulence of *Cryptococcus neoformans*. *Eukaryot. Cell* 3: 1067-1075. DOI: 10.1128/EC.3.5.1067-1075.2004

Dupré-Crochet, S., Erard, M. and Nübe, O. (2013). ROS production in phagocytes: why, when, and where? *J. Leukoc. Biol*. 94: 657-670. DOI: 10.1189/jlb.1012544

Jersmann, H. P. A., Dransfield, I. and Hart, S. P. 2003. Fetuin/α_2-HS glycoprotein enhances phagocytosis of apoptotic cells and macropinocytosis by human macrophages. Clin. Sci. 105: 273-278. DOI: 10.1042/CS20030126

Kock, J. L. F., Sebolai, O. M., Pohl, C. H., van Wyk, P. W. J. and Lodolo, E. J. (2007). Oxylipin studies expose aspirin as antifungal. *FEMS Yeast Res*. 7: 1207-1217. DOI: 10.1111/j.1567-1364.2007.00273.x

Kock, J. L. F., Strauss, C. J., Pohl, C. H. and Nigam, S. (2003). The distribution of 3-hydroxy oxylipins in fungi. *Prostaglandins Other Lipid Mediat*. 71: 85-96. PMID: 14518554

Latifi, A., Ruiz, M. and Zhang, C. C. (2009). Oxidative stress in cyanobacteria. *FEMS Microbiol.* 33: 258-278. DOI: 10.1111/j.1574-6976.2008.00134.x

Leippe, M., Andra, J. and Muller-Eberhard, H. J. (1994). Cytolytic and antibacterial activity of synthetic peptide derived from amoebapore, the pore-forming peptide of Entamoeba histolytica. *Proc. Natl. Acad. Sci.* 99: 2602-2606. PMID: 8146160

Levitz, S. M. and Boekhout, T. (2006). *Cryptococcus*: The once-sleeping giant is fully awake. *FEMS Yeast Res.* 6: 461-462. DOI: 10.1111/j.1567-1364.2006.00113.x

Luberto, C., Martinez-Marino, B., Taraskiewicz, D., Bolanos, B., Chitano, P., Toffaletti, D. L., et al. (2003). Identification of App1 as a regulator of phagocytosis and virulence of *Cryptococcus neoformans*. *J. Clin. Invest*.112: 1080-1094. DOI: 10.1172/JCI18309

Madu, U. L., Ogundeji, A. O., Mochochoko, B. M., Pohl, C. H., Albertyn, J., Swart, C. W., et al. (2015). Cryptococcal 3-hydroxy fatty acids protect cells against amoebal phagocytosis. *Front. Microbiol.* 6: 1-12. DOI: 10.3389/fmicb.2015.01351

Malanovic, N. and Lohner, K. (2016). Antimicrobial peptides targeting Gram-positive bacteria. *Pharmaceuticals.* 9: 59. DOI: 10.3390/ph9030059

Molmeret, M., Horn, M., Wagner, M., Santic, M. and Abu-Kwaik, Y. (2005). Amoebae as training grounds for intracellular bacterial pathogens. *Appl. Environ. Microbiol*. 71: 20-28. DOI: 10.1128/AEM.71.1.20-28.2005

Noble, R. and Gibson, Q. H. (1970). The reaction of ferrous horseradish peroxide with hydrogen peroxide. JBC 245: 2409-2413.

Pal, D., Dasgupta, S., Kundu, R., Maitra, S., Das, G., Mukhopadhyay, S. et al. (2012). Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. *Nat. Med*. 18: 1279–1285. DOI: 10.1038/nm.2851

Sebolai, O. M., Pohl, C. H., Botes, P. J., Strauss, C. J., van Wyk, P. W. J., Botha, A. and Kock, J. L. F. (2007). 3-Hydroxy fatty acids found in capsules of *Cryptococcus neoformans. Can. J. Microbiol*. 53: 809-812. DOI: 10.1139/W07-045

Sebolai, O. M., Pohl, C. H., Botes, P. J., van Wyk, P. W. J. and Kock, J. L. F. (2008). The influence of acetylsalicylic acid on oxylipin migration in *Cryptococcus neoformans* var. *neoformans* UOFS Y-1378. *Can J. Microbiol*. 54: 91-96. DOI: 10.1139/w07-114

Sebolai, O. M., Pohl, C. H., Kock, L. J. F., Chaturvedi, V. and Del Poeta, M. (2012). The presence of 3-hydroxy oxylipins in pathogenic microbes. *Prostaglandins Other Lipid Mediat*. 97: 17-21. DOI: 10.1016/j.prostaglandins.2011.11.001

Siddiqui, R. and Khan, N. A. (2012). *Acanthamoeba* is an evolutionary ancestor of macrophages: a myth or reality? *Exp. Parasitol.* 130: 95-97. DOI: 10.1016/j.exppara.2011.11.005

Steenbergen, J. N. and Casadevall, A. (2003). The origin and maintenance of virulence of the human fungus *Cryptococcus neoformans*. *Microb. Infect*. 5: 667-675. DOI: 10.1016/S1286-4579(03)00092-3

Steenbergen, J. N., Shuman, H. A. and Casadevall, A. (2001). *Cryptococcus neoformans* interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. *Proc. Natl. Acad. Sci*. 98: 15245-15250. DOI: 10.1073/pnas.261418798

