Exploring the role of bacterial endosymbionts in modulation of innate immune responses during infection by *Rhizopus microsporus*.

by

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The cure for pain

is in the pain

~Rumi

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Abbreviations

AcN	acetonitrile
Ca_2CO_3	calcium carbonate
CBS	Centraalbureau voo schimmelcultures
cDMEM	complete Dulbecco's modified eagles' medium
CFUs	Colony forming units
CFW	calcofluor white
CO_2	carbon dioxide
Con-A	Concanavalin A
Ctrl	control
ddH_2O	double distilled water
DKA	diabetic keto acidosis
DMSO	dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
EDTA	Ethylenediaminetetra acetic acid
ESF	Endosymbiont free
FA	Formic acid
FB	Fluorescence brightener
FBS	Foetal bovine serum
FITC	Fluorescein isothiocynate
Fmlp	N-formyl –Methionyl-leucyl Phenylalanine
GFP	Green fluorescent protein
GRP	glucose regulated protein
h	hour
H_2O_2	water
HILIC	Hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
IFNy	Interferon gamma
Ig	immunoglobulin
IU	international unit
KH_2PO_4	potassium dihydro-phosphate
LB	Lysogeny broth

LC-MS/MS	Liquid chromatography mass spectrometry
LPS	lipopolysaccharides
M-CSF	monocyte colony stimulating factor
MF	molecular formula
Min	minute
mL	millilitre
mM	milli moles
mm	millimetre
MOI	multiplicity of infection
MS	mass spectrometry
NA	nutrient agar
Na_2HPO_4	Sodium phosphate
NaCl	sodium chloride
$NaNO_2$	sodium nitroxide
NMR	nuclear magnetic resonance
NRPS	non-ribosomal polyketide synthase
NRRL	Northern regional research laboratory
ns	non-significant
OD	optical density
PAMPs	pathogen associated molecular patterns
PBMCs	peripheral blood mononuclear cells
PBS	Phosphate buffered salt
PCR	Polymerase chain reaction
PDA	potatoe dextrose agar
PFA	Paraformaldehyde
pH	power of hydrogen
PKS	Polyketide synthetase
PMA	phorbol 12-myristate 13-acetate
PMSF	phenyl methyl suformyl fluoride
PRP	protein rich plasma
PRR	pattern recognition receptor
ps	penicillium streptomycin
PTU	Phenylthiourea
PTU	phenyl-2-thiourea

PVP	polyvinyl pyrrolidine
Rcf	relative centrifugal force
rDNA	ribosomal DNA
RP	reverse phase
Rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
SAB	Sabouraud broth
SDA	sabouraud dextrose agar
SDS	sodium dodecyl sulfate
sf DMEM	serum free Dulbecco's modified eagles' medium
SN	supernatant
TBE	Tris borate EDTA
TFA	Trifluoric acid
TRITIC	tetra methyl rhodamine
UCLA	University of California Los angels
UV	ultra violet
v/v	volume per volume
w/v	weight per volume
WGA	wheat germ agglutinin
YPD	yeast peptone dextrose
µg	micro gram
µm	micro meter
DIC	differential interference contrast
DMEM	Dulbecco's modified eagles' medium

Abstract

Mucormycosis is a life-threatening mold infection with overall mortality rates of 50%, yet fatality levels reach 100% in patients with disseminated disease, prolonged neutropenia, or brain involvement. The disease is caused by a group of filamentous fungi, the mucorales and particularly targets patients with immunosuppression due to diabetic ketoacidosis (DKA), iron overload, severe trauma, neutropenia, corticosteroid treatment, or organ transplantation.

These predisposing conditions are linked to defects in key aspects of the innate immunity, particularly defects in phagocytic effector functions by macrophages and neutrophils, suggesting phagocytic activity is crucial to disease control. Yet, we currently have a very limited understanding of the interaction between mucormycete infecting spores and phagocytes.

In this thesis, we show that the early events during phagocyte- spore interaction may determine infection by *R. microsporus.* We report that spore metabolism modulates macrophage effector functions including phagocytic uptake of spores, phagosome acidification following uptake and cytoskeletal organisation via a secreted factor.

We also demonstrate the secreted factor is not produced by *R. microsporus* but a bacterial endosymbiont *Ralstonia pickettii*. This is a close relative of Paraburkholderia species belonging to the family Bukholderiaceace that produce rhizo-toxins already characterised and implicated in plant pathology but not yet in human disease. However, although the secreted compound here shares chemical and functional attributes with the rhizo-toxins, it is not a rhizo-toxin.

Thesis Overview

Infections due to invasive opportunistic moulds such as the mucormycetes continue to emerge especially in patients with various immunosuppressive threats including but not limited to organ transplants, HIV/AIDS, haematological malignancies, metabolic acidosis, iron overload, corticosteroid therapy, neutropenia, and trauma. Mucormycosis is now the second most invasive mould infection after aspergillosis. It presents with hallmark symptoms of angio-invasion, thrombosis and necrosis. These infections are difficult to manage because of challenges associated with poor diagnosis, expensive yet unsuccessful therapy and anti-fungal drug resistance. Among the risk factors is a defect in phagocytic effector functions which seems crucial for disease progression. Bettering our understanding of the mechanisms underlying spore-phagocyte interactions is key if we are to improve prognosis. This thesis is written in a traditional style including chapter 1(introduction), chapter 2 (methods and materials) and chapters 3-6 (results and discussion). Three projects are presented and a discussion for each chapter follows immediately to explain the data. The work presented in this thesis is mainly focused on *R. microsporus* but data from other mucoralean fungi is also included for comparisons.

Chapter 3 explores spore-macrophage interactions, describing some of the key approaches developed in this project. A major of the chapter is a finding that metabolic spores inhibit phagocytic uptake by macrophages via a secreted factor.

Chapter 4 is a follow up on the observations made in chapter 3 where further characterisation of the secreted factor is made, highlighting that the factor is secreted by a bacterial endosymbiont rather than the fungus.

Chapter 5 is also a build-up on chapters 3 and 4, mainly exploring bacterial symbiosis and its impact on pathogenicity of *R. microsporus* both *in vitro* and *in vivo*.

Chapter 6 is additional work and possible hypotheses for future directions.

Chapter 7 is a final discussion for the thesis

CHAPTER ONE: Introduction

1.1 Mucorales and Mucormycosis

1.1.1 Overview

Fungi have increasingly gained medical importance in recent times and are now known to cause a range of diseases worldwide (Skiada et al., 2012, Blyth et al., 2014, Roden et al., 2005). However, this is in parallel with the increasing numbers of at-risk individuals. Clinically, manifestations of diseases due to fungi have ranged from mild superficial to aggressively fatal and disseminated infections. Nevertheless, the taxonomic diversity of the disease-causing agents continues to expand as fungi that were primarily known to be saprobes like the mucorales, are now regarded major and emerging aetiological agents (Skiada et al., 2012, de Hoog et al., 2014, Mohindra et al., 2014).

Indeed, mucoralean fungi are the causative agents of a fatal disease called mucormycosis. The disease is characterized by hallmark symptoms of angio-invasion, thrombosis and tissue necrosis (Chayakulkeeree et al., 2006, Neblett Fanfair et al., 2012, Ribes et al., 2000). The infection is very aggressive, hard to treat and individuals with a defect in phagocytic effector functions show increased susceptibility (Ribes et al., 2000).

1.2 The mucorales

The mucorales are a group of traditional fungi that together with chytridiomycetes diverged early in the fungal kingdom and thus are regarded as basal fungi (Lee et al., 2010, Mendoza et al., 2015). They are best known environmentally as agricultural produce spoilers and clinically as laboratory contaminants especially some members of the *Rhizopus* genus (de Hoog et al., 2014).

They form a sub-family within the phylum mucormycotina (formally zygomycotina) that is phylogenetically sandwiched between the lower fungi (i.e. the slime moulds and flagellate

fungi) and higher fungi (i.e. the ascomycota, basidiomycota and deuteromycetes groups) (Grigoriev et al., 2014, Spatafora et al., 2016, de Hoog et al., 2014) (**Figure 1)**.

Mucoralean fungi are non-motile and are morphologically characterized by formation of thalli networks of coenocytic (a septate) mycelia with root like structures called rhizoids, aerial sporangiophore on which sporangia and spores are formed asexually; or non-enveloped zygospores during sexual reproduction (Lee et al., 2010, Lee and Heitman, 2014, Mendoza et al., 2015, Hoffmann et al., 2013, Spatafora et al., 2016).

The phylum mucormycotina contains nine orders with mucorales as the most studied among them (Lee et al., 2010). Majority of these fungi are saprophytic in nature and propagate by both sexual and asexual means (Lee et al., 2010, Mendoza et al., 2015).

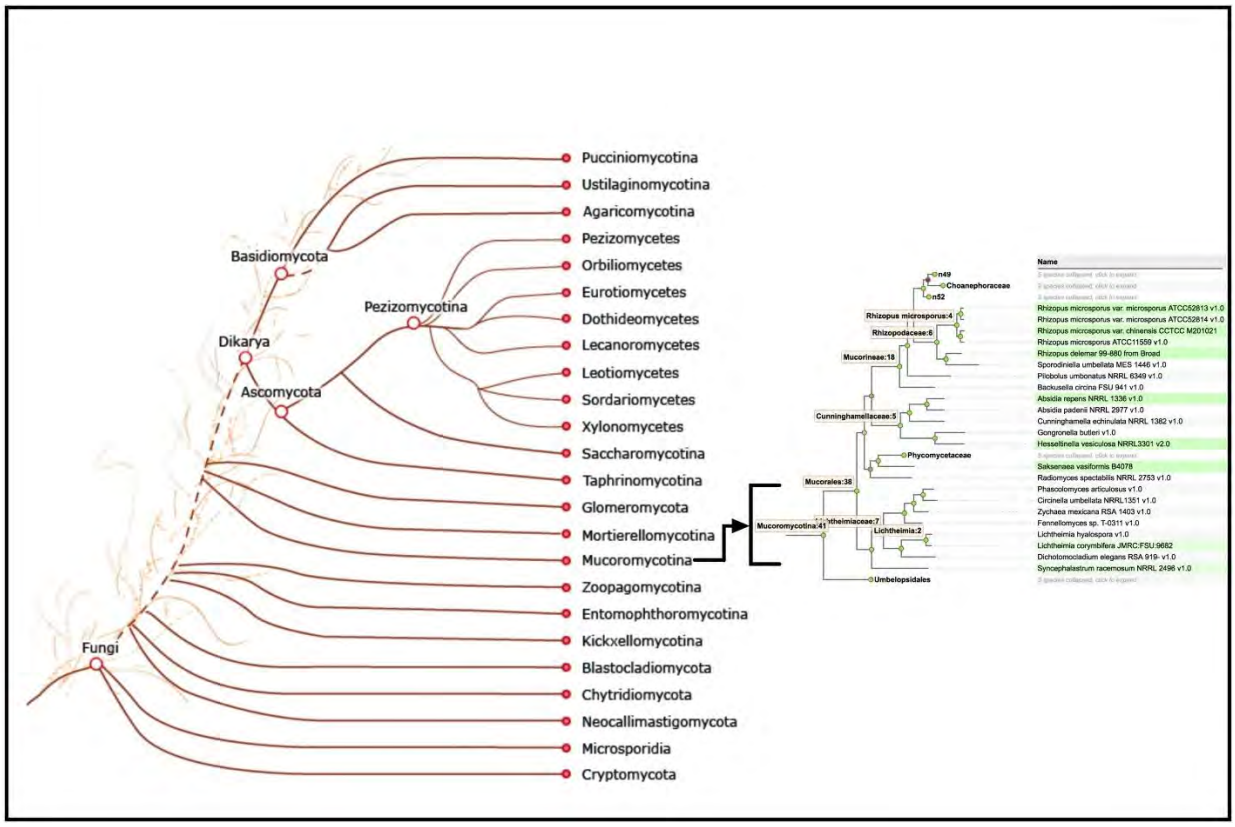


Figure 1: The Mucoromycotina is sandwiched between lower and higher fungal phyla.

The Mucoromycota fungi are phylogenetically sandwiched between lower slime and flagellate fungi and higher fungi such as the dikaryon, ascomycota and basidiomycotina. This family is composed of several orders including Rhizopodaceae, Mucorineae and Cunninhamellaceae among others as indicated. Under these are several species as indicated. The phylogenetic tree is adopted from MycoCosm data base (Grigoriev et al., 2014).

1.3 Reproduction in mucorales

The term zygomycotina has been used interchangeably to describe mucorales in the past and this is also reflected in their sexual reproductive cycle as mentioned above. However, mucorales reproduce by both sexual and asexual means. These concepts of reproduction in mucoralean fungi have been described since the 1800 particularly in *Syzygites megalocarpus* (Blakeslee, 1904, Lee and Heitman, 2014).

1.3.1 Asexual reproduction

During asexual reproduction, offspring arise from a single parent. Spores germinate under suitable conditions to form a thallus network of mycelia. From these, aerial hyphae emerge from which sporangia form. This is now called a sporangiophore. It is on the sporangia that mitospores called sporangiospores later develop via mitosis (**Figure 2**). Mitospores are formed inside sporangioles and released when mature by disintegration of the sporangium wall or as a whole sporangiole that breaks off the Sporangiospore. It has been suggested that this form of reproduction is more rapid in development and drives dispersal and perhaps infection (Lee et al., 2010, Mendoza et al., 2015, Lee and Heitman, 2014).

Figure 2: Mucoralean fungi reproduce by both sexual and asexual means.

Diagrammatic representation of asexual and sexual reproduction in mucormycetes. Mucormycetes reproduce by both sexual and asexual means. Asexual reproduction yields the ubiquitous sporangiospores responsible for dispersal and infection while sexual reproduction gives rise to the overt and the more genetically diverse zygopores (Image by Benjamin Cummings, 2005).

1.3.2 Sexual reproduction

Sexual reproduction is by physical blending or fusion of morphologically similar gametangia tips to form a thick and ornamented wall called a zygo-sporangium following a mixture of multinucleated contents. This is also called the teleomorphic phase. The Greek term, *Zygos* which means balance of scales is used to describe the resultant joining (the yoke) (Lee and Heitman, 2014, Johannes Wöstemeyer, 2007, Alexander Idnurm, 2007). In mucoralean fungi, sexual reproduction can be homo or hetero-thallic. Whilst homothallism requires conjugation of mating hyphal types (gametangia) from a single thallus, two different but sexually compatible thalli are required during heterothallism. Zygosporangia develop thick walls and are the equivalent of resting spores (**Figure 2)** (Lee and Heitman, 2014).

1.3.2.1 The sex *loci* in mucorales

The sex locus in mucoralean fungi was first discovered in *Phycomyces blakesleeanus* and is still poorly characterized when compared with the two dikaryon lineages, Ascomycetes and Basidiomycetes (Idnurm et al., 2008, Lee and Heitman, 2014, Poggeler et al., 2011, Coelho et al., 2017, Gryganskyi et al., 2010, Wetzel et al., 2012). Unlike in animals and some plants where sex identity is determined by large sex specific chromosomes, genomic studies reveal sexual reproduction in fungi to be controlled by a small, specialized region of the genome called the mating type (*MAT*) or sex locus (Idnurm et al., 2008, Lee et al., 2010, Lee and Idnurm, 2017).

The *MAT* locus may contain two or more alleles that encode key domain transcription factors including homeodomain or the high mobility group (HMG) proteins (Idnurm et al., 2008, Lee and Heitman, 2014, Lee et al., 2010). Studies on *P. blakesleeanus* show that the sex locus of mucoralean fungi comprise a single HMG gene flanked at both ends by conserved putative triose phosphate transporter (*tptA*) and RNA helicase (*rnhA)* genes to form a unique syntenic

helicase gene cluster (TPT/HMG/RNA) (**Figure 3**) (Idnurm et al., 2008, Lee and Idnurm, 2017). Each of these mating types encodes an allelic HMG gene: *sexP* for the (+) and *sexM* for the (-) mating types (Idnurm et al., 2008).