Tsitsigiannis, D. I. and Keller, N. P. (2007). Oxylipins as developmental and host-fungal communication signals. *Trends Microbiol*. 15: 109-118. DOI: 10.1016/j.tim.2007.01.005

van Oss, C. J., Gillman, C. F., Bronson, P.M. and Border, J. R. (1974). Opsonic properties of human serum alpha-2-hs glycoprotein. *Immunol*. *Comm*. 3: 329-335. PMID: 4142783

van Wyk, P. W. J. and Wingfield, M. J. (1991). Ascospores ultrastructure and development in *Ophiostoma cucullatum. Mycologia* 83: 698-707. DOI: 10.2307/3760427

Voelz, K. and May, R. C. (2010). Cryptococcal interactions with the host immune system. *Eukaryot. Cell* 9: 835-846. DOI: 10.1128/EC.00039-10

Wang, H., Zhang, M., Bianchi, M., Sherry, B., Sama, A. and Tracey, K.J. (1998). Fetuin (alpha2-HS-glycoprotein) opsonizes cationic macrophage deactivating molecules. *Proc. Natl. Acad. Sci.* 95: 14429-14434. PMID: 9826717

Zaragoza, O., Chrisman, C. J., Castelli, M. V., Frases, S., Cuenca-Estrella, M., Rodríguez-Tudela, J. L. et al. (2008). Capsule enlargement in *Cryptococcus neoformans* confers resistance to oxidative stress suggesting a mechanism for intracellular survival. *Cell Microbiol*. 10: 2043-2057. DOI: 10.1111/j.1462-5822.2008.01186.x

Zaragoza, O., Rodrigues, M. L., De Jesus, M., Frases, S., Dadachova, E. and Casadevall, A. (2009). The capsule of the fungal pathogen *Cryptococcus neoformans. Adv. Appl. Microbiol.* 68: 133-216. DOI: 10.1016/S0065-2164(09)01204-0

3.7 FIGURE LEGENDS

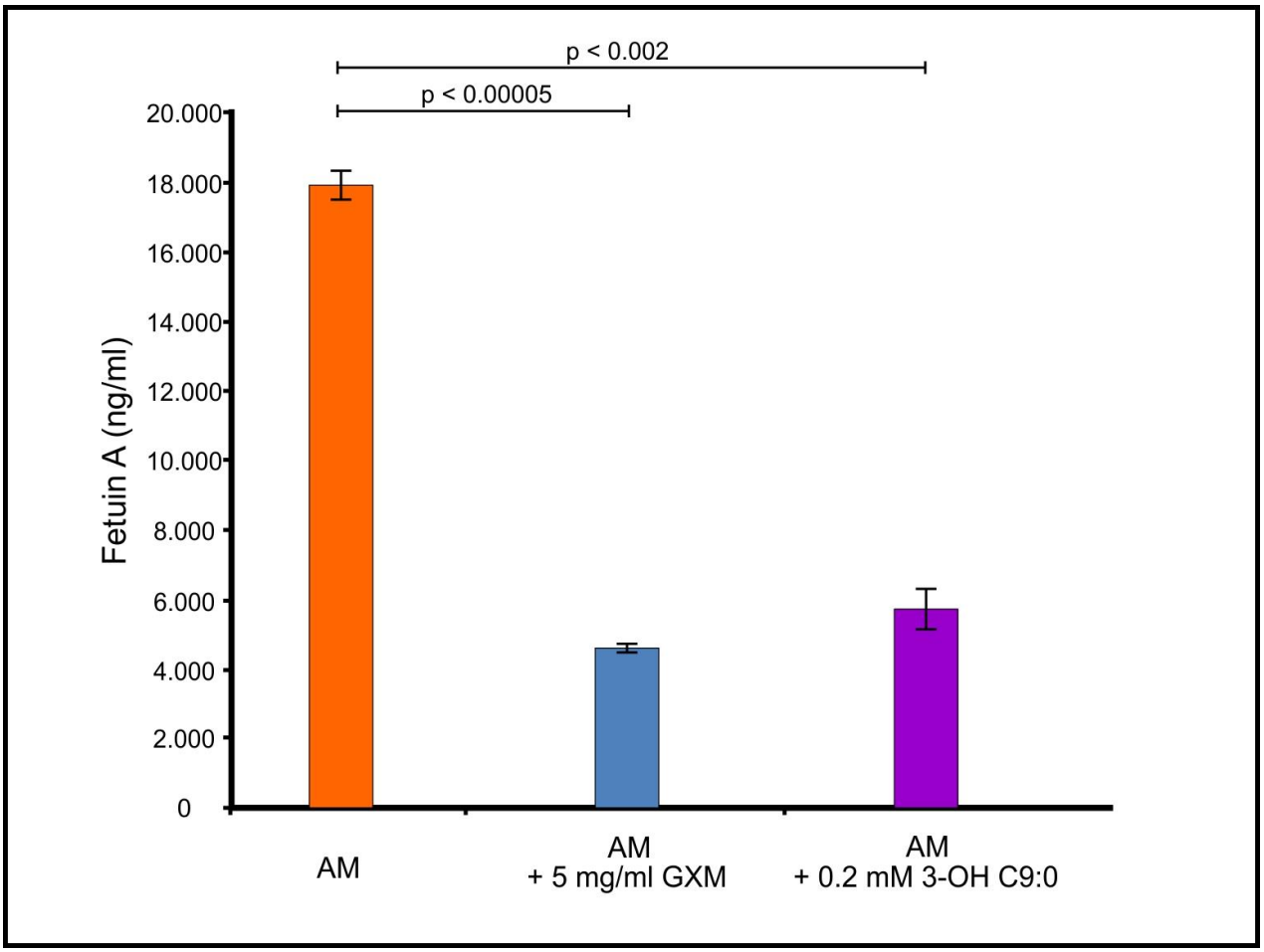

Figure 1. The ELISA quantitative results showing the levels of fetuin A present across the different experimental conditions i.e. GXM-treated amoeba (AM) and 3-OH C9:0-treated amoeba, including the control i.e. non-treated amoeba cells. The results indicate that 3-hydroxy C9:0, similar to GXM, may possess an anti-phagocytic quality. GXM = Glucuronoxylomannan; 3-OH C9:0 = 3-hydroxy C9:0.