This seems to be a bona fide sex locus in *P. blakesleeanus* because haploid strains contain either the *sexP* or *sexM* in the genome and only expressed through mating the two mating types resulting into formation of zygospores. Secondly, *sexP* strains will only mate with (-) mating types and *sexM* strains with (+) mating types and no mating occurs between strains of the same mating type. Lastly, strains with chromosomes represented twice for the *sexP* and *sexM* genes appear to be self-fertile. Similar observations have been made in *M. circinelloides* and *R. delemar* (Idnurm et al., 2008, Gryganskyi et al., 2010, Lee and Idnurm, 2017, Lee et al., 2008). The overall structure of the sex locus in mucorales is highly conserved but genomic studies on other mucoralean fungi seem to show that specific differences may exist as demonstrated in **Figure 3**. Particularly, the direction of transcription of the *sexP* and *sexM* genes differs in some species such as *P. blakesleeanus* (Idnurm et al., 2008). In addition, the presence of repetitive elements on the *sexP* locus like with *P. blakesleeanus*, presence of an additional open reading frame (ORF) *arbA* that encodes an Ankyrin –RCC1-BTB-POZ domain protein such as in *R. oryzae* (*sex P*) and *S. megalocarpus* (-) sex locus), and a partial gene inversion in the *rnh A* gene (*S. megalocarpus*) have been identified by bioinformatics and genomic mapping (**Figure 3**) (Idnurm et al., 2008, Lee and Heitman, 2014)**.**

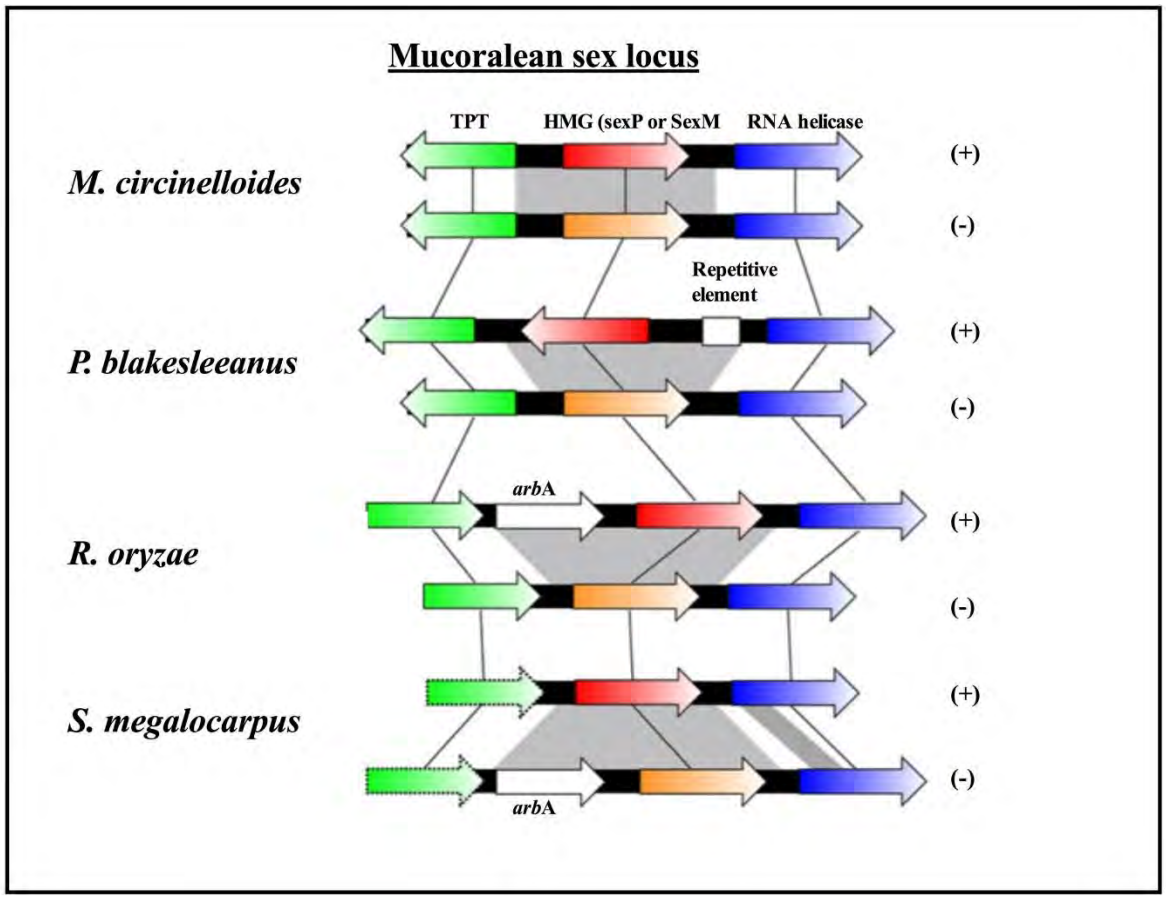


Figure 3: Mucoralean fungi sex locus encodes sexP (+) and sexM (-).

The sex loci of four different mucoralean fungi is shown. The (+) sex locus encode the sexP gene whilst (-) encodes the sexM gene. Both of these are flanked by tptA and rnhA genes. There are key defining features that differentiates between loci of the different mucoralean fungi: including the direction of *sexP* and *sexM*, the presence and absence of repetitive element and an additional ORF encoding 10nkyrin-RCC1-BTP-POZ; and partial *rnh*A gene inversion. (Image adapted from Lee et al., (Lee and Heitman, 2014).

1.3.2.2 Sex and infection in mucoralean fungi

Asexual spores are ubiquitously available in the environment and are the main source of dispersion and infection usually through inhalation (Mendoza *et al*., 2015, Kennedy *et al*., 2016). Infection by other modes such as traumatic implantation and ingestion are also common. In regard to sexual zygospores, a particular mating type may skew their ability to elicit an infection. This has been noted among *Mucor* species. For instance, *Mucor amphibiorum*, a causative agent of ulcerative mucormycosis, a (+) mating type is more virulent than the (-) mating type species (Lee and Heitman, 2014, Stewart and Munday, 2005). Similar observations have been made with *Mucor piriformis,* the causative agent of Mucor rot in plants (Stewart and Munday, 2005, Lee and Heitman, 2014, Kennedy *et al*., 2016).

Mating types are also linked to spore dimorphism and virulence in *M. circinelloides,* where (+) types are more virulent than their (-) counterparts. However, further investigations now show that the sex*M* gene may not solely be responsible for dimorphism in spores as mutants for this gene can still produce large spores (Lee and Heitman, 2014, Li *et al*., 2011, Lee *et al*., 2010). We can perhaps suggest that spore size may be controlled by the Sex*P* gene or other genetic *loci* acting either independently or in concert with the sex*M* gene.

The sex paradigm here can easily be related to that of the basidiomycete, *Cryptococcus neoformans* where the alpha mating type predominates both in the environment and clinical niches. Whether this unisexual bias in *Cryptococcus* can be extrapolated to explain the virulence predominance expressed by (+) mating type in the mucorales remains to be explored. Nonetheless, as efforts are made to understand virulence dynamics of mucormycetes, it is apparent to note that sex types may significantly influence aetiology by mucoralean fungi and such implications should be keenly looked for in future studies. However, for this study we have mostly employed asexual spores.

1.4 Clinical features of mucormycosis

1.4.1 Epidemiology of mucormycosis

Mucoralean fungi are the aetiological agents of an infection called mucormycosis. This is a rare but emerging opportunistic invasive filamentous fungal disease. Mucormycosis is fatal and is associated with high (30-90 %) mortality rates worldwide (**Table 1**) (Brown *et al*., 2012). Fatality rates of the disease are increased if not diagnosed early enough to allow aggressive treatment and management (Lewis and Kontoyiannis, 2013).

Individuals at risk include a cohort of immunosuppressed patients, particularly those with uncontrolled hyperglycaemic ketoacidosis, trauma, malignancies (haematological (HM) and non-HM), iron overload, neutropenia or organ transplant. The disease can also target healthy individuals, especially those inoculated via traumatic injuries (Petrikkos et al., 2014, Lewis and Kontoyiannis, 2013, Kyriopoulos et al., 2015, Brown et al., 2012).

Mucormycosis can manifest in at least six different forms, including rhino-cerebral, disseminated, pulmonary, cutaneous and gastrointestinal forms (Petrikkos et al., 2012, Singla et al., 2018). Clinical manifestations are often unique and aggressive, with gruesome and almost irreversible complications, which usually occur during late stages of the infection (**Figure 4**). Early signs of the infection can be non-specific and almost indistinguishable from other invasive fungal infections (IFIs) (Lewis and Kontoyiannis, 2013).

Although the disease is associated with severe clinical consequences, its epidemiology is still poorly documented. So far, only a handful of surveillance studies exist, and these places incidence of mucormycosis (including cases by entomophthorales) at 1.2 to 1.7 cases per million persons per year (Rees et al., 1998, Petrikkos et al., 2014). The caveat with these statistics is that majority of the studies have been done in Europe which is only about a fifth of the world's population. Indeed, more studies are needed if the worldwide incidences of mucormycosis are to be appreciated.

Table 1: Worldwide distribution of mycoses

Disease	Location	Estimated life-threatening infections / year at that location	Mortality rates (% in infected populations)
Opportunistic invasive mycoses			
Aspergillosis (*A. fumigatus*)	Worldwide	>200, 000	30-95
Candidiasis (*C. albicans*)	Worldwide	>400,000	46-75
Cryptococcosis (*C. neoformans*)	Worldwide	>1,000,000	20-70
Mucormycosis (*R. oryzae*)	Worldwide	>10,000	30-90
Pneumocystis (*Pneumocystis jerovecii*)	Worldwide	>400,000	20-80
Endemic dimorphic mycoses			
Blastomycosis *(Blastomyces dermatitidis*)	Mid-western and Atlantic USA	~3000	<2-86
	South western USA	~25,000	<1-70
		~25,000	28-50
Coccidioidomycosis (*Coccidioides immitis*)	Mid-western USA	~4,000	5-27
	Brazil		
Histoplasmosis (*Histoplasma capsulatum*)		>8,000	2-75
	South east Asia		
Paracoccidioidomycosis (*Paracoccidioides barasilliensis*)			
Penicillosis (*Penicillium marneffei*)			

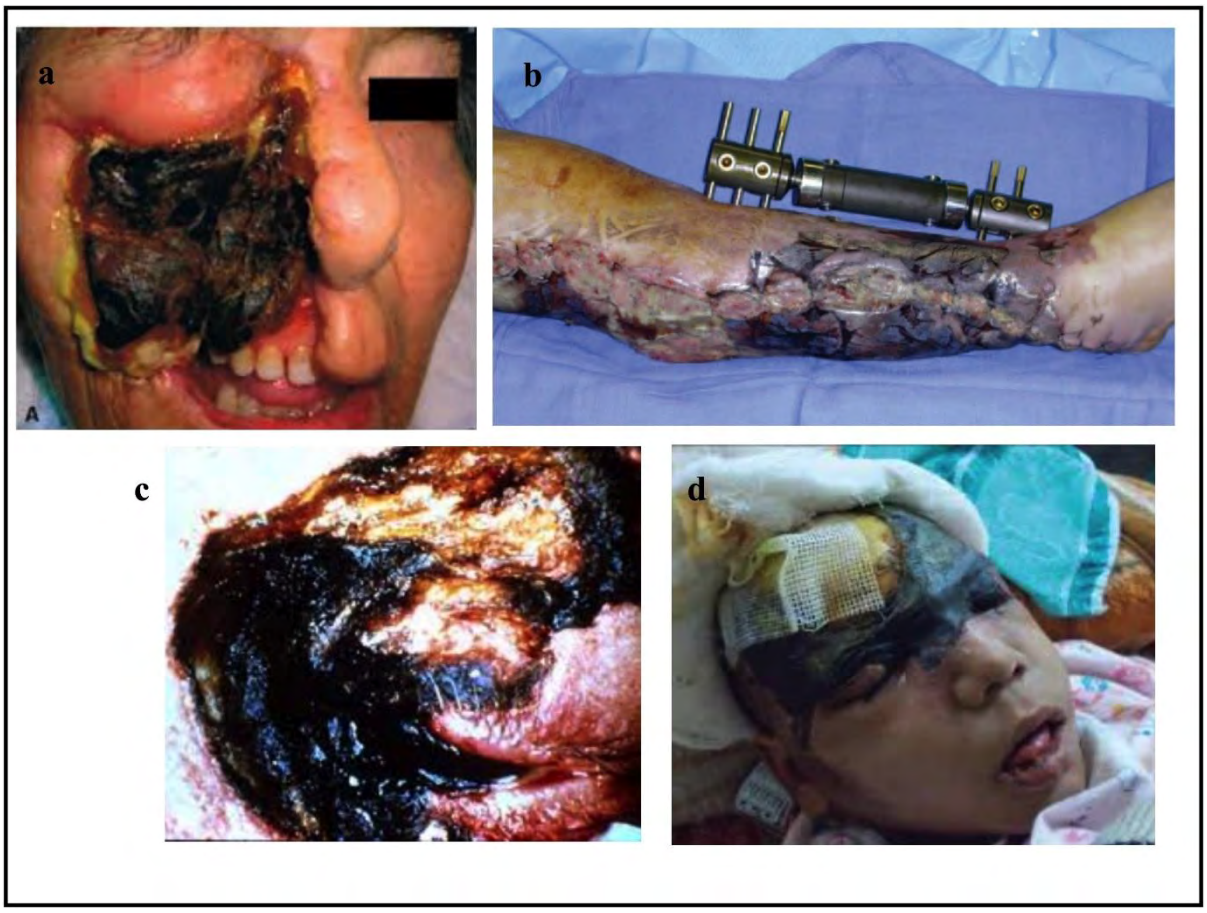


Figure 4: Mucormycosis manifests clinical symptoms characteristic of tissue death.

Clinical manifestations of mucormycosis are characterised by angio-invasion which culminates into tissue necrosis and manifestations range from superficial to disseminated infections. (**a** and **d**) Depict cutaneous presentation, one of the most prevalent forms of mucormycosis. (**b** and **c**) Depict gross presentation of invasive mucormycosis in a patient infected via traumatic injury (Images taken from (Zaman et al., 2015).

Review of literature reporting incidence of mucormycosis from the past 10-15 years generally shows an increase in mucormycotic cases. Some of which highlight similar predisposing factors as mentioned earlier while others report new risks for mucormycosis. For instance, recently the exposure to drugs such as voriconazole with limited or no anti-mycotic activity is reported as being a major and new emerging risk factor for mucormycosis (Petrikkos et al., 2014, Mendoza et al., 2015, Moreira et al., 2016, Riley et al., 2016, Kontoyiannis et al., 2000, Kontoyiannis et al., 2016, Petrikkos and Drogari-Apiranthitou, 2011, Bonifaz et al., 2012, Saegeman et al., 2010, Bitar et al., 2009, Ambrosioni et al., 2010, Meis and Chakrabarti, 2009, Roden et al., 2005).

In addition to the forms mentioned above, soft tissue involvement is also a common presentation of mucormycosis. The aetiological agents of this and the afore mentioned forms are predominantly *Rhizopus*, *Lichtheimia*, and *Mucor* species (Petrikkos et al., 2014, Mendoza et al., 2015, Moreira et al., 2016, Riley et al., 2016, Kontoyiannis et al., 2000, Kontoyiannis et al., 2016, Petrikkos and Drogari-Apiranthitou, 2011, Bonifaz et al., 2012, Saegeman et al., 2010, Bitar et al., 2009, Ambrosioni et al., 2010, Meis and Chakrabarti, 2009, Roden et al., 2005). Other aetiological agents such as *Cunninghamella bertholletiae* are also occasionally reported but predominantly in health individuals.

This background clearly highlights the clinical relevance of mucormycosis; and the need to better our understanding of epidemiological impact, aetiology, pathogenesis, diagnosis and treatment is now apparent in order to improve prognosis.

1.4.2 Aetiology of mucormycosis

Mucoralean fungi are a group of rapidly growing, heterotrophic, terrestrial thermotolerant moulds (Chayakulkeeree et al., 2006, Kontoyiannis and Lewis, 2006). Though largely regarded

as opportunists, some may infect immunocompetent individuals (Chayakulkeeree et al., 2006, Mendoza et al., 2015).

Rhizopus species are the most prevalent, responsible for about half of the reported cases (Roden et al., 2005, Petrikkos et al., 2014). Common examples include, *Rhizopus arrhizus, R. 16icrospores,* and *R. oryzae*. Other genera often implicated include *Mucor*, *Lichtheimia* (formally *Absidia*), *Cunninghamella, Rhizomucor, Apophysomyces, Mortierella, Saksenaea, Syncephalastrum* and *Cokeromyces* (**Figure 5**) (Roden et al., 2005, Zaki et al., 2014, Antoniadou, 2009). *Lichtheimia* and *Mucor* are the second and third most frequent causes of mucormycosis. *Cunninghamella, Lichtheimia* and *Apophysomyces* species cause disease in immunocompetent individuals (Simitsopoulou et al., 2010, Lake et al., 1988). *R. pusillus* is the most common member of the *Rhizomucor* genus, and debate as to of whether *Mortierella* is a true mucoralean fungus continues, as members of this genus are unable to sporulate like mucorales do (Chayakulkeeree et al., 2006).

Figure 5: The Common aetiological agents of mucormycosis.

Aetiology of mucormycosis is dominated by members of the *Rhizopus*, *Mucor and Lichtheimia* genera but occasionally infections due to members of *Cunninghamella, Mortierella* and *Rhizomucor* are also reported. Images were taken from Mycology Online-University of Adelaide, Zygomycetes and Wikipedia websites.

1.4.2.1 *Rhizopus microsporus* as an aetiological agent of mucormycosis.

R. microsporus is among the emerging aetiological agents of mucormycosis. This agent falls within the most prevalent genera in *Rhizopus*, with *R. oryzae* as the commonest cause of mucormycosis (Roden *et al*., 2005, Ribes *et al*., 2000). However, recent clinical reports particularly from middle income countries such as India show a changing trend in the aetiology of mucormycosis, importantly highlighting the increasing incidences due to *R. microsporus* (Pandey et al., 2018, Ribeiro et al., 2012, Lyskova et al., 2013). It has so far been implicated in patients with but not limited to diabetis, haemopoetic stem cell transplantation (HST) and traumatic cases (West et al., 1995, Pandey et al., 2018, Wang et al., 2018, Ville et al., 2016). The commonly implicated strains of *R. microsporus* are *R. microsporus var microsporus, R. microsporus var oligoporus, R. microsporus var rhizopodoformis and R. microsporus var azygosporus* (Pandey et al., 2018, West et al., 1995, Ribeiro et al., 2012, Sato et al., 2016). Thus, this changing trend shows how relevant it is to explore virulence attributes of *R. microsporus*. In this project, we focus mainly on a clinical isolate of *R. microsporus* as the model organism for this study.

1.4.3 Pathogenesis mucormycosis

Normally the immune system will recognize, contain, and selectively eliminate threats posed by infecting particles such as mucormycete propagules. However, this ability is impaired under certain conditions facilitating colonization by the pathogens. For mucormycosis, development heavily depends on underlying factors which can be host or pathogen based. Examples of host-based are mentioned (Section 1.4.1) above whereas pathogen-based factors will be detailed later on.

It appears that most of the host factors are highly linked to defective phagocytic effector functions and nutritional imbalance. For instance, diabetic keto-acidosis (DKA), steroid

therapy and iron overload affect the ability of macrophages to contain and prevent spore germination following uptake (Mendoza et al., 2015, Gebremariam et al., 2016, Gebremariam et al., 2014, Andrianaki et al., 2018b, Ibrahim et al., 2012).

1.4.4 Host factors for mucormycosis

1.4.4.1 Diabetes and metabolic acidosis

Until recently, diabetes and metabolic acidosis were regarded the most prevalent risk factors for mucormycosis, estimated in about 36-88% of mucormycosis cases. This has recently been overshadowed by an over-representation of haematological malignancies, especially in Europe (Mendoza et al., 2015, Nithyanandam et al., 2003, Ibrahim and Kontoyiannis, 2013, Kontoyiannis et al., 2012, Petrikkos et al., 2014, Chen et al., 2017, Anane et al., 2009). As far as diabetes is concerned though, its role in disease progression is usually associated with elevation of serum ketone levels, a condition known as diabetic ketoacidosis (Nithyanandam et al., 2003, Gebremariam et al., 2016, Petrikkos et al., 2014, Ribes et al., 2000, Roden et al., 2005, Gen et al., 2013, Chen et al., 2017, Trabelsi et al., 2005, Anane et al., 2009). The condition results in acidification with subsequent decline of blood pH (Mendoza et al., 2015, Gebremariam et al., 2016, Boelaert et al., 1988). The shift in pH impacts on a series of physiological events; most notably the deposition of iron from its carrier proteins such as ferrin, transferrin and lactoferrin (Mendoza et al., 2015, Boelaert et al., 1989, Artis et al., 1982, Boelaert et al., 1993).

Iron is an essential nutrient for both the host and fungus. Its release increases its availability to the pathogen, promoting pathogenicity by enhancing fungal growth (de Locht et al., 1994, Verdonck et al., 1993, Mendoza et al., 2015, Weinberg, 1971, Weinberg, 1994, Boelaert et al., 1993, Ibrahim et al., 2008c, Gebremariam et al., 2016). Under normal conditions, the ferrin proteins are responsible for physiological iron homeostasis, regulating its availability and toxicity in host micro-environments (Weinberg, 1971, Mendoza et al., 2015). The anomaly can

clinically be managed by iron chelating agents such as deferoxamine and deferasirox. However, whilst deferasirox is a potent chelator, deferoxamine is a xeno-siderophore of iron as explained in the next section below (Windus et al., 1987).

1.4.4.2 Iron overload

Iron is an essential metal and the second most abundant after aluminium. It exists in two ionization states of Fe^{2+} or Fe^{3+} (Howard, 1999). The metal is required for a range of biological systems as a cofactor for several enzymes involved in redox processes (Howard, 1999, Johnson, 2008, Condon et al., 2014, Zhang et al., 2013). In context, iron is essential to both the host and fungal pathogens and thus it is apparent that both compete for the nutrient for survival (Nairz et al., 2010, Nevitt, 2011, Condon et al., 2014).

Human hosts are able to limit the availability and access to micronutrients by pathogens through a process called nutritional immunity (Leon-Sicairos et al., 2015, Condon et al., 2014). In regard to iron homeostasis, the host has evolved mechanisms such as expression of glycoproteins like ferrin, transferrin and ferritin that physiologically bind and transport iron. In order to circumvent host iron chelation, pathogens including fungi have evolved subtle mechanisms such as non-ribosomal secreted iron chelators (siderophore), reductive iron assimilation or possession of ferritin-like proteins that enable them access host reserves (Howard, 1999, Weinberg, 1971, Frederick et al., 2009, Condon et al., 2014).

To the contrary though, opportunistic fungal pathogens such as mucormycetes demonstrate reduced ability to evade host chelation. However, their access to highly restricted nutrients such as iron can be made possible under certain conditions such as diabetic ketoacidosis (DKA), deferoxamine therapy, multiple blood transfusions or dyserythropoiesis (Mendoza et al., 2015, Chayakulkeeree et al., 2006, Boelaert et al., 1989). This subsequently improves growth, survival and may also boost virulence. Indeed, several studies allude to the importance

of impaired iron homeostasis in the development of mucormycosis both clinically and in animal models (Mendoza et al., 2015, Chayakulkeeree et al., 2006, Gebremariam et al., 2016, Boelaert et al., 1988, Boelaert et al., 1993).

In addition, the role of iron in expression of glucose regulated protein (GRP) 78 and its fungal ligand the CotH3 has been characterised. Both initiate and promote invasion of endothelial cells by the *R. oryzae*. Glucose, iron and beta hydroxyl-butyrate, conditions synonymous during DKA promote over expression of these proteins which compromises survival of mice and treatment with an anti-CotH3 antibody rescues the mice making CotH a possible anti-fungal target (Gebremariam et al., 2014, Gebremariam et al., 2016).

1.4.4.3 Trauma and blast injuries

Mucoralean fungi often target soft tissues such as sinuses, brain, lungs, gastrointestinal tract and the skin. In rare cases the maxilla may also be infected but not often due to a rich blood supply to these regions which will most certainly prevent infection by most fungal agents with exception of the more virulent ones (Auluck, 2007).

However, during trauma to soft tissues, opportunistic fungi including mucormycetes will thrive among immunocompetent and immunosuppressed individuals (Kaur et al., 2014, Kennedy et al., 2016, Kontoyiannis and Lewis, 2006, Chayakulkeeree et al., 2006, Mendoza et al., 2015, Saez et al., 2014, Wang et al., 2018, Gkegkes et al., 2018, Skiada and Petrikkos, 2013, Mayayo et al., 2013, Warkentien et al., 2015, Lelievre et al., 2014, Weddle et al., 2012).

This is because traumatic injury promotes implantation of fungal propagules into deeper layers of the tissues possibly leading to accessibility of the endothelial cells by fungal spores. These host cells facilitate infection because they possess GRP78 receptor that binds CotH3, a highly expressed ligand on mucormycete spores that facilities spore binding with host cells as mentioned (Kaur et al., 2014, Kennedy et al., 2016, Kontoyiannis and Lewis, 2006, Chayakulkeeree et al., 2006, Mendoza et al., 2015, Gebremariam et al., 2014).

The commonest clinical form in these patients is invasive cutaneous mucormycosis often linked with co-morbidities such as Morel Lavallee lesions. Morel Lavallee lesion is a closed delving soft tissue injury resulting from an abrupt separation of skin and subcutaneous tissue (Singla et al., 2018, Jundt et al., 2018, Ingram et al., 2014, Kronen et al., 2017, Warkentien et al., 2015, Neblett Fanfair et al., 2012).

Nonetheless, colonization by mucoralean fungi can still be facilitated by other confounding factors such as inoculum size or any of the other mentioned risk factors (Section 1.4.1). Meanwhile in other traumatic patient types such as those with burns, use of broad spectrum antibiotics and/or topical anti-bacterial increases the risk whereas nosocomial mucormycosis is also reported among patients with surgical wounds and catheterization (Chayakulkeeree et al., 2006, Zhao et al., 2012, Davuodi et al., 2015, de Chaumont et al., 2014, Kaur et al., 2014)

1.4.4.4 Immunosuppressive and other risk factor for mucormycosis

Several immunosuppressive conditions that trigger mucormycotic infections have already been mentioned and the list continues to grow, with mortality rates of between 60-100% now associated with immunosuppressive disorders (Chayakulkeeree et al., 2006) (Gurevich et al., 2012, Mousset et al., 2010, Page et al., 2008, Hamdi et al., 2014, Petrikkos et al., 2014, Lewis and Kontoyiannis, 2013, Rodriguez-Gutierrez et al., 2015, Singh et al., 2007, Vinh et al., 2009, Deyo et al., 2017, Sriperumbuduri et al., 2017).

Interestingly, HIV/AIDs is not a common risk for mucormycosis. Its link to the disease is so far associated with intravenous drug use (Chayakulkeeree et al., 2006). Other risk factors recently identified have included neonatal prematurity, systemic lupus erythematosus (SLE), nephrotic syndrome, malnourishment and prolonged use of broad-spectrum antimicrobials including antifungals such as voriconazole (Chayakulkeeree et al., 2006, Gomes et al., 2011, Sriperumbuduri et al., 2017, Fingerote et al., 1990).

1.4.5 The mucormycete related virulence attributes

Whilst host factors are somewhat known, pathogen associated factors for mucormycotic disease development are poorly understood. This knowledge gap has affected our understanding of aetiology, diagnosis, treatment and management, but most importantly the pathogenicity of mucoralean fungi. Thus, examining host-mucormycete interactions is key for underpinning the principles governing disease manifestations.

From present data so far, we can tell that aetiology and risk factors are quite diverse but with common clinical presentations of the disease. This may suggest similar mechanisms in virulence attributes of the aetiological agents. Yet only a handful of virulence factors for mucormycetes have been identified to date including but are not limited to inoculum dose, spore size and dimorphism, hyphal biomass, and ability for attachment to matrix proteins as detailed below (Mendoza et al., 2015, Ibrahim et al., 2012, Gomes et al., 2011)

1.4.5.1 Spore size, inoculum size, and hyphal biomass as virulence attributes for mucormycetes

Mucoralean fungal spores are the main source of infection. The hypothesis that spore size may contribute towards virulence has been proposed (Iwen et al., 2005) . The size of these spores' ranges between 3-11 μm (Ribes et al., 2000, Richardson, 2009). Spore size is linked to their tropism into specific sites of infection, especially in the respiratory tract.

It is believed that smaller spores of size <5μm are deposited in the lower airway whilst larger ones of size >7μm remain trapped in the upper respiratory tract (Richardson, 2009, Chayakulkeeree *et al*., 2006, Li *et al*., 2011, Gomes *et al*., 2011, Iwen *et al*., 2005). Infections due to *Apophysomyces elegans* of spore size 5-8 μm, which causes mainly upper respiratory

mucormycosis, as opposed to *Rhizopus pusillus* of size 3-5μm, causes lower tract disease and has been used to support this paradigm (Gomes et al., 2011, Antachopoulos *et al*., 2010).

Additionally, size is also correlated with spore germination and virulence. For instance, reports show large spores metabolically germinate faster than small spores. This is further demonstrated during infection where large spores, but not small ones, are able to germinate inside macrophages (Gomes et al., 2011, Li et al., 2011). This can be compared to titan and goliath cells of *Cryptococcus* and *Candida* species, respectively which are believed to evade phagocytosis (Zaragoza et al., 2010, Okagaki et al., 2010, Mendoza et al., 2015, Dambuza et al., 2018).

However, if we are to consider large spores as being more virulent, then we may fail to explain why the aetiology of mucormycosis is dominated by *Rhizopus* species which produces spores of small size less than 5μm (Ribes et al., 2000, Petrikkos et al., 2014, Roden et al., 2005). Similarly, so the hypothesis on influence of size on tropism can also be thrown into question.

On the other hand, infection by hyphal biomass can also occur. When this happens, Polymorphonuclear neutrophil (PMN) mediated killing is tasked to damage and eliminate the fungal mass. However, PMN killing can be overcome if the inoculum is heavy enough to impair PMN killing ability (Li et al., 2011, Mendoza et al., 2015, Chayakulkeeree et al., 2006, Honda et al., 1998, Antachopoulos et al., 2010, Simitsopoulou et al., 2010, Chamilos et al., 2008c). This pathogenicity feature is thought to be genus specific though. For instance, *Cunninghamella bertholletiae,* whose infections progress much faster than other aetiologies, are believed to largely benefit from impaired PMN effector functions (Chayakulkeeree et al., 2006, Simitsopoulou et al., 2010). This may partially explain why neutropenic patients are prone to mucormycotic infections.

1.4.5.2 Attachment to matrix proteins

Attachment on a target cell is the first step of infection for most pathogens including most mucoralean fungi. Mucormycete spores have a high affinity for matrix proteins such as laminin and Type IV collagen *in vitro* (Ibrahim et al., 2005, Mendoza et al., 2015, Bouchara et al., 1996). Mucormycetes also show affinity and specificity for endothelial cells *in vitro,* as demonstrated with *R. oryzae,* due to the presence on endothelial cells of the glucose-regulated protein GRP78, a specific receptor for the spore surface ligand CotH (Mendoza et al., 2015, Li et al., 2011, Ibrahim et al., 2005, Liu et al., 2015, Gebremariam et al., 2016, Gebremariam et al., 2014, Liu et al., 2010). Studies in a mouse model show that GRP78, a member of the heat shock protein (Hsp70) chaperone family, is overexpressed during hyperglycaemia and increased iron levels; and CotH expression by *Rhizopus* spores is also increased under similar conditions. Indeed, a mutant of *R. oryzae* for CotH, exhibits reduced ability to invade and damage endothelial cells over-expressing GRP 78; and fails to sustain an infection in DKA mouse model (Liu et al., 2015, Mendoza et al., 2015, Liu et al., 2010, Gebremariam et al., 2014). Indeed, treatment with anti-CotH antibodies prevents *R. oryzae* from establishing an infection in a DKA mouse model, proving that the CotH ligand can be targeted as a virulence factor (Gebremariam et al., 2014, Gebremariam et al., 2016)

1.4.5.3 Spore germination as a virulence attribute of mucormycetes

Fungal spores are ubiquitous in the wider environment and their colonisation of environmental niches is initiated following germination. Similarly, so this physiological and an indispensable process is crucial for the establishment and manifestation of an infection. For instance, this is true with mucoralean fungi where germination of spores to hyphae manifests symptoms such as angio-invasion, tissue necrosis, thrombosis and dissemination (Sephton-Clark et al., 2018). In context, shifts in gene expression levels have been noted following initiation of fungal germination. For instance, over expression of genes for respiration, cell wall remodelling and

cytoskeleton have all appeared to be key for germination (Sephton-Clark et al., 2018, Soll and Sonneborn, 1971, Thevelein et al., 1982, Tsukahara, 1980). Indeed, the transition from dormancy to vegetative forms allows for the onset of disease. *In vitro* this can take as little as 6 h, thus allowing the fungus to colonise favourable environments. Studies on *R. delmar* and *Nosema bombycis* reveal significant differences in transcriptional signatures in dormant and germinated spores (Liu et al., 2016, Sephton-Clark et al., 2018). However, the metabolomics of germinating spores is still poorly understood and exploration of these aspects in now invaluable. In this study, we shall explore the impact of spore germination on host fungal interactions.