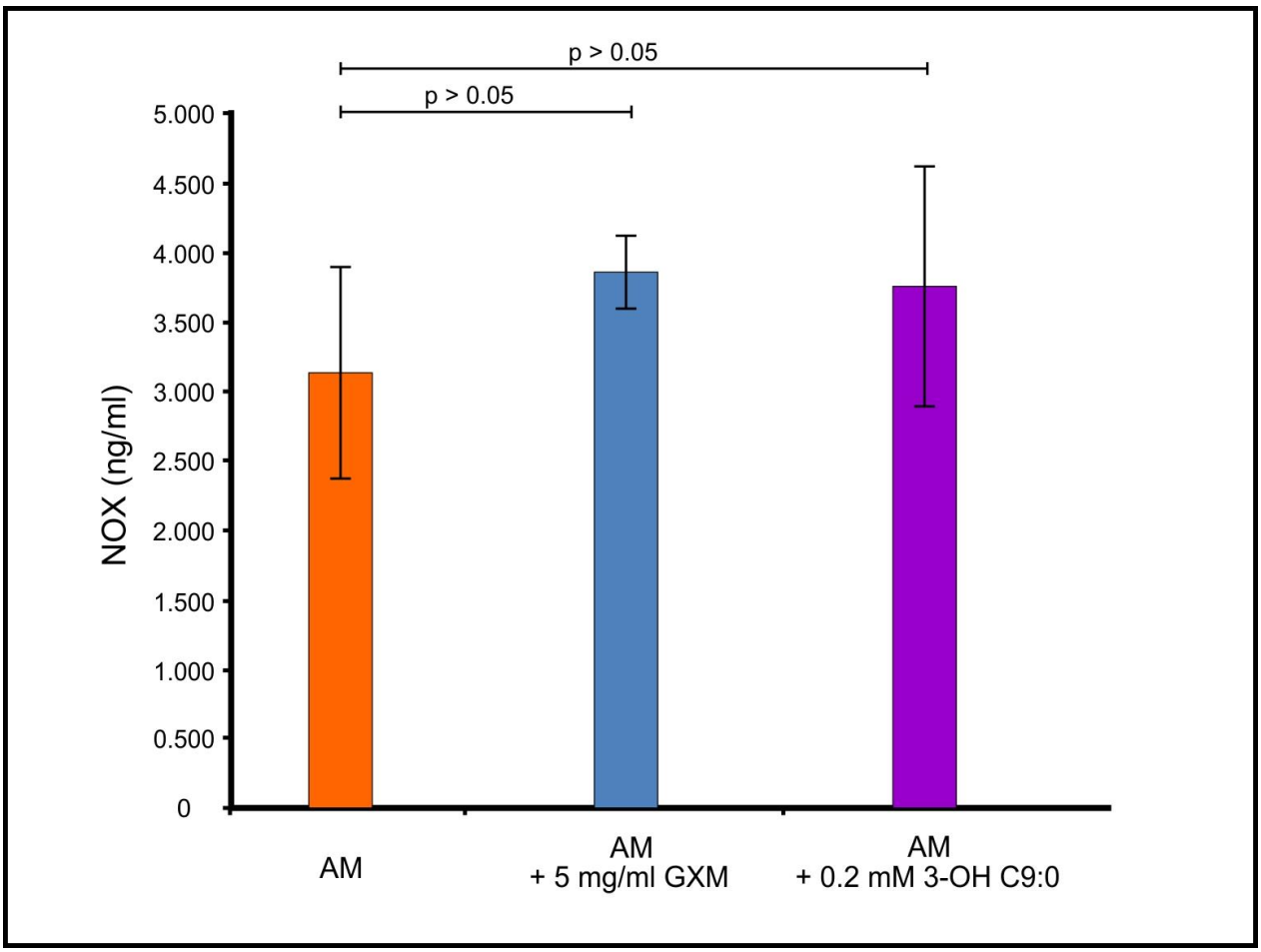

Figure 2. The ELISA quantitative results showing the levels of NADPH oxidase-1 (NOX) present across the different experimental conditions i.e. GXM-treated amoeba (AM) and 3-OH C9:0-treated amoeba, including the control i.e. non-treated amoeba cells. There was no significant ($p > 0.05$) difference between the control results and the experimental conditions.

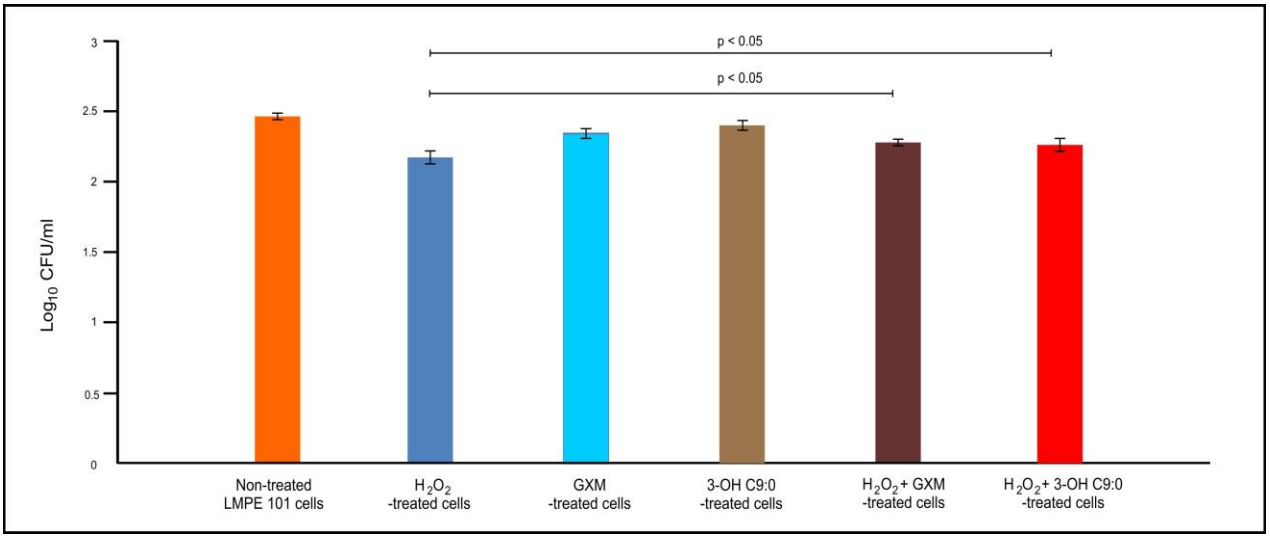

Figure 3. The survival assay results showing the number of colony forming units (CFUs) that were enumerated after the acapsular strain *C. neoformans* LMPE 101 was exposed to different treatment conditions. The results suggest that 3-hydroxy C9:0, similar to GXM, protect cells against oxidative damage.

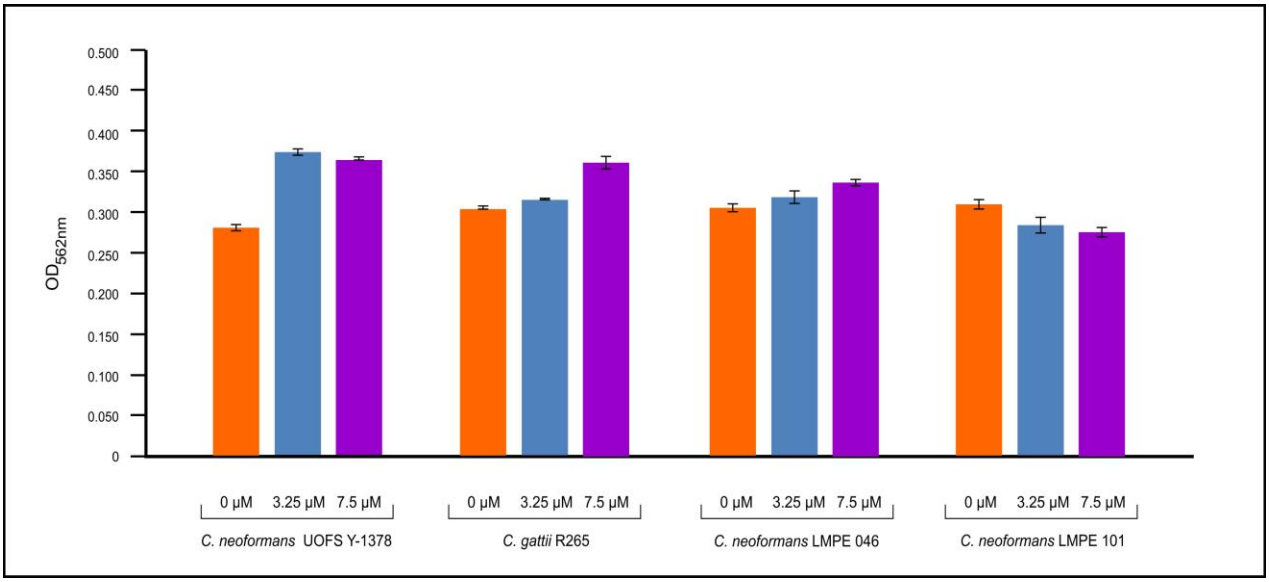

Figure 4. Results of the effect of amoebapore on the growth of different cryptococcal strains i.e. *C. neoformans* UOFS Y-1378 (has 3-hydroxy C9:0), *C. gattii* R265 (has no 3-hydroxy C9:0), *C. neoformans* LMPE 046 (has 3-hydroxy C9:0) and *C. neoformans* LMPE 101 (is acapsular and has no 3-hydroxy C9:0). When considering the growth results, it is evident that the strain with 3-hydroxy C9:0 was much more resistant to the effects of amoebapore when compared to the other three strains that do not have 3-hydroxy C9:0.