1.4.5.4 Bacterial symbiosis as a virulence attribute

It is common for fungi to host bacterial symbionts in what can be termed as purely mutualistic, addictive and transmissible relationship. Mucormycetes, particularly of the *Rhizopus* genus, have been identified to harbour and maintain subtle interactions with endo-hyphal bacteria. (Ibrahim et al., 2008b, Dolatabadi et al., 2016, Araldi-Brondolo et al., 2017, Lackner et al., 2009a, Lastovetsky et al., 2016, Frey-Klett et al., 2011, Gee et al., 2011, Moebius et al., 2014a, Compant et al., 2008, Bonfante and Desiro, 2017, Mondo et al., 2017).

1.4.5.5 Bacterial symbiotic influence on Mucoralean fungi (non-pathogenic mechanisms)

Bacterial endosymbionts are known to establish associations such as facultative, addictive and mutualistic relationships with several hosts. A diverse range of hosts have been revealed to establish such relations including but not limited to plants, insects and fungal hosts (Cooper et al., 2017, Mioduchowska et al., 2018, Papa et al., 2017, Sazama et al., 2019). These associations can influence the hosts capacity to adapt to various environments in addition to rendering them susceptible or resistant to pathogens and antimicrobials (Cooper et al., 2017, Nazir et al., 2014). So far, we note that bacterial symbionts can modulate fungal phenotypes

on various levels including influence on fungal reproduction, metabolism, virulence, thermo-tolerance, antifungal resistance and their evolution among other effects (Spraker et al., 2016, Araldi-Brondolo et al., 2017, Mondo et al., 2017, Murray et al., 2017, Nazir et al., 2014, Salvioli et al., 2016, Hoffman et al., 2013, Shaffer et al., 2017, Lastovetsky et al., 2016, Frey-Klett et al., 2011, Partida-Martinez et al., 2007c).

For instance, Mondo et al (2017) has recently described that bacterial endosymbiont influences fungal sexuality by an endosymbiont gaining transcription control of the fungal *RAS2* gene which encodes GTPases key for fungal reproduction in *R. microsporus* (Mondo et al., 2017). This is also implicated in the reproductive addiction that exists between this fungus and the proteobacteria *Burkholderia* (Mondo et al., 2017). Yet, what remains unclear is whether these impacts contribute towards clinical manifestation of mucormycosis.

1.4.5.6 Bacterial symbiotic influence on mucoralean fungi (pathogenic mechanisms)

Endo-hyphal bacteria contribute towards plant-fungal pathophysiology. Some *Burkholderia* species are endosymbionts of *R. microsporus* and are associated with toxinogenic manifestation of rice blight disease. For instance, the toxins rhizoxin, implicated in rice blight; and rhizonin, a hepatotoxic cyclopeptide are both produced by bacteria belonging to the genus *Burkholderia*. Rhizoxin is a potent anti-mitotic agent that acts by binding beta tubulin of eukaryotic cells, while the mode of action for rhizonin is not yet known but implicated in hepatotoxicity (Scherlach et al., 2006, Partida-Martinez and Hertweck, 2005, Takahashi et al., 1987, Iwasaki et al., 1984, Fukushima et al., 1982, Partida-Martinez et al., 2007a). However, either toxins are yet to be implicated in the pathogenesis of human mucormycosis.

Together with other clinical observations, the hypothesis that bacterial endosymbionts might play a role in the pathogenesis of mucormycosis has been proposed by Chamilos *et al.* (2007). He postulates that whilst mucoralean fungi are equally ubiquitous and virulent as *Aspergillus*, prevalence of mucormycosis among the immunosuppressed was rare in the past perhaps due

to the wide usage of antibiotics in the management predisposed patients. In their observations, they point out the predominance *Rhizopus* species as aetiological agents for mucormycosis. Given the close association between *Rhizopus* and bacterial endosymbionts, they highlight that pathogenicity of these mucoralean fungi might be modulated by bacterial presence (Chamilos et al., 2007).

Indeed, there are other lines of evidence that also support this hypothesis. For instance, Sugar *et al* reports that ciprofloxacin, a quinolone with no intrinsic antifungal activity, can act synergistically with antifungals to improve the survival of mice during mucormycosis. This mode of treatment has been used against *Candida* spp but out-put has been attributed to inhibition of fungal topoisomerases (Sugar and Liu, 2000, Sugar et al., 1997, Chamilos et al., 2007). Sugar et al further shows that elimination of the endosymbiont from the fungus by quinolones reduced fungal pathogenicity and increased antifungal efficacy. However, so far there is no evidence where administration of quinolones alone rescues mice from fungal infection. Taken together, Chamilos *et al* suggests that with use of antibiotics in management of immunosuppressed individuals, mucormycotic cases were kept in check until perhaps later when antibacterial resistance developed (Chamilos *et al.*, 2007).

In regard to, Ibrahim *et al* (2008) recently reported that endosymbionts, though widely found among mucoralean fungi do not contribute towards pathogenesis of mucormycosis. Showing no difference in the survival of DKA mice infected with parent or endosymbiont free strains of *R. oryzae* (Ibrahim *et al*., 2008b). Partida-Martinez *et al* (2008), also through screening of clinical isolates of mucormycetes for endosymbionts, reports absence of toxin producing bacteria employing metabolic, molecular and microscopic screening techniques (Partida-Martinez *et al*., 2008).

From this stand point, it is fair to say that the role of bacterial endosymbionts in pathogenesis of fungal diseases including mucormycosis is not well characterized especially with data from

only two studies suggesting otherwise. It is too soon to neglect bacterial endosymbionts without an exhaustive account. The existing evidence of possible modulation of fungal phenotypes by these symbionts is intriguing enough to demand through dissection of possible influence on fungal; and this thesis will further investigate this point.

1.4.5.7 The fungal cell wall and its' role in immunological responses

The fungal cell wall is a recognised living cellular organelle that has the capacity to change and adopt to demands of the ever-changing environments apart from maintaining cellular integrity. Importantly it is the primary site of interaction with the host cells (Lee and Sheppard, 2016). Studies on the fungus *C. albicans* reveal that its cell wall is mainly composed of three major generic structures including mannans, ß-glucans and chitin as shown in (**Figure 6)** (Gow et al., 2017). However, on the other hand the cell wall of filamentous fungal organisms is poorly studied. Recent studies of the filamentous fungi such as *Aspergillus fumigatus* show that over 90% of its cell wall is made of polysaccharides (Latge et al., 2017). These include the (alpha and beta) 1,3 glucans, chitin, galactomannan and beta (1,3) (1,4) glucans, whose biosynthesis has been associated with three major enzymes i.e. the transmembrane synthases, the transglycosidases and glycosyl hydrolases (Latge et al., 2017, Bernard and Latge, 2001). Recently the role of melanin is being better appreciated as a filamentous fungi immune modulation tool especially with *A. fumigatus* (Chai et al., 2010, Stappers et al., 2018). All of these structures (also called the pathogen associated molecular patterns (PAMPs) play major roles during infection because recognition of these structures by the host pattern recognition receptors (PRRs) can orient the host immune responses (Dubey et al., 2014, Bernard and Latge, 2001, Gow et al., 2017). On the other hand, the study into the mucormycete cell wall is lagging behind. But so far, a few studies reveal the uniqueness that exists between the mucormycete and other well characterised fungal cell walls (Melida et al., 2015). Studies on *Rhizopus oryzae* and *Phycomyces blakesleeanus* show the abundance of fucose-based polysaccharides similar

to algal fucoidans (Melida et al., 2015). These are unexpected polymers usually associated with low glucans and increased chitin expressions levels (Melida et al., 2015). However, conserved production of mannans in Zygomycetes and Ascomycetes has been noted (Burnham-Marusich et al., 2018). To a lesser extent, the cell wall of the mucormycete spores remains poorly studied, but a study by Andrianaki *et al*, reveals the role of melanin in inhibiting macrophage functions such as phagosome maturation (Andrianaki et al., 2018b).

Figure 6: The fungal cell wall is made of three main generic structures.

The fungal cell wall is made of four major generic morphological structures including mannans, Beta-glycan and chitin. Image taken from J. Ene and N. Gow.

1.4.5.8 Stages of mucormycete infection of host cells

Susceptibility to mucormycosis is strongly linked to the underlying conditions that predispose one this fungal disease. For instance, iron deposition is a marked factor for hypersensitivity to mucormycosis (Ibrahim et al., 2010, Shirazi et al., 2015). However, the lack of functional phagocytes also underlines how vital these cells are in the host defence against mucoralean fungi (Roilides et al., 2012, Inglesfield et al., 2018). For mucoralean fungi to be successful pathogens must adhere to, invade while evading host defence mechanisms. Thus, understanding how this happens in crucial to underpinning control of mucormycosis. Unfortunately, there is no enough information available on how mucoralean fungi invade host cells in the various sites including lungs, sinus, intestinal epithelial and soft tissue to elicit an infection.

So far, the stages of infection have been suggested to involve; **Firstly**, interaction of infecting propagules with extracellular matrix components (Section 1.4.5.2). These are usually extracellular protein matrices that separate epithelial and endothelial cells from the stroma, providing support and acting as barriers for the invading organisms (**Figure 7**) (Timpl et al., 1979). **Secondly**, breaching the endothelial barrier leads to the interface between the infecting particles with the host defence mechanisms particularly innate phagocytes and complement system. For instance, inhalation of mucoralean spores does not lead to development of disease because macrophages phagocytose spores and inhibit their germination **(Figure 7**) (Waldorf and Diamond, 1984). In susceptible individuals mucormycete can easily evade macrophage mediated killing such as generation of oxidative metabolites, cationic peptides and defensins (Ibrahim, 2011, Diamond et al., 1978, Waldorf and Diamond, 1985). **Thirdly**; interaction and evasion of the endothelium (angio-invasion). This has been suggested by some authors as the most crucial stage in the pathogenesis of mucormycosis also referred to as the hallmark for the infection (Ribes et al., 2000, Spellberg et al., 2005a). This angio-invasion subsequently results

into vessel thrombosis and tissue death further exacerbating the infection following cut off of leukocyte and antifungals to the foci of the infection. Additionally, this stage of infection is also underlined by the presence of host GRP78 and the fungal CotH ligand (Gebremariam et al., 2014). However, the interaction mechanisms between mucormycete propagules and host cells still remains to be fully characterised.

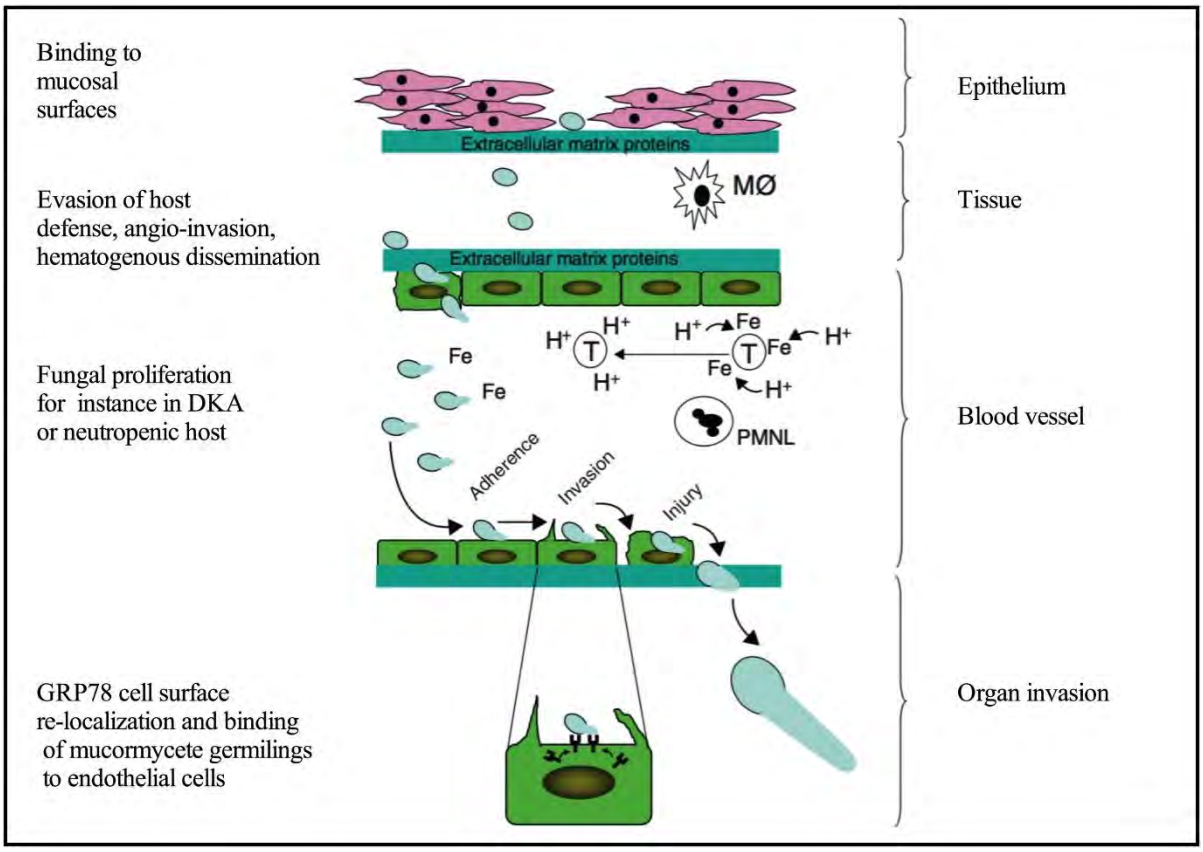


Figure 7: Mucoralean fungi invades host through evasion of host defence mechanisms.

Mucormycosis is initiated through inhalation, ingestion or direct implantation into an abraded skin of fungal particles. The spores then bind host exposed matrix proteins, this can be aided by secreted fungal proteases. Once inside the tissue, the spores can evade macrophage mediated killing effector functions. This leads to invasion of the endothelium, in susceptible individuals the spores quickly thrive and disseminate to vital organs (Image taken from (Ibrahim, 2011).

1.4.6 Diagnosis of Mucormycosis

Early diagnosis is important in clinical management of mucormycosis but can be challenging because early symptoms are usually non-specific and are often indistinguishable from other fungal infections (Kontoyiannis and Lewis, 2011). Unfortunately, there are hardly any serological biomarkers, and recently developed tools such as ß-D-glucan and *Aspergillus* galactomannan tests are yet to prove applicable to mucormycotic diagnosis (Farmakiotis et al., 2018). Additionally, immunodiffusion techniques often used for the diagnosis of close relatives, the entomophthoramycetes, are yet to prove useful for mucormycosis and have shown vulnerability to cross-reactivity (Kaufman et al., 1990, Yangco et al., 1986, Mendoza et al., 2015).

Thus, mucormycotic diagnosis is achieved primarily by microscopy (direct or fluorescence), culture and histology (such as haematoxylin and eosin (H&E), Grocott –Gomori methenamine-silver nitrate and period acid Schiff (PAS)) (**Figure 8).** Histology is a more definitive tool compared to direct microscopy and culture; in addition, both techniques are prone to environmental contamination. Indeed, sensitivity of culture results can be questionable, but repeated culturing and isolation from sterile sites does allow positive diagnosis (Kontoyiannis and Lewis, 2011, Kontoyiannis et al., 2000). Note that a positive direct examination does not always correlate with culture results.