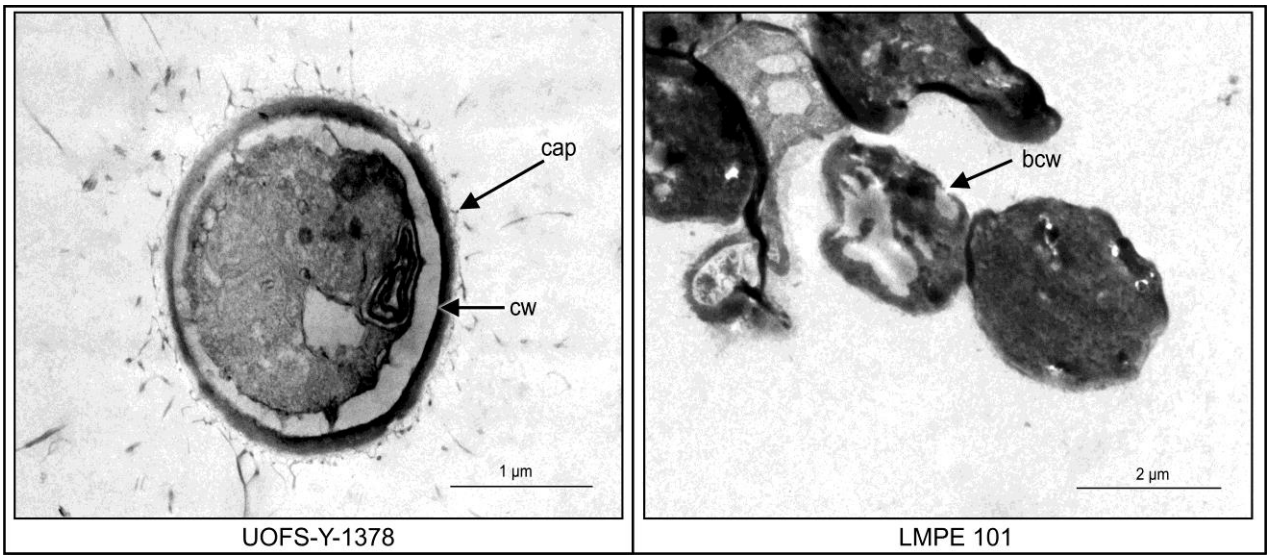

Figure 5. A comparison of the effect of amoebapore on the ultrastructure of *C. neoformans* UOFS Y-1378 (has 3-hydroxy C9:0) and *C. neoformans* LMPE 101 (is acapsular and has no 3-hydroxy C9:0). When considering the strain 1378 results, it is conceivable that the expressed resistance may be due to the inability of amoebapore to create pores on its cell wall which is opposite to the results of the strain LMPE 101.

3.8 SUPPLEMENTARY FIGURES

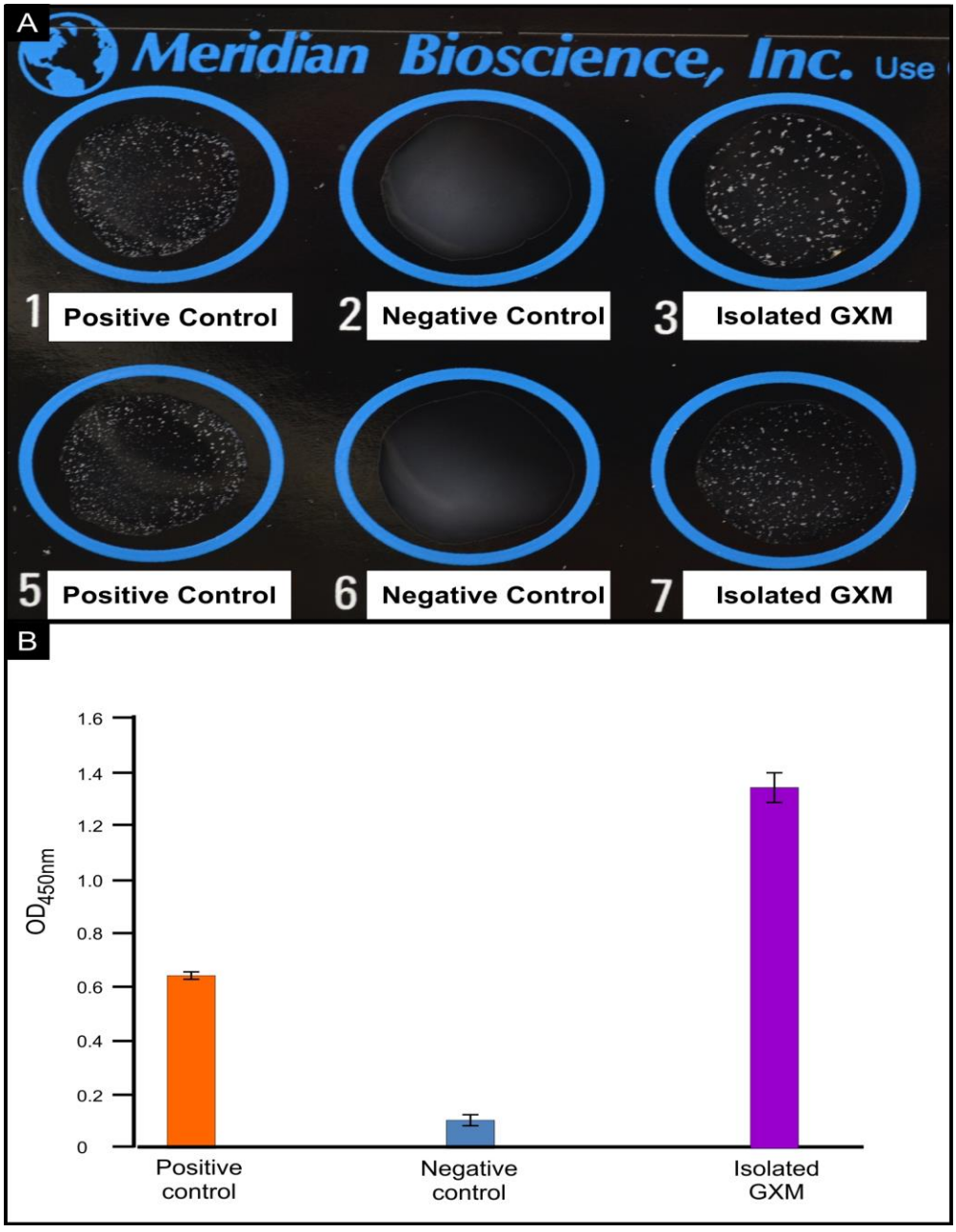

Figure S1. (A) shows a positive agglutination test for the isolated GXM while (B) also shows a positive ELISA result for the isolated GXM. Both these tests confirm the successful isolation of GXM in our study after it was harvested using irradiation.