Recently, improved and more advanced diagnostic tools have also been invented to improve clinical diagnosis of mould infections. These have included aggressive computed tomography (CT) guided fine needle aspiration. This is superior to many of the conventional techniques such as staining, polymerase chain reaction, and antigen and enzyme biomarkers, but is not routinely applicable due to several challenges including limited access to CT scanners, lack of awareness of infection risk among diagnosis doctors, CT data interpretation, cost, rapid rate of disease progression among others (Lass-Florl et al., 2007, Kontoyiannis and Lewis, 2011).

It is important differentiation of mucormycetes from other moulds is promptly made for proper and specific management of mucormycosis. Usually, molecular approaches such as PCR are used for this purpose but are not widely applicable especially in resource limited settings (Schwarz et al., 2006, Kasai et al., 2008). It would also appear that a physician's early suspicion of the disease can also be as important during diagnosis of mucormycosis (Kontoyiannis and Lewis, 2011).

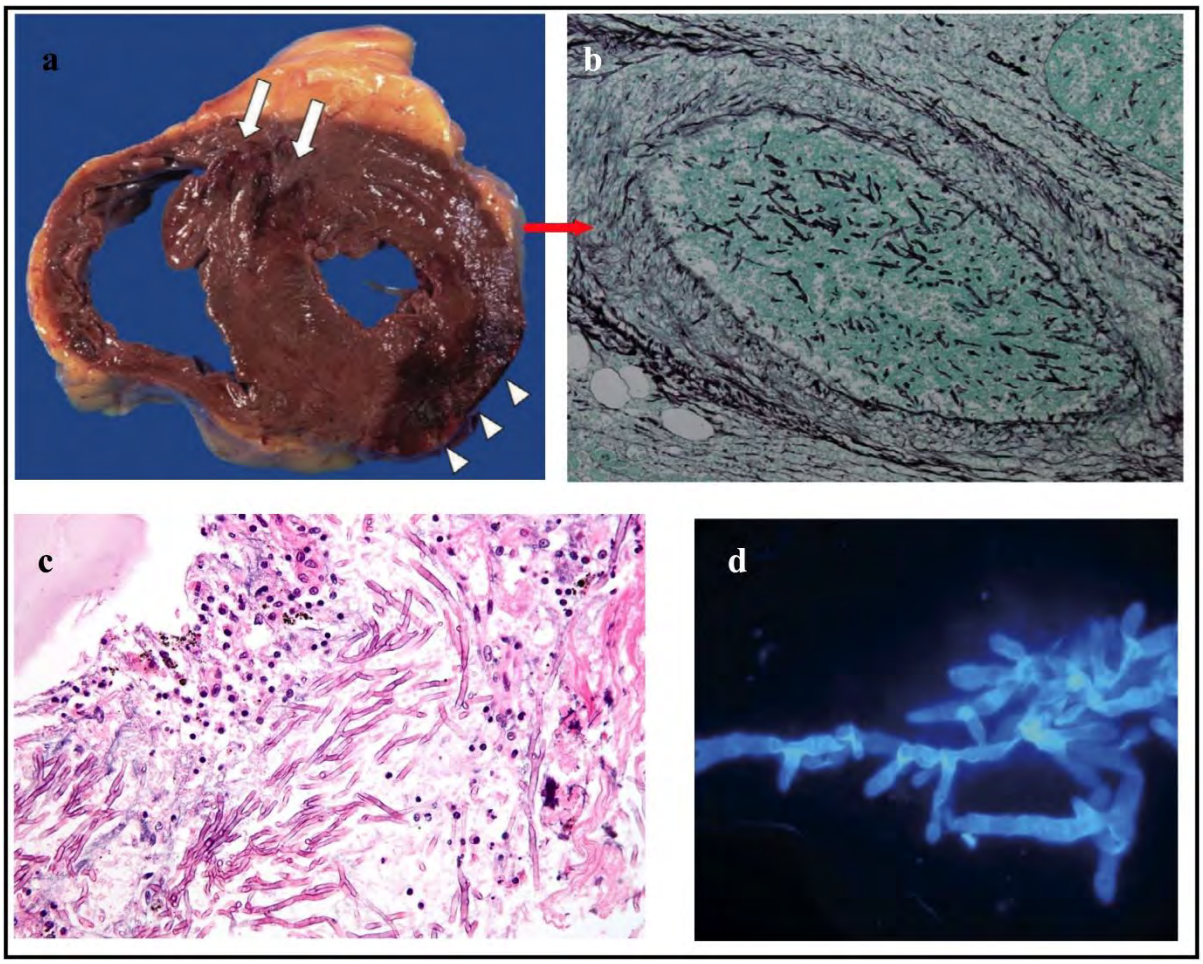


Figure 8: Tools for diagnosis of clinical mucormycosis

Clinical materials and some of the common tools used in the diagnosis of mucormycosis including (**a**) shows pathological tissue sample demonstrating sections of fungal infestation shown by arrows (**b**) demonstration of Grocott sliver staining of a pathological tissue sample (**c**) demonstration of haematoxylin and eosin staining of an infested pathological tissue sample and, **(d**) demonstration of calcofluor white staining of fungal elements in pathological samples. Images are adopted from clinical reports of mucormycosis (Taken and adapted from Lass-Florl and Joshita et al, (Lass-Florl, 2009, Joshita et al., 2008).

1.4.7 Treatment of Mucormycosis

Mucormycosis is a challenging infection to treat and proper management of the disease is dependent on four major factors including timely diagnosis, reversal of the underlying predisposing factors, surgical debridement, and aggressive administration of antifungal therapy (Chamilos et al., 2008b, Mendoza et al., 2015, Kontoyiannis and Lewis, 2011, Guevara et al., 2004). A timely diagnosis significantly impacts prognosis, since early surgical removal of focal lesions can be triggered to prevent dissemination to vital organs (Mendoza et al., 2015). Reversal of predisposing factors helps to boost the host's immunity (Mendoza et al., 2015, Abe et al., 1990, Ibrahim et al., 2007), and surgical debridement of infected tissues is widely used in mucormycotic management because the disease causes blood vessel thrombosis and necrosis that cut off blood supply to the infected sites. This also negatively impacts penetration by antifungals to the site (Roden et al., 2005). Indeed, a combination of surgical debridement and antifungal treatment improves prognosis compared to use of antifungals alone, whilst a surgical remedy can be applied independently (Langford et al., 1997, Tedder et al., 1994, Mendoza et al., 2015, Pelton et al., 2001, Ochi et al., 1988).

1.4.7.1 Antifungal treatment remedy for Mucormycosis

Mucoralean fungi are resistant to most antifungal agents but do show sensitivity to the polyenes which act by irreversibly binding ergosterol in the fungal cell membrane causing leakage of cell contents. These include drugs such as Amphotericin B deoxy cholate (AmB), however, treatment with this class requires administration of high doses to counter resistance. Mechanism of amphotericin B resistance can be via alterations in sterol content, re-orientation, masking or reduced ergosterol content and previous exposures to azoles (Spellberg et al., 2005b, Mendoza et al., 2015).

AmB is also associated with toxicity, but several modifications such as lipid modifications to Liposomal AmB (LAmBisome) or amphotericin B lipid complex (Abelcet) have been made that reduce toxicity though are also associated with reduced efficacy and penetration (Ibrahim et al., 2003, Noel, 1999, Reed et al., 2008, Gleissner et al., 2004, Mendoza et al., 2015, Groll et al., 2000, Ibrahim et al., 2008a, Lewis et al., 2010, Hamill, 2013, Gebremariam et al., 2017b). Occasionally, other antifungal classes such as azoles (e.g. itraconazole, fluconazole, voriconazole, triazole and posaconazole) have also been used to manage mucormycosis. These act by inhibiting the cytochrome P450-dependent 14-alpha-lanosterol demethylase of the fungal membrane leading to an accumulation of toxic cell membrane sterols and inhibit ergosterol production. This in turn causes impaired growth, replication and cell death. Mechanisms of resistance to azoles include, alteration or over-expression of lanosterol (14-alpha) demethylase, and presence of energy dependent efflux systems (Sun et al., 2002, Espinel-Ingroff et al., 2001, Cuenca-Estrella et al., 2006, Mendoza et al., 2015, Dannaoui et al., 2002).

In regard to azoles, Itraconazole has limited activity against *Rhizopus* species but has shown to have *in vitro* efficacy against *Lichtheimia* species (Sun et al., 2002, Mendoza et al., 2015). Fluconazole and the second generations broad spectrum azoles (e.g. triazole and voriconazole) have no *in vitro* activity against mucorales, whilst voriconazole, often used as a prophylactic treatment in at risk patients for invasive fungal diseases. Indeed, there have been reports for occurrence of mucormycosis in during treatment of aspergillosis and other mold infections with voriconazole (Blin et al., 2004, Shindo et al., 2007, Pongas et al., 2009, Vigouroux et al., 2005, Marty et al., 2004, Kobayashi et al., 2004, Oren, 2005, Sharifpour et al., 2018).

In vitro investigations show similar findings, where simultaneous pre-treatment with all AmB, caspofungin acetate and voriconazole increases virulence of mucoralean fungi in both murine and drosophila models and the mode of resistance has been linked to ergosterol alterations

(Lamaris et al., 2009, Lewis et al., 2011). Labelling voriconazole as risk for acquiring mucormycosis in at risk individuals.

Posaconazole demonstrates in *vitro* activity against mucormycetes but efficaciously inferior to AmB against *Rhizopus oryzae* and *Mucor circinelloides* in a murine model (Spreghini et al., 2010, Dannaoui et al., 2003, Barchiesi et al., 2007, Luo et al., 2013, Enoch et al., 2011). Clinically, posaconazole can be an alternative in patients intolerant to or as a step down from polyenes (Mendoza et al., 2015, Enoch et al., 2011, Salas et al., 2012, Rodriguez et al., 2008). Isavuconazole and echinocandins (which target 1, 3-beta glucan synthase) are also activate against mucormycetes but with a narrow spectrum (Guinea et al., 2010, Guinea et al., 2008, Pfaller et al., 2013, Mendoza et al., 2015).

Together, this demonstrates that antifungal mono-therapy is not enough for treatment of mucormycosis, and that combination therapy may improve prognosis when both intra- and interclass antifungals are used (Lewis et al., 2010, Perkhofer et al., 2008, Simitsopoulou et al., 2008, Gebremariam et al., 2017b, Gebremariam et al., 2017a, Luo et al., 2014).

1.4.7.2 Other treatment remedies for mucormycosis

Non-antifungal approaches can also be used in synergy to supplement treatment with antifungals. Such remedies include chelating agents such as iron chelators (e.g. deferoxamine and deferasirox). However as mentioned (Section 1.4.4.2) deferoxamine predisposes one to mucormycosis by acting as a xeno siderophore for iron whilst deferasirox is fungicidal against mucoralean fungi (Boelaert et al., 1993). A siderophore is a small, high affinity iron-chelating agent produced by fungi or bacteria to sequester iron across membranes; and a xeno siderophore would be a siderophore used by an organism other than the one that produces it (Boelaert et al., 1988, Arizono et al., 1989, Kaneko et al., 1991, Rex et al., 1988, Windus et al., 1987). A combination of deferasirox and liposomal AmB improves survival and reduces fungal

burden in a murine model, though clinical trials of this combination were inconclusive (Donnelly and Lahav, 2012, Ibrahim et al., 2007, Spellberg et al., 2009, Spellberg et al., 2012). Lastly, other strategies have included use of barbaric oxygen in conjunction with standard therapy and FK506. The hyperbaric oxygen therapy is often used after surgical debridement particularly in cases of cutaneous disease and rhino-cerebral disease. High oxygen concentrations may improve neutrophil killing of mucoralean fungi or inhibit fungal germination (Almannai et al., 2013, Ribeiro et al., 2013). FK506, is 23-membered macrocyclic polyketide. It possesses several biological activities such neuroprotective, neuro-regenerative anti-inflammatory and antifungal activities. It acts by targeting the heat shock protein 90 (Hsp90) heavily involved in fungal survival and control calcineurin protein during stress responses and cell wall repair. Thus, FK506 operates by targeting Hsp90-calcineurin pathway (Ban et al., 2016, Lamoth et al., 2015)

1.5 Immunity to Filamentous Fungal Infections

Immunity is a balanced state of having primed or adequate defences to fight infection by unwanted biological invaders while having tolerance to self-antigens to avoid autoimmune disorders. Antifungal immune responses can be dynamic depending on the individual organism characteristics such as morphology (conidia, spores or hyphae) and the site of infection. Primarily we are exposed to moulds such as mucoralean fungi through inhalation of infecting particles called spores. If not adequately cleared, disease progression often happens especially when a host's recognition system or subsequent immune responses are broken down also known as immunosuppression (Blanco and Garcia, 2008, Romani, 2011). Antifungal immune responses are divided into innate or adaptive immunity (Deepe and Bullock, 1990, Janeway, 2005, Roitt and Delves, 2001, Powers-Fletcher et al., 2016).

1.5.1 Innate Antifungal Immunity

This is the first line of defence and largely not as specific as the adaptive. It is constitutive and includes physical barriers such as the skin, mucous membrane surfaces, normal microbial flora and several defensins including cell membranes, cellular receptors and humoral factors (Blanco and Garcia, 2008, Romani, 2011, Medzhitov and Janeway, 1997, Janeway, 2005). Beyond these is a host of other innate effector faculties such as hematopoietic phagocytes, including tissue resident macrophages, neutrophils, dendritic cells; and non-hematopoietic cells such as endothelial and epithelial cells (Janeway, 2005, Taylor-Smith, 2017, Leopold Wager et al., 2016, Roitt and Delves, 2001, Brown, 2011). The phagocyte including neutrophils, monocytes and macrophages are a key component of innate antifungal immunity because they facilitate intra and extracellular fungal containment and killing (Brown, 2011, Powers-Fletcher et al., 2016). Respectively, monocytes and macrophages recognise, engulf and contain fungal particles attenuating fungal growth through antimicrobial secretions and nutrient deprivation, while neutrophils and monocytes will kill and damage conidia, spore germilings or fungal hyphae by secretion of defensins and through oxidative mechanisms (Andrianaki et al., 2018b, Powers-Fletcher et al., 2016, Aimanianda and Latge, 2010, Luther et al., 2007, Schaffner et al., 1982, Boyle et al., 2012). Indeed, underlying conditions such as leukaemia, aplastic anaemia, corticosteroid therapy, HIV/AIDs and auto immune diseases which can impair phagocyte counts and functions especially neutrophils can predispose one to infections by invasive fungal diseases including mucormycosis. For instance, corticosteroid therapy is known to impede oxidative burst and reduce lysosomal activity subsequently decreasing immune-cell mobilization (Duong et al., 1998, Ng et al., 1994, Powers-Fletcher et al., 2016).

However, an interaction between these phagocytes and fungal particles is also facilitated by pattern recognition receptors (PRRs). These can be soluble or cell-associated and recognise conserved microbial structures called pathogen associated molecular patterns (PAMPs). For

instance, fungi possess cell wall antigens such as the capsule, glucans, glycoproteins, glucans, nucleic acids and melanin. These can trigger specific immune responses. PRRs are also expressed on other cells including dendritic and various nonprofessional immune cells such as endothelial cells, fibroblasts and epithelial cells (Janeway, 2005, Roitt and Delves, 2001, Garcia-Carnero et al., 2018, Blanco and Garcia, 2008, Salek-Ardakani et al., 2012, Powers-Fletcher et al., 2016).

Four families of PRRs have been identified so far and include C- type lectin receptors (CLR), Toll like receptors (TLR), nucleotide binding oligomerization domain (NOD)-like receptors (NLRs), and retinoic acid inducible gene (RIG)-I-like receptors (RLRs) **Table 2**. Among all, CLRs and TLRs appear to be the empirical receptors associated with innate-antifungal immunity (Powers-Fletcher et al., 2016, Levitz, 2010).

The CLRs are a group of both soluble and transmembrane receptors. Several including Dectin-1, Dectin-2, dendritic cell (DC)-specific intracellular-adhesion molecule (ICAM) 3-grabbing non-integrin (DC-SIGN), macrophage inducible C-type lectin (Mincle) and soluble mannose-binding lectin (MBL) receptors. These interact with chitin, *N*-linked mannans and both alpha and beta glucans (Powers-Fletcher et al., 2016, Wang et al., 2011)

Yet, TLRs, are a family of about 10 transmembrane receptors and appear to be one of the main recognition receptors for fungal pathogens through sensing beta glucan, mannans and nucleic acids. Specifically, TLR-2, -3, -4, -6, and -9 have been named in this regard (Braedel et al., 2004, Powers-Fletcher et al., 2016). It is important to note that, different fungal morphologies may impact on the type of immune responses via interaction with TLRs. For instance, whilst an interaction between *Aspergillus* conidia and macrophage via a TLR-4 dependent mechanism leads to production of pro-inflammatory cytokines (TNF-alpha) and interleukin (IL)-1ß, interaction with hyphae via TLR-2 predominantly yields inflammatory cytokine IL-2. Through

this, *Aspergillus* species are capable of evading the host's innate immunity (Netea et al., 2003, Braedel et al., 2004, Powers-Fletcher et al., 2016).

Table 2: Fungal PAMPs and cognate PRRs

PAMPs on all fungi	**PRRs**	
Mannans	Mannose receptor (CD206), DC-SIGN (CD209), Lingering (CD207), and dectin-2, mincle (CLEC4E) for alpha mannans, scavenger receptors CD5, CD36 and SCARF1.	(Kim et al., 2011, Wang et al., 2011, Powers-Fletcher et al., 2016, Means et al., 2009)
1,3 β-glucans	Dectin-1 (CLEC7A) and complement receptor 3 (CR3 (CD11c/CD18))	(Kim et al., 2011, Stier et al., 2014)
1,6 β-glucans	Dectin -1, and CR3	(Stier et al., 2014)
Chitin	Mannose receptor (CD206), Dectin-1, TLR2	(Bueter et al., 2013)
Ligands specific to some fungi		
α glucans of *Pseudallescheria boydii*	Dectin-2 (CLEC6A)	(Levitz, 2010)
BAD1 of *Blastomyces dermatitidis*	CR3 (CD11c/CD18)	(Levitz, 2010)
HSP60 of *Histoplasma capsulatum*	CD18	(Levitz, 2010)
Phospholipomannan of *Candida albicans*	TLR2	(Netea et al., 2006, Levitz, 2010)
O-linked mannoses of *C. albicans*	TLR4	(Netea et al., 2006, Levitz, 2010)
Opsonic ligands		
C3b	CR1 (CD35)	(Levitz, 2010)
iC3b	CR3 (CD11b/CD18) and CR4 (CD11c/CD18)	(Levitz, 2010)
IgG	FcγRI (CD64), FcγRIIA (CD32) and FcγRIIIA (CD16)	(Levitz, 2010)

1.5.2 Adaptive Anti-fungal Immunity

Innate immunity plays a key role in activating the adaptive system. Several factors are involved including production of chemotactic factors such as complement peptides, chemokines, cytokines or leukotrienes produced by host cells but can also be pathogen specific. The linkage between the innate and adaptive immune responses against fungal infections is mediated mainly by dendritic cells. This is done through antigen presentation of fungal antigens, expression of lymphocyte costimulatory molecules, secretion of cytokines and migration to lymphoid tissues (Ramirez-Ortiz and Means, 2012, Roy and Klein, 2012, Powers-Fletcher et al., 2016). Resultant signals from these interactions may vary depending on the type of fungal antigen presented subsequently impacting the adaptive T cell and antibody production and response.

For instance, a T-helper type 1 (Th1) or Th-17 activation leads to a pro-inflammatory antifungal cellular immune response through $CD4^+$ differentiation. This causes production of anti-fungal specific cytokines including Th1 derived IFN-gamma and IFN-alpha; and / or a Th17 derived IL-17 and IL-22. While Th1/Th17 activation is pro-inflammatory, Th2 (e.g. IL-4, IL-5 and IL-10) is anti-inflammatory thus, a strong Th1/Th17 response is protective against fungal infections.

This also shows that T-cell immunity is crucial against fungal infection. Thus, conditions affecting T-cell functions such as anti-rejection therapy used during solid organ transplantation predisposes one to fungal infections (Espinosa and Rivera, 2012, Schlitzer et al., 2018, Rivera et al., 2006, Rivera et al., 2011, Wuthrich et al., 2016, Wuthrich et al., 2011).

Finally, a few studies on invasive fungal diseases show that fungus specific antibodies may confer protection. Some *in vitro* and *in vivo* studies have demonstrated antibody mediated killing through opsonisation with subsequent enhancement of T-cell function; and antifungal

vaccine protection. Majority of the antibodies so far are restricted to recognition of cell surface molecules (Hernandez-Santos et al., 2013, Edwards, 2012, Powers-Fletcher et al., 2016).

1.5.3 Phagocytosis, a key component of antifungal innate immunity

Phagocytes recognise and engulf infectious particles via an actin polymerization dependent process called phagocytosis (Aderem and Underhill, 1999, Nicola et al., 2008, Stuart and Ezekowitz, 2005, Underhill and Goodridge, 2012). This process is also important during physiological body development and tissue remodelling (Aderem and Underhill, 1999). Phagocytosis is an intrinsically primed process among professional phagocytes such as macrophages, neutrophils and dendritic cells. Other cells such as B-lymphocytes and epithelial cells may also possess limited phagocytic attributes and these are collectively called 'paraprofessional' or 'non-professional' cells (Lovewell et al., 2014, Aderem and Underhill, 1999, Hallett and Dewitt, 2007).

The process is subtle and highly explicit, often triggered by the engagement between specific PRRs and conserved PAMP domains (Stuart and Ezekowitz, 2005). This trigger a flow of phagocytic based information including signal transductions, actin-based motility, membrane trafficking, and phagocytosis is complete when a target particle is engulfed in a cellular compartment called a phagosome (**Figure 9**) (Aderem and Underhill, 1999, Underhill and Goodridge, 2012, Underhill and Ozinsky, 2002, Stuart and Ezekowitz, 2005).

Following engulfment, a series of downstream machinery, notably the formation of a phagolysosome, are activated where key defensive factors such as oxygen radicles, nitrogen oxide, antimicrobial proteins, defensins, binding proteins and hydrogen ion transporters are also recruited (Stuart and Ezekowitz, 2005, Diamond, 1993, Brakhage et al., 2010, Diamond et al., 1978). This subsequently culminates in the critical peak of phagocytosis called phagolysosome maturation leading to particle internalisation and degradation, antigen presentation, inflammatory responses (**Figure 9**) and activation of the adaptive system

(Diamond, 1993, Nicola et al., 2008, Stuart and Ezekowitz, 2005, Underhill and Ozinsky, 2002, Underhill and Goodridge, 2012).

However, given the importance of this process, some pathogens have developed mechanisms to evade phagocytosis (Aderem and Underhill, 1999). For instances, *Listeria monocytogenes* escapes phagosomes into the cytosol following uptake, while *Mycobacterium tuberculosis* can prevent the fusion between phagosomes and lysosomes. The antigenic capsules possessed by some bacterial and fungal pathogens also attenuates phagocytic processes (Aderem and Underhill, 1999). It is also important to note that certain therapeutic regimens such as corticosteroid therapy may interfere with phagocytic mechanisms. This has proved true for mucormycete infections (Ribes et al., 2000).

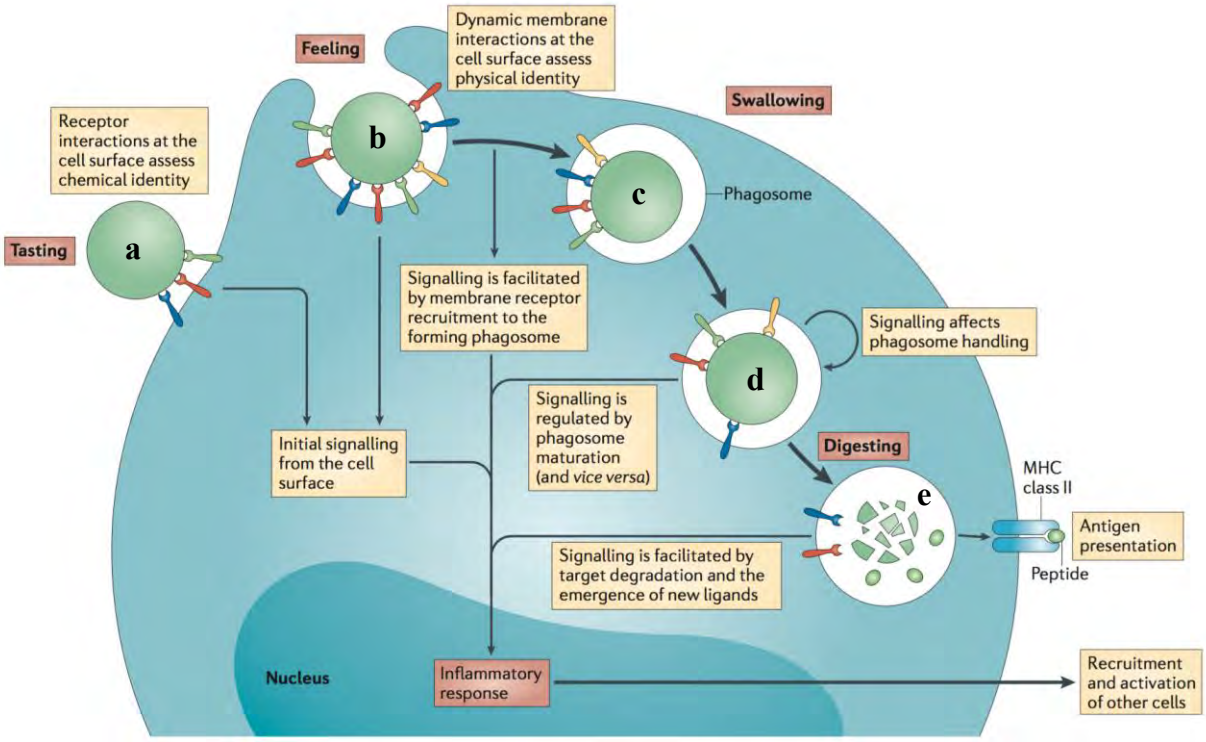


Figure 9: Phagocytosis of foreign particles such as fungal spores is governed by strict host information flow.

Under health conditions phagocytes will recognise mucormycete spores by conserved germ line PRRs that respond to spore PAMPs such as the cell wall glycoproteins. (**a**) This engagement then sets in motion a cascade of events including **(b)** an actin dependent phagocytic uptake of the spore leading to **(c-d)** containment, internalisation which is decorated with phagolysosome acidification and maturation **€** this subsequently leads to degradation of the fungal spore with activation of inflammatory responses and antigen presentation. The image was taken and adopted from Underhill et al (2012).

1.5.4 Innate Immunity against Mucoralean Fungi

Despite a detailed description of anti-fungal immunity here our understanding of innate responses against mucoralean fungi remains limited. However, if we are to compare with immune responses against other invasive fungal diseases such as Aspergillosis, perhaps similar conclusions can be drawn. So far, similar response patterns have been reported. For instance, the role of phagocytes such as neutrophils and macrophages in recognition, uptake and containment of mucoralean fungal spores has also been shown by several authors employing both *in vitro* and *in vivo* approaches **(Figure 10)** (Voelz et al., 2015, Inglesfield et al., 2018, Aimanianda and Latge, 2010, Brakhage et al., 2010, Li et al., 2011, Andrianaki et al., 2018b, Chamilos et al., 2008a).

Importantly, both *Aspergillus* and mucoralean fungal species are able to evade phagocyte effector functions. Indeed, *A. fumigatus* resting conidia evades macrophage recognition due to masking by a spore protein coat, the rod let; while mucoralean fungi such as *R. oryzae* and *M. circinelloides* are able to avoid macrophage recognition and killing (Aimanianda et al., 2009, Aimanianda and Latge, 2010, Brakhage et al., 2010, Li et al., 2011, Andrianaki et al., 2018b). Whole genome expression data in a fly model also suggests that infection with mucormycete spores selectively regulates genes involved in pathogen recognition, stress responses, detoxification, steroid metabolism or tissue repair, including several other genes with no known functions (Chamilos et al., 2008a, Gomes et al., 2011). What is intriguing here is that 37% of the genes down regulated in the fly model are also involved in tissue repair. Consistent with this, mucormycosis is clinically dominated by angio invasive and necrotic foci (Chamilos et al., 2008a).

Thus, we ought to critically explore phagocyte-mucoralean fungal interaction mechanisms to better our understating of both molecular and cellular mechanisms involved during mucormycosis pathogenesis.

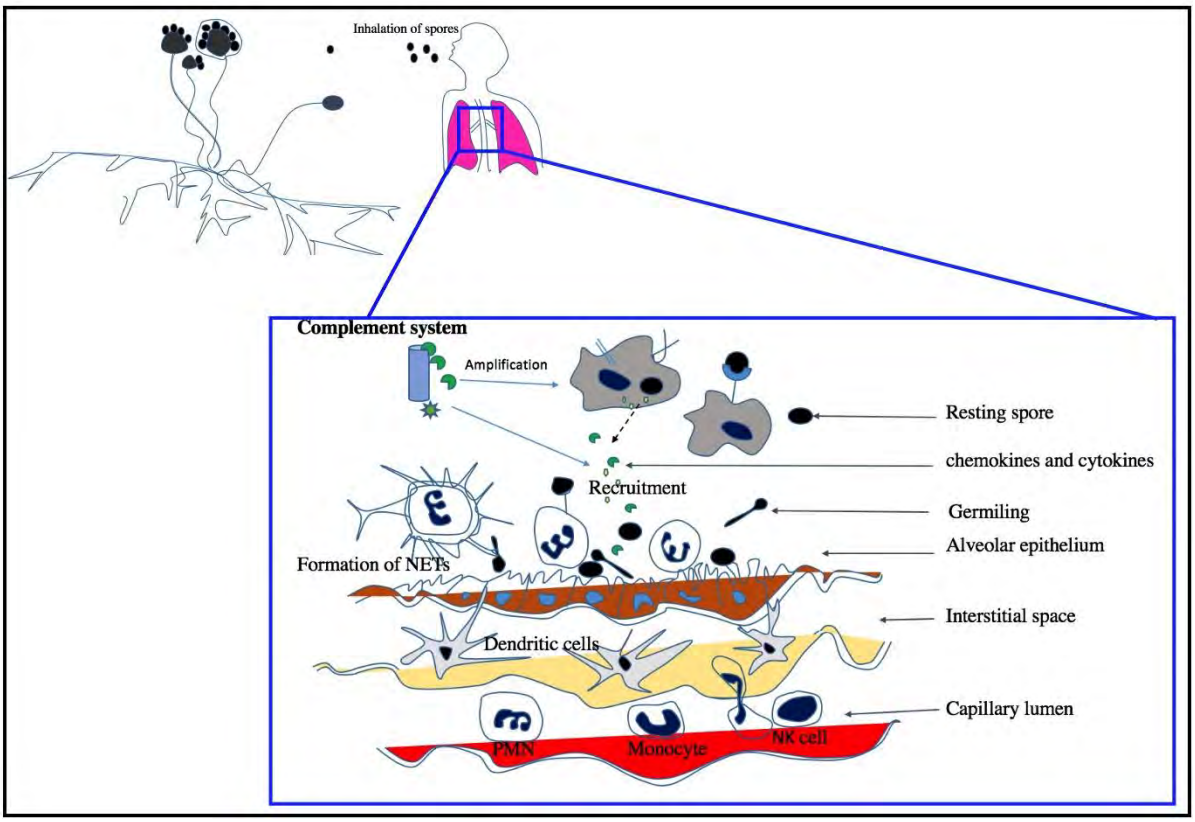


Figure 10: The innate immune system is crucial in containing mucormycete infecting propagules.

Following inhalation of mucormycetes, innate cells such as macrophages recognise spores and phagocytose them. Following phagocytosis, macrophages release cytokines which amplify immune response by activating the complete system and recruitment of immune cells such as neutrophils and dendritic cells which later activate the adaptive immune system.