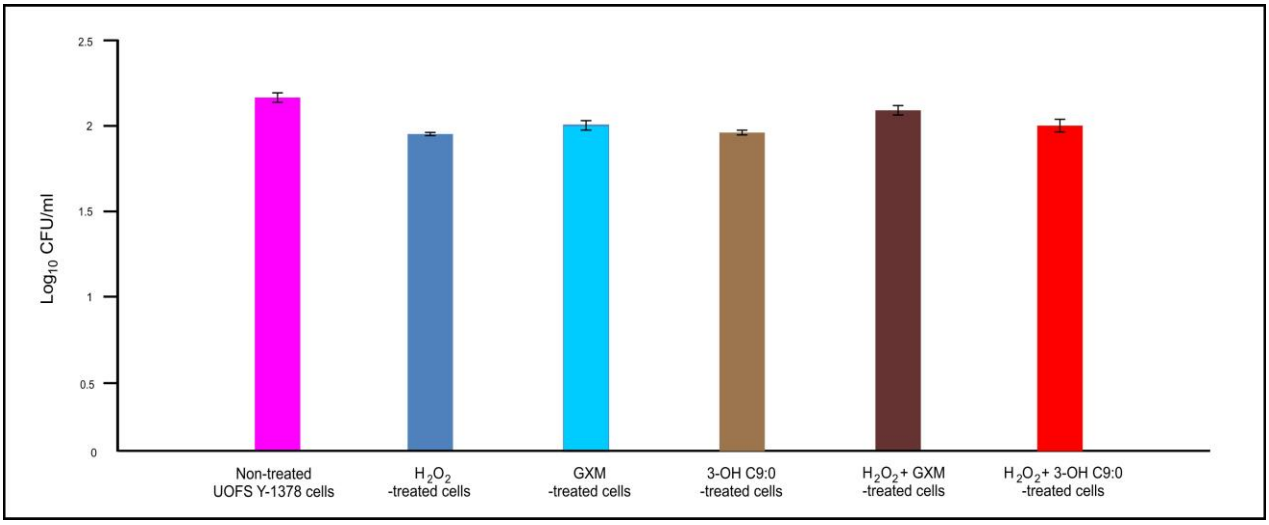

Figure S2. The survival assay results showing the number of colony forming units (CFUs) that were enumerated after the strain *C. neoformans* UOFS Y-1378 was exposed to different treatment conditions. The results suggest that 3-hydroxy C9:0, similar to GXM, protect cells against oxidative damage.

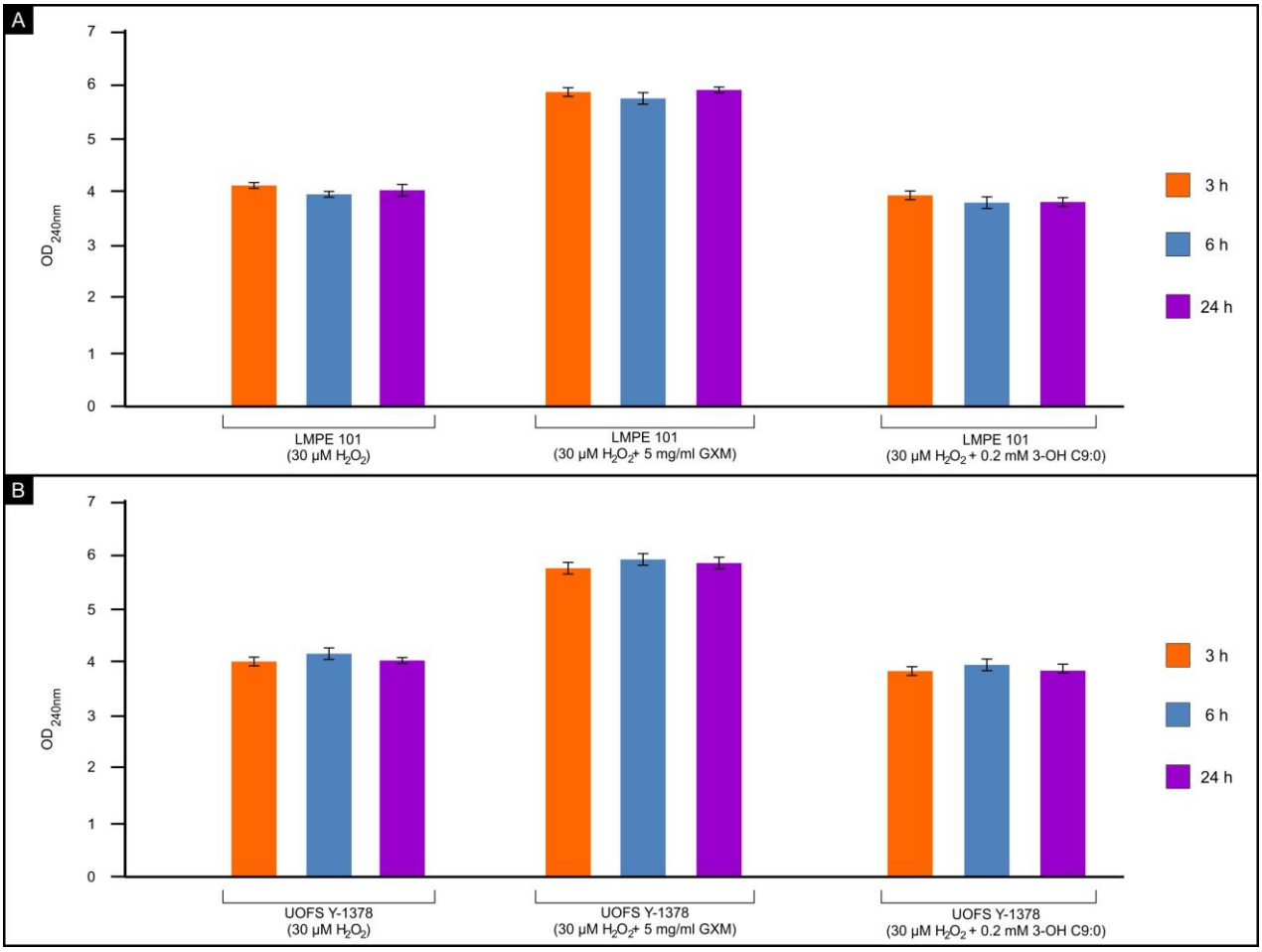

Figure S3. The fate of hydrogen peroxide in media containing hydrogen peroxide-treated cells as well as hydrogen peroxide-treated cells in the presence of GXM or 3-hydroxy C9:0. Panel (A) shows results for the strain *C. neoformans* LMPE 101 while Panel (B) shows results for the strain *C. neoformans* UOFS Y-1378.

DISSERTATION SUMMARY

In this dissertation; an attempt was made to elucidate the role of 3-hydroxy C9:0 in determining the fate of cryptococcal cells when acted upon by amoebae. First, it was sought to determine the physiological concentrations of 3-hydroxy C9:0 that were secreted by *C. neoformans* UOFS Y-1378. Through using LCMS, it was established that cells secreted 0.2 mM of these molecules. Thus, in the design of some studies 1 mM was used in order to establish if a dose-dependent response would be established. The direct effect of these molecules on amoebal growth was next investigated. Here, it was clear that at physiological concentrations, these 3-hydroxy fatty acids did not negatively affect the growth of amoebae. This finding was critical as it pointed that these molecules probably effected a signalling mechanism in amoebae that may promote the survival of cryptococcal cells when interacting with amoebae. Could the concerned mechanism involve phagocytosis? To answer this question, co-culture experiments were designed wherein cryptococcal cells were deliberately fed to amoebae and in some experiments 3-hydroxy C9:0 was added to the co-culture media. It became evident that cryptococcal cells, which did not naturally produce 3-hydroxy C9:0, were more readily: 1) internalised, and 2) phagocytosed by amoebae. Interestingly, when 3-hydroxy C9:0 was artificially added (to the co-culture media) the cryptococcal cells became resistant to amoeba. This response was dose-dependent. Additionally, the strain UOFS Y-1378 (naturally produces 3-hydroxy C9:0) was more resistant to amoeba when compared to the other strains that did not produce 3-hydroxy C9:0. These results suggested that these molecules protected cells against amoebal phagocytosis. To investigate the manner/mechanism(s) through which cells were protected, a number of further experiments were designed. In one experiment, fetuin A was analysed. This protein is reported to be pivotal in enhancing the phagocytic

efficiency of macrophages. Thus, could such a molecule be present in amoeba and important be manipulated by 3-hydroxy C9:0? The data showed that amoebae produced a fetuin A-like molecule. The levels of this molecule were low, similar to levels obtained in the presence GXM, in the presence of 3-hydroxy C9:0. This suggested that 3-hydroxy C9:0 may lower the number of cryptococcal cells that could potentially be internalised by amoebae. Thus, this molecule may be anti-phagocytic. Additionally, it seems that 3-hydroxy C9:0 may also protect cryptococcal cells that could be internalised and exposed to the harsh internal environment of amoebal food vacuole. This assertion was tested after experiments were designed mimicking the internal food vacuole environment. To be specific, it was demonstrated that an acapsular strain that was devoid of 3-hydroxy C9:0 (which in theory should be susceptible to hydrogen peroxide) was resistant when exposed to hydrogen peroxide in the presence of 3-hydroxy C9:0. Moreover, strains that were devoid of 3-hydroxy C9:0 were more susceptible to amoebapore (amoebal anti-microbial peptide found inside the food vacuole) compared to a strain that naturally produces these 3-hydroxy acids. When considering all these results, it is reasonable to suggest that it seems that 3-hydroxy C9:0 protected cells against amoebal phagocytosis. This, therefore, highlights the production pathway of these molecules as targets for developing drugs that may impair the pathogenesis of cryptococcal cells. It was thus not surprising to note that when the strain UOFS Y-1378 was exposed to aspirin, a known inhibitor of 3-hydroxy fatty acid production, its cells became susceptible to amoebal phagocytosis.

The findings recorded in the dissertation also have implications for the fate of cryptococcal cells when acted upon by macrophages. The findings suggest that cells may

deploy 3-hydroxy C9:0, possibly in concert with the capsule, in order to evade immuno-processing leading to a diseased-state in a susceptible host. In clinical settings, it is well known that without the prospect of treatment a diseased-host could die within three months when immunocompromised. Thus, the idea would be administering a cheap drug like aspirin could assist in combating cryptococcal infections.

Key words: 3-Hydroxy C9:0; Amoeba; Amoebapore; Aspirin; Capsule (GXM); *Cryptococcus*; Fetuin A; Hydrogen peroxide; Internalisation; Phagocytosis.