1.6 Aims and Strategy

Mucormycosis is the second most invasive filamentous fungal infection after Aspergillosis. The disease targets a cohort of individuals with certain underlying conditions (Section 1.4.1) and is associated with several challenges including poor diagnosis, costly but often unsuccessful treatment, and high level of antifungal resistance leading between 40-90% mortality rates (Section 1.4). This has raised awareness of the medical importance of the disease worldwide, and substantial efforts are being made to understand its pathogenesis in recent years. Indeed, we now have a better understanding of the factors that predispose one to the infection, but the virulence attributes of mucormycetes remain elusive. One of the major risk factors for mucormycosis is a defect in phagocytic effector function. Yet, studies on other filamentous fungi such as *Aspergillus* and mucormycetes highlight the importance of innate immunity, particularly phagocytes, in containing the infecting particles (Voelz et al., 2015, Inglesfield et al., 2018, Aimanianda and Latge, 2010, Brakhage et al., 2010, Li et al., 2011, Andrianaki et al., 2018b, Chamilos et al., 2008a). However, it is possible that while host immune response to filamentous fungi is conserved, different fungi including the mucoralean fungi may possess variable mechanisms for evading host immune responses. All taken together, our understanding of host-mucormycete interactions is very limited, yet with recent technological advancements both molecular and cellular mechanisms involved can be navigated. The research presented in this thesis deepens our understanding by exploring the role played by phagocytes particularly macrophages against mucormycete infection. Future research will not only focus on molecular interactions but also other confounding factors such as signalling pathways mediated by both hosts and pathogens.

The **first study** focuses on the host, where interactions between macrophages and mucormycete infecting spores are explored. Studies on *Aspergillus* have shown that

macrophages are important in containing infecting particles especially in the early stages of infection. However, it has also been established that recognition and uptake of *Aspergillus* conidia by macrophages may depend on their metabolic state. This part of the thesis demonstrates interaction mechanisms between mucormycete spores and macrophages, describing some of the key protocols applicable for mucormycete study.

The **second study** of the thesis is a follow up on observations made in the first study that pre-germinated spores show reduced phagocytic uptake compared to resting spores. This is opposite to what has been found with *Aspergillus.* This section therefore reports that mucormycete metabolic spores secrete a supernatant factor that significantly affects phagocytic uptake by macrophages. The chapter characterises the impact of the supernatant factor on other phagocytic effector functions. The second part attempts to characterise and identify the supernatant using physical and chemistry approaches.

The **third and final study** is a follow up of observations made in previous two studies. This fronts the hypothesis that the supernatant factor in question is secreted by a bacterial symbiont that resides inside the fungal cytosol. It is not uncommon for mucormycetes, especially *R. microsporus,* to harbour bacterial endosymbionts. Although endo-hypha bacteria have been implicated in plant pathology, their contribution to clinical mucormycosis is yet to be fully established. A few studies have already shown that bacterial symbionts may play no role in clinical manifestations of the disease, yet to the contrary others demonstrate that bacterial symbionts significantly modulate fungal phenotypes. Here, we identify the bacteria and explore the influence of bacterial symbiosis on fungal pathogenicity.

CHAPTER TWO: Methods and Materials

2.1 Chapter Overview

Some of the methods presented in this chapter were performed in collaboration with other Lab groups. Specifically, metabolomics (Dunn Lab, School of Biosciences, UoB), Chemistry work (Trillo Lab and Mass spectrometry, School of Chemistry, UoB); and Mouse work (Ashraf Ibrahim's Lab, University of California Los Angeles (UCLA, USA)). For all these, we prepared the samples and also participated in some experimental and data analyses, particularly HPLC and survival data analyses.

2.2 Ethics

All zebrafish care and experimental procedures were conducted according to Home Office legislation and the Animals (Scientific Procedures) Act 1986 (ASPA) under the Home Office project license 40/3681 and personal licenses l13220C2C to Kerstin Voelz and lCDB92D64 to Herbert Itabangi.

Blood work: Blood was performed after completion of Hepatitis B vaccination. Whole blood was collected according to the Hall ethical approval for blood work. Buffy coat and whole blood derived cells were destroyed 7 days after initial blood isolation.

2.3 Strains and growth conditions

2.3.1 Mucoralean strains and cultivation

Mucormycete strains (**Table 3**) were routinely cultured on sabouraud dextrose agar (SDA) (EMD Millipore co-operation) or potato dextrose agar (PDA) to induce sporulation at room temperature. For metabolic activation assay, mucormycete spores were cultivated in conical flasks containing 100 - 250 mL of sabouraud broth or serum free Dulbecco's modified eagle's media (sfDMEM) supplemented with 1 % penicillin/streptomycin (stocks of 100 units / mL penicillin and 100 units / mL streptomycin); and 1 % L-glutamine (stock of 2 mM / mL) at 37 ^{o}C with shaking (200 rpm) for 1, 2, 4, or 6 hr. For isolation of bacterial endosymbiont, fungi were fermented in 250 mL flasks containing VK media (1 % corn starch, 0.5 % glycerol, 1%

gluten meal, 1 % dried yeast, 1 % corn steep liquor, 1 % $CaCO_3$) and pH adjusted to 6.5, as previously described by (Scherlach et al., 2006). HL5 (40 % peptone, 20 % yeast extract, 38 % glucose, 1.4 % KH_2PO_4, 1.4% Na_2HPO_4) medium was also occasionally used. Mucormycete stocks were frozen down in 20 % glycerol and stored at – 80 ° C. Media and other chemical requirements were purchased from Sigma-Aldrich unless otherwise indicated.

2.3.2 Other fungal strains and cultivation

Both *Candida albicans* (SC5314) and *Saccharomyces cerevisiae* were also used in this project as phagocytic targets. The yeasts were grown either on yeast peptone dextrose (YPD) media plates or broth at 30 ° C with shaking at 200 rpm. Stocks were maintained in 20 % glycerol at -80° C. Both strains were obtained from the Hall Lab, University of Birmingham.

2.3.3 Bacterial strains and cultivations

Burkholderia cepacia (obtained from Queens University Belfast) and bacterial endosymbionts isolated from mucormycetes during the project as shown in **Table 3** were cultured on nutrient agar plates (0.5 % peptone, 0.5 % NaCl, 1.5 % Agar and 0.3 % yeast extract) at 30° C. For molecular manipulations bacteria were grown in LB broth media at 30° C with shaking 80 rpm. For bacterial supernatants, the bacteria were grown in the respective media such as *sf* DMEM, VK or HL5 at 30 with shaking 80 rpm and processed. Stocks of the bacteria were frozen down in 40% glycerol and stored at -80° C.

Table 3: Fungal and bacterial strains used in the project.

No.	Name	Strain id	Source
1	*Rhizopus microsporus*	FP469-12.6652333	Clinical isolate from Queen Elizabeth Hospital Trauma centre by Dr Deborah Mortiboy
2	Cured *Rhizopus microsporus*	(FP469-12.6652333)	This project
3	*Ralstonia pickettii*	Bacterial endosymbiont	Isolated from *R. microsporus* (FP469-12.6652333)
4	*Rhizopus microsporus*	CBS 631.82	Obtained from CBS collection centre
5	Cured *Rhizopus microsporus*	Cured CBS 631.82 strain	Created in the lab for this project
6	*Ralstonia pickettii*	Bacterial endosymbiont	Isolated from *R. microsporus* CBS 631.82.
7	*Rhizopus chenensis*	NA	Clinical isolate from Marc Hospital
8	*Rhizopus delmar*	RA 99-880	
9	*Lichtheimia corymbifera*	FP454-9.6002134	Clinical isolate
10	*Cunninghamella bertholletiae*	NB2	Obtained from Ashraf Ibrahim (UCLA)
11	*Mucor circinelloides f. lusitanicus*	FP624-CBS-277490	Obtained from CBS
12	*Mucor circinelloides f. lusitanicus*	FP623-NRRL-3631	Obtained from NRRL culture centre
13	Cured *Rhizopus chenensis*	NA-ESF	This project
14	Cured *Rhizopus delmar*	RA 99-880-ESF	This project
15	Cured *Lichtheimia corymbifera*	FP454-9.6002134	This project
16	Cured *Cunninghamella bertholletiae*	NB2	This project
17	Cured *Mucor circinelloides f. lusitanicus*	FP624-CBS-277490-ESF	This project
18	Cured *Mucor circinelloides f. lusitanicus*	FP623-NRRL-3631-ESF	This project
19	*Candida albicans*	SC5314	Obtained from Hall Lab
20	*Saccharomyces cerevisiae*	AM13/0001	Obtained from Aberdeen (Donna MacCallum)
21	*Burkholderia cepacia*	Clinical isolate from cystic fibrosis	Obtained from Queens University Belfast (Hall lab strain bank)
22	*Micrococcus luteus*	Bacterial endosymbiont	Isolated from *Mucor circinelloides f. lusitanicus* FP624-CBS-277490
23	*Micrococcus luteus*	Bacterial endosymbiont	*Mucor circinelloides f. lusitanicus* FP623-NRRL-3631
24	*Sphingomonas wittichii*	Bacterial endosymbiont	Isolated from *Cunninghamella bertholletiae* (NB2)

2.3 Harvesting Mucoralean Fungal Spores

Mucormycetes sporangiospores generated from hyphae for 14-24 days were harvested by flooding the surface of the culture plate with 10 mL of PBS, washed 3x and counted with a haemocytometer. Spores were used within 14-24 days of sporulation as previously described by Voelz *et al*., 2015. Spores prepared in this way are defined here as "resting spores".

For experiments that required metabolically active spores, the spores were incubated in Sabouraud broth media or sf DMEM media at 37^{o} C with shaking at 200 rpm for respective time points, collected by centrifugation, and washed with 1x PBS. Spores prepared this way were defined here as pre-germinating, germinating, and swollen or metabolically active spores".

For experiments requiring UV or fixed killed spores, the spores were suspended in 20 mL 1xPBS and irradiated twice for 15 min in a UV PCL-crosslinker at 1200 μJ/cm^2, and cooled on ice between treatments as previously described by Voelz *et al*., 2015. Successful killing was confirmed by plating for CFUs. For fix killing, the spores were suspended in an appropriate volume of 4% paraformaldehyde (PFA) and incubated for 30 min at room temperature with or without shaking.

Latex beads were also processed similarly to spores including, washing, with PBS, counting and re-suspension.

2.4 Supernatant collection

Fungal supernatants, sporangiospores were harvested as described above, counted and adjusted to 4x10^8 spores / mL in 250 mL of sfDMEM, VK or HL5 medium and incubated at 37^{o}C with shaking (200 rpm) for 4 h. Following incubation, the preparation was centrifuged at 3990 rpm for 5 mins, supernatant collected and filter sterilized through a 0.45 μm (Millipore) syringe filter before assays commenced.

Bacterial supernatants, a loopful of bacterial colony was sub cultured in Lysogenic broth (LB) agar overnight at 30 ºC and 150 rpm, colonies washed in 1x PBS, and adjusted to an optical density of 0.2 at 600 nm. To 250 mL of the respective medium including sfDMEM, VK or HL5, 200 µL of the bacteria suspension was added and incubated at 30 ºC and 200 rpm (Scherlach et al., 2006).

For pH measurement of the supernatants, the pH of the supernatants was measured using pH meter (Mettler Toledo). Three different replicates were collected measured. For accuracy, the meter was calibrated prior to taking the reading. Mettler Toledo displays pH corrected to the nearest 2 decimal points.

For supernatant stability experiments, supernatants were collected and stored in the fridge at 4° C and its activity against phagocytosis evaluated when freshly collected and at other indicated time points for up to a period of 4 weeks.

For inactivation experiments, the supernatants were boiled at 100 ºC for 1 h, digested with 50 µg / mL proteinase K at 37º C for 1 h or oxidized with 1 nmoles /mL of sodium periodate ($NaIO_4$) at 37ºC with mild shaking for 45 min. Phenylmethylsulfonyl fluoride (PMSF) was used to inhibit proteinase K following protein digestion.

For size exclusion experiments, 15 mL of supernatant was filtered through an Amicon® tube (Sigma-Aldrich cat number UFC801024) equipped with a 3 kDa ultra-centrifugal filter. Samples were centrifuged for 30 min at 3000 g and both the 'flow through' (*i.e.* filtrate) and 'concentrated portions of the supernatant' (*i.e.* retained by the filter) were kept for phenotypic assay.

Media and chemicals were purchased from Sigma-Aldrich unless otherwise indicated and HL5 (Formedium Limited) was obtained from Jason King (University Sheffield)

2.5 Bacterial symbiosis experiments

2.5.1 Generation of endosymbiont free strains

For the generation of endosymbiont-free fungal strains, the fungi were cultivated in the presence of ciprofloxacin at a concentration of 60 µg / mL. Absence of the bacteria was confirmed through bacterial isolation, fluorescence microscopy by SYTO9 staining, and polymerase chain reaction (PCR) amplification of the 1.5 kb band for bacterial 16S rDNA as described in the later sections.

For passage experiment, the fungus was continuously cultivated on ciprofloxacin and spores collected between age 14-21 days for a period of 5 months. Spores harvest and counts were performed as in (Section 2.3). However, it is important to note that only phenotypic and not genetical characteristics were monitored at this stage and details are provided in Chapter 5.

2.5.2 Fermentation of *R. microsporus* for bacterial isolation

To a 250-mL conical flask containing 100 mL of VK media, 200-500 spores were added aseptically and incubated at 30°C with shaking at 80 rpm for 2-7 days (Scherlach et al., 2006). Following cultivation, a small mycelial pellet was aseptically taken from a 2-day old liquid fungal culture and submerged into 500 µL of 2000 units / mL Lyticase enzyme (Sigma-Aldrich) in distilled water and incubated at 25° C for 2.5 h. Using mechanical stress (pipetting or vortexing) the mycelia were broken down and then centrifuged at 13200 rpm for 30 mins. Aseptically, 10-20 µL of the supernatant were plated on nutrient agar (NA) and incubated at 30° C until a bacterial or fungal growth was observed. Bacterial colonies were then sub-cultured in LB broth and maintained at -80°C.

2.5.3 Substrate experiment

The influence of the bacteria on the fungus's ability to produce the supernatant factor was also investigated. This was aimed at establishing whether the endosymbiont was a key signal for

the fungus to secrete the compound in question. Accordingly, fungal substrates were prepared in different growth conditions including SDA, HL5, DMEM and VK medium as described (Section 2.2). Spores or hyphal masses were harvested, washed, mashed using a bead beater with sterile soil beads (Qiagen) or heat killed by autoclaving, to yield live mashed or heat killed fungal substrate. The respective substrates (~0.1 g) were collected into sterile 50 mL falcon tubes and 20 mL of bacterial suspension at OD 0.2 and incubated at 30 with shaking (50-80 rpm) for 24 h. After incubation, the tubes were centrifuged to collect the supernatant, and filtered before assays commenced.

2.6 Susceptibility treatment of spores

Mucormycete spores (with or without the endosymbiont) were tested for susceptibility towards a range of stress conditions. The settings mimicked different degrees of oxidative (0.25, 0.5, 1, and 5 mM hydrogen oxide (H_2O_2)), Nitrosative (1, 5, and 20 mM sodium nitrite ($NaNO_2$) (Fisher scientific)), cell wall (0.005, 0.01, 0.05% sodium dodecyl sulphate (SDS) (Fisher scientific)), osmotic (0.05, 0.1, 0.3 M sodium chloride (NaCl)) and antifungal pressure (0.5, 1, 2.5, 5 µg / mL Amphotericin B (AmB)) stress. The spores were grown, harvested, washed and counted as described before and adjusted to 10^5 cells /mL in *sf* DMEM containing the stress factor at the respective concentrations in a centrifuge tube. To each well of a 48 well plate, 1 mL of the spores' suspension was dispensed; and incubated at 37° C and 5% CO_2 for 24 h. To evaluate the influence of the stress factor on the spores, serial dilutions were plated and colony forming units (CFUs) counted after 24 h or 48 h for endosymbiont-free spores.

2.7 Tissue culture

2.7.1 Media and growth conditions

Both a macrophage-like cell line J774.1 and human primary monocyte-derived macrophages were used for experimental work. In addition, human isolated neutrophils and erythrocytes

were also employed during the project. The J774.1 cell line is a transplantable murine reticulum cell sarcoma exhibiting phagocytic effector properties of macrophages derived from a female BALB/c/NIH mouse (Ralph et al., 1975). The murine cell line was thawed and cultivated routinely in complete Dulbecco's modified eagle's media (*c*DMEM) supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin (stocks of 100 units/mL penicillin and 100 units/ mL streptomycin); and 1% L-glutamine (stock of 2 mM / mL) at 37^{o} C, 5 % CO_2 (Smith et al., 2015, Ma et al., 2006, Voelz et al., 2009). J774 macrophages were used between passage 4 and 15.