VERHANDELING OPSOMMING

In die verhandeling is 'n poging aangewend om die rol van 3-hidroksie C9:0 in die bepaling van die lot van *Cryptococcus*-selle, in die teenwoordigheid van amoebae, uit te klaar. Eerstens is gepoog om die fisiologiese konsentrasies van 3-hidroksie C9:0, wat afgeskei word deur *C. neoformans* UOVS Y-1378, te bepaal. Deur gebruik te maak van LCMS, is daar vasgestel dat 0.2 mM van hierdie molekule uitskei word deur die selle. In die studie is 1 mM gebruik ten einde vas te stel of daar 'n dosis-afhanklike reaksie is. Die direkte effek van hierdie molekules op die groei van amoebas is vervolgens ondersoek. Hieruit het dit geblyk dat 3-hidroksie vetsure, teen fisiologiese konsentrasies, nie ’n negatiewe invloed op die groei van amoebas het nie. Hierdie bevinding is van kritieke belang aangesien dit daarop dui dat hierdie molekules waarskynlik 'n sein-meganisme bewerkstellig in amoebas, wat die oorlewing van *Cryptococcus*-selle kan bevorder, wanneer hulle interaksie met amoebas ondergaan. Kan die betrokke meganisme fagositose behels? Om hierdie vraag te beantwoord, is ko-kultuur eksperimente ontwerp waarin *Cryptococcus*-selle doelbewus vir amoebas gevoer is in die teenwoordigheid en afwesigheid van 3-hidroksie C9:0 in die media. Dit het geblyk dat *Cryptococcus*-selle, wat nie van nature 3-hidroksie C9:0 produseer nie, meer geredelik: 1) geïnternaliseer, en 2) gefagositeer word deur amoebas. Dit is interessant dat wanneer 3-hidroksie C9:0 kunsmatig by die ko-kultuur media gevoeg word, die *Cryptococcus*-selle weerstand bied teen amoeba en die respons was dosis-afhanklike. Daarbenewens was stam UOVS Y-1378 (wat natuurlik 3-hidroksie C9:0 produseer) meer bestand teen amoeba in vergelyking met die ander stamme wat nie 3-hidroksie C9:0 produseer nie. Hierdie resultate het aangedui dat hierdie molekules selle teen fagositose deur amoebas beskerm. Om die wyse/meganisme(s) waardeur dit selle beskerm word te ondersoek, is 'n aantal verdere

eksperimente ontwerp. In een eksperiment, is Fetuïen A ontleed. Hierdie proteïen is deurslaggewend in die verbetering van die fagositiese doeltreffendheid van makrofae. Kan so 'n molekule ook in amoebas teenwoordig wees en gemanipuleer word deur 3-hidroksie C9:0? Die data het getoon dat amoebae ŉ Fetuïen A-agtige molekule produseer. Die vlakke van hierdie molekule was laag in die teenwoordigheid van 3-hidroksie C9:0, soortgelyk aan vlakke wat in die teenwoordigheid GXM opgemerk is. Dit dui daarop dat 3-hidroksie C9:0 die aantal *Cryptococcus*-selle wat potensieel geïnternaliseer kan word deur amoebae mag verlaag. Dus, kan hierdie molekuul antifagosities wees. Verder blyk dit dat 3-hidroksie C9:0 ook *Cryptococcus*-selle kan beskerm wat geïnternaliseer en blootgestel is aan die harde interne omgewing van amoeba se voedselvakuool. Hierdie stelling is getoets nadat eksperimente ontwerp is wat die interne voedselvakuool omgewing naboots. Om spesifiek te wees, dit is bewys dat 'n kapsellose stam wat sonder 3-hidroksie C9:0 was (wat in teorie vatbaar vir waterstofperoksied moet wees) bestand was wanneer dit blootgestel is aan waterstofperoksied in die teenwoordigheid van 3-hidroksie C9:0. Verder was stamme sonder 3-hidroksie C9:0 meer vatbaar vir amoeba-porieë (anti-mikrobiese peptied gevind in die voedselvakuool) in vergelyking met 'n stam wat natuurlik hierdie 3-hidroksie vetsure produseer. By die oorweging van al hierdie resultate is dit redelik om voor te stel dat dit blyk dat 3-hidroksie C9: 0 beskerm selle teen fagositose deur amoebas. Dit beklemtoon dus die produksie weg van hierdie molekules as teikens vir die ontwikkeling van middels wat die patogenese van *Cryptococcus*-selle kan verminder. Dit was dus nie 'n verrassing om te sien dat wanneer die stam UOVS Y-1378 blootgestel is aan aspirien, 'n bekende inhibeerder van 3-hidroksie vetsuur produksie, dit vatbaar geraak het vir fagositose deur amoeba nie.

Die bevindinge in die verhandeling het ook implikasies vir die lot van *Cryptococcus*-selle se interaksie met makrofae. Die bevindinge dui daarop dat selle 3-hidroksie C9:0, moontlik in samewerking met die kapsel, mag gebruik ten einde immuno-verwerking te ontduik, wat kan lei tot 'n siektetoestand in 'n sensitiewe gasheer. Uit ’n kliniese oogpunt is dit algemeen bekend dat, sonder die vooruitsig van die behandeling, 'n immuunonderdrukte siek gasheer binne drie maande kan sterf. Dus, is die idee dat die toediening van 'n goedkoop middel soos aspirien, kan help in die stryd teen *Cryptococcus*-infeksies.

Sleutelwoorde: 3-Hydroksie C9:0; Amoeba; Amoebapoor; Aspirien; Kapsule (GXM); *Cryptococcus*; Fetuïen A; Waterstofperoksied; Internalisering; Fagositose.