Human primary peripheral monocytes (PBMCs), neutrophils or erythrocytes were isolated from Buffy coats or whole blood obtained locally from healthy volunteers. The buffy coats were supplied by a local NHS blood transfusion unit at Queen Elizabeth Hospital. To isolate mononuclear cells from buffy coats, the buffy sample was diluted two-fold with PBS and 30 mL centrifuged over a 15 mL Ficoll – Paque Plus cushion at 800 relative centrifugal force (rcf) for 20 min at 20^{o} C. The mononuclear layer was then collected, treated with ice cold lysis buffer (1L = 8.3g NH_4Cl, 1g $KHCO_3$, 0.04g Na_2EDTA dehydrate) and then washed with ice cold PBS (+/- calcium and magnesium, pH 7.2) to remove platelets. Monocytes were then isolated through seeding and adherence in a plastic 24 multi-well plate at a concentration of $4x10^6$ cells / 400 mL in Rosewell Park Memorial Institute 1640 (RPMI 1640) adhesion medium (Life Technologies) supplemented with 2% AB human serum, 1% of 2 mM L-glutamine and 1% 100 units/mL of penicillin/streptomycin at 37^{o}C and 5 % CO_2 for 1 h. The non-adherent monocytes and lymphocytes were removed with pre-warmed PBS and adhered cells differentiated into macrophages in RPMI 1640 supplemented with 10% AB human serum, 1% of 2 mM L-glutamine, 1% 100 units/mL of penicillin/streptomycin and 20 ng/mL macrophage colony stimulating factor (M-CSF) at 37^{o} C and 5 % CO_2 for 3 days. After 3 days, non-viable and non-adhered cells were washed with pre-warmed PBS and incubated in 1 mL of

differentiation media minus M-CSF at 37° C and 5% CO_2 for 3-4 days before assays commenced. This protocol is a revised version of that by Voelz et al (Voelz et al., 2009).

For isolation of PBMCs and neutrophils from whole blood, 6 mL of undiluted blood was centrifuged over a double layer of Percoll made of different densities (6 mL of 1.098 under 6 mL of 1.079) at 150 g for 8 min then 1200 g for 10 min. Top band was taken for monocytes and processed as described above whilst the lower second band was aspirated for neutrophil isolation and treated with red cell lysis buffer as described above, washed with room temperature PBS, re-suspended in adhesion RPMI 1640 media, checked for viability with 1% trypan blue and counted before assays commenced. Chemicals and media were purchased from Sigma-Aldrich unless otherwise indicated.

2.7.2 Freezing down cells

Cells were grown in sterile T75 tissue flasks until an approximate 80-90 % confluence was achieved. The cells were then mechanically detached off the tissue flask floor with a cell scrapper into 10 mL of *c*DMEM and transferred into a 15-mL centrifuge tube. The cells were centrifuged at 1000 rpm, 20° C for 7-10 min. The supernatant was discarded and the pellet re-suspended in an appropriate volume of freezing media containing 50% FBS, 40% *c*DMEM and 10% Dimethyl sulfoxide (DMSO). The suspension was aliquoted into 2 ml cry vials and tubes frozen down at -80° C overnight in Mr. Frosty™ (Nalgene Cryo1° C or Coolcell® LX controlled-rate alcohol free) freezing containers, before transferring to liquid nitrogen. The freezing down process was performed rapidly to avoid damage to the cells by DMSO.

2.7.3 Defrosting cells from liquid nitrogen

Aliquot tubes were briefly thawed in a water bath at 37° C and quickly transferred into a 15-mL centrifuge tube with 10 mL of *c*DMEM to avoid damage by DMSO. The cells were then

centrifuged at 1000 rcf, 20° C for 7-10 min, supernatant discarded and pellet re-suspended in pre-warmed cDMEM and transferred to sterile T75 flasks for cultivation at 37° C and 5% CO_2.

2.7.4 Sub-culturing J774 phagocytes

For routine maintenance and cultivation, cells were passage into fresh media after attaining an ~80-90 % confluence. Accordingly, the old media was discarded and replaced with pre-warmed cDMEM culture media, cells mechanically detached from the flask floor, diluted appropriately for instance in fractions of $^1/_2$, $^1/_4$, $^1/_8$ or $^1/_{10}$, and cultured in fresh media in tissue flasks.

2.7.5 Phagocytosis assay

Multiplicity of infection (MOI) determination: Appropriate infection doses for both J774 macrophages and PBMCs to be used here were determined before phagocytosis assays commenced. This was performed by co-incubating macrophages with different concentrations of spore doses to make infection ratios of 1:1, 1:2, 1:3, 1:4 and 1:5. Seeding concentrations for macrophages were 10^5 for J774 and ~$2x10^5$ (for a 24 well plate) for PBMCs. These preparations were then incubated for periods of 1, 1.5 and 2 h to determine the appropriate exposure time adequate for phagocytic uptake by the two cell types. **Note;** that MOI of 1: 5 for both cell lines and incubation periods of 1 h for J774.1 macrophage and 2 h for PBMCs were later established appropriate for application during project and were used as will be described in later section below.

Phagocytosis assay applications: One day prior to the experiment, 1 mL of 10^5 cells/mL J774.1 murine macrophage-like cells were seeded in a 24 well plate in pre-warmed (37ºC) complete Dulbecco's modified eagles' media (cDMEM) supplemented with 10% Foetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine. Monolayers were incubated at 37ºC and 5% CO_2 for 12 h. Following incubation, the overnight media was

aspirated and the cells were washed 2x with pre-warmed PBS, and then refreshed with 1 mL pre-warmed serum free DMEM (sfDMEM) and incubated as before for 1 h. Then, the media was removed and the cells were washed 2x with PBS. Resting or germinated spores stained with FITC (Section 2.9) were added as 1 mL sfDMEM suspension of $5x10^5$ spores/mL for a multiplicity of infection (MOI) of 1:5.

Co-cultures were incubated as described above for 1 h to allow spore uptake. Phagocytosis was terminated and cells subsequently fixed with 4 % PFA for 15 min, washed 3x with PBS and counter-stained with Con-A or calcofluor white (CFW) (Section 2.9). Note, cells were stained before fixing as fixing enhances CFW permeability. Cells were maintained in 500 mL PBS and imaged using a Nikon Ti microscope.

For experiments requiring use of UV killed or metabolically activated spores, latex beads, *C. albicans* or *S. cerevisiae*, the particles were prepared as described in 2.1.2 and 2.1.4. For live imaging, cells were maintained in sfDMEM+ 50 nm LysoTracker Red DND-99 (Invitrogen, molecular probes) under humidified conditions as described under microscopy section.

For spore viability experiments, macrophages were lysed with 1 mL sterile water and aggressively washed to collect adherent cells. Then, 5 µL of the lysate was plated out on SDA and CFUs were counted. For supernatant effect experiments, the cells were treated with 75% supernatant or 3 pmoles/mL rhizoxin for 1 h and washed 3x before challenging with a phagocytic target.

For PBMCs, the phagocytosis assay was performed using RPMI 1640 supplemented with 2% AB human serum, 1% of 2 mM L-glutamine and 1 % 100 units/mL of penicillin/streptomycin at 37ºC and 5 % CO_2 for 2 h as described above.

Phagocytosis scoring, percentage phagocytosis was determined by counting the number of macrophages (that phagocytosed 1 or more spores and the those that didn't) before computing the number of those that had phagocytosed spores divided by the total number of both

categories multiplied by 100. Accordingly; macrophages were counted and recorded from individual images taken until a final total of 1000 macrophages were realised each induvial technical repeat. For each of these data points, percentage phagocytosis was calculated, tested for normality using D'Agostino & Pearson omnibus normality test. Summary means of the data points were derived and statistically analysed using parametric nor non-parametric test depending on the normality findings. For each phagocytosis experimental set up, at 3 biological with subsets of 3 technical repeats were performed.

2.8 Other assays

2.8.1 Effect of *R. microsporus* spores or supernatant on erythrocytes

Supernatants and spores were harvested as already described (sections 2.3 and 2.4) and erythrocytes obtained from health volunteers as already described (section 2.8). The red blood cells (RBCs) were washed and re-suspended in Dulbecco's PBS (Sigma-Aldrich) at 1000 g, 25^{o} C for 7 min. To an Eppendorf tube containing 800 µL of fungal supernatants or control samples, 200 µL of the RBC suspension was added and incubated overnight at room temperature.

Following incubation, the tubes were centrifuged to collect the supernatant and optical density of the supernatants measured at OD 550 nm. To determine the effect of the spores on the RBCs, the cells were co-incubated with RBCs in 24 well plate and live cell time lapse images acquired under phase contrasts as described in (section 2.8 and 2.9). Control samples sfDMEM, PBS as negative and RBC lysis buffer as positive controls. Optical density from at least 3 biological with 3 subsets of technical repeats were measured. The technical repeats' data points were then tested for normality, mean OD derived before analysis with parametric or non-parametric statistical tests were performed.

2.8.2 Cell viability assay using trypan blue staining

We employed a trypan blue assay as one of the means to assess the effect of the supernatant factor on macrophage cell viability. Accordingly, macrophages were seeded in a 24 well plate at a concentration of 10^5, incubated overnight at 37° C and 5 % CO_2. Following incubation, cells were treated with the supernatant for 2 h, washed with pre-warmed PBS and 500 mL of 1% trypan blue (1:1 v/v trypan blue and sf DMEM) was added, incubated at 37° C and 5% CO_2 for 10 min. Cell viability was assessed on the ability or inability to take up trypan blue stain, dead cells stained blue, whilst live cells remained unstained. The experimental set-up included 3 biological repeats with 3 subsets of technical repeats. From these images were randomly taken and up to a total of 300 macrophages counted per each biological repeat. Thus, the percentage cell survival was computed from the number of unstained cells divided by the total number (stained and unstained) multiplied by 100.

2.8.3 Cell viability assay using lactate dehydrogenase (LDH)

LDH assay is another method of evaluating cell viability. The assay is based on the principle that injury or toxicity to a tissue causes cells to release lactate dehydrogenase, a fairly stable enzyme. The enzyme is an oxidoreductase that catalyses interconversion of pyruvate and lactate. Here we utilized an LDH kit purchased from Sigma-Aldrich (Kit no. MAK066-1KT) to determine the effect of supernatants, resting spores, swollen spores, and rhizoxin on the viability of macrophages. Procedures of the assay were based on the manufacturer's instructions described in the kit insert. Test readings were done using a FLUO-star Omega plate reader at 450 nm and calculations done as directed by the insert.

2.8.4 Neutrophil migration assay (Chemotaxis)

To model Neutrophil chemotaxis, we employed the 2D chemotaxis assay using the ibidi- micro (μ) - slide chemotaxis kit (Ibidi cells in focus (kit no. 80322)). Detailed protocol instructions

were followed as provided for in the insert by the kit manufacturer. Accordingly; cells we loaded into the ibidi chamber and allowed to migrate towards supernatant, media (negative) or FMLP (positive) controls while being followed by time-lapse imaging under the phase contrast channel (section 2.9.3) at a time interval of 2 min for 2 h. Cell movements were tracked manually in Image-J(Fiji)-Mtrack2. Cell tracks from pooled cells tracked in 3 separate experiments are shown. The tracks were analysed using statistical features of ibidi chemotaxis tool. Plotted data are obtained using the accumulated distance, velocity, euclidian distance, Forward Migration Index (FMI), and directionality. Differences between the test and controls is statistically determined using raileys test. However, some data was eliminated from this analysis because of a difference in tracking slice numbers. For two of the biological repeats where 61 slices were used as opposed to 31 used in the initial experiment.

2.9 Microscopy

2.9.1 Fluorescence staining of Mucoralean Spores

For experiments requiring stained spores, the spores were counted, adjusted to $2x10^7$ in PBS and stained with 100 μg/mL fluorescein isothiocynate isomer 1 (FITC) (Sigma-Aldrich) in 0.1M sodium bicarbonate buffer (pH 7.45) (Sigma-Aldrich), 25-50 μg/mL Rh-TRITC-Concanavalin A (Thermo-fisher scientific) or 250 μg/mL calcofluor white (Sigma-Aldrich) in PBS for 30 min with shaking at room temperature.

2.9.2 Cytotoxic assay

J774A.1 cells were seeded onto round 13 mm coverslips in 24 well plates and cultured as described (Section 2.7). Prior to staining, cells were treated with supernatants or 3 pmoles / mL rhizoxin and synchronised on ice for 10 min.

Media was then replaced with pre-warmed sfDMEM and cells incubated for 30 min. Samples were then fixed with 4% PFA for 10 min, permeablised with 0.1% Triton X for 5 min and

washed 3x with PBS. To stain for beta tubulin, coverslips were firstly immersed in 1% bovine serum albumin (BSA) for 1 h and further incubated overnight in PBS containing 5 µg/mL anti-beta tubulin ab18207 (Abcam) primary antibody and 0.1% BSA. Samples were then washed and incubated with 2 µg/mL Alexa Fluor 488 goat anti-rabbit secondary ab 150081(Abcam) for 1 h.

For F-actin staining, samples were incubated with 3 Nano moles phalloidin conjugated to Alexa Fluor 488 (Thermofisher Scientific) for 15 min. After cytoskeleton staining, cells were counter-stained with 2 µg/mL Hoechst (4'6 Diamichino-2-phenylindol for 15 min) where appropriate and mounted onto glass slides with ProLong ant fade mountant (Thermofisher Scientific).

2.9.3 Image acquisition

Live-cell time lapse images were acquired under phase contrast at 14X magnification on a Nikon Ti microscope (20x magnification combined with a 0.7x de-magnifier) equipped with a QICAM Fast 1394 CCD camera (Q-Imaging). Fixed-cell fluorescence imaging was, unless stated otherwise, performed at 63x magnification on a Zeiss Axio Observer Z1 equipped with structured illumination (Apotome) using a Flash 4 sCMOS camera (Hamamatsu).

2.9.4 Image processing

Mean staining intensity score, cell size and shape

Spores (refer to section 2.9.1**),** Images were processed in image-J to measure mean staining intensity or cell size for each induvial spore per image. Up to 300 spores were analysed from 3 biological repeats.

Macrophages (actin, tubulin or phagosome maturation) (refer to section 2.9.2**),** Images were processed with either Image-J or Metamorph software to determine mean staining intensity or cell shape. Up to 300 cells were analysed from at least three biological repeats.

These data points were tested for normality using D'Agostino & Pearson omnibus normality test employing parametric or non-parametric statistical tests.

2.9.5 Transmission Electro-microscopy (TEM)

This work was done in collaboration with University of Aberdeen's Microscopy Core Facility. Images were acquired by Xin Zhou and Dr Elizabeth R Ballou using a JEM-1400 Plus Electron Microscope. The experiments were performed on resting or swollen spores processed as described in (Section 2.3).

2.10 Molecular techniques

2.10.1 Fungal and bacterial DNA isolation

DNA isolation from fungal or bacterial strains for polymerase chain reaction was performed with a DNeasy® powerlyzer® soil Kit (Qiagen kit no.120888-50) for fungal strains or a Dneasy® powerlyzer® microbial kit (Qiagen) for bacterial strains. DNA was obtained from 10^7 spores or a sizeable collected bacterial pellet by homogenised with beads in a screw-cupped tubes using a bead beater (Bertin technologies) at speed 6500 for 1 min following the manufacturer's instructions.

2.10.2 Polymerase chain reaction (PCR)

To detect the presence of the bacterial endosymbiont, we employed universal primers as applied elsewhere by (Ibrahim et al., 2008b, Partida-Martinez et al., 2007a) that amplify the 1.5 kb band for 16S rDNA from genomic DNA isolated from mucormycetes. The primers used were Forward: 5'CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG3', Reverse; 5'CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT 3'. PCR conditions using the Phusion kit (Bio Labs) were 35 cycles, at 95^{o} for 2 min, 60^{o}C for 30s, and 72^{o}C for 1 min (**Table 4 and 5**), for both reactions, and products were analysed by means of UV trans-

illumination through gel electrophoresis. PCR kits purchased from Bio labs and primers from Eurofins.

Table 4: PCR reaction components using Phusion high fidelity DNA polymerase

Components	**50µL**
5X Phusion HF buffer	10 µL
10 mM dNTPs	1.0 µL
10 µM Forward primer	0.5 µL
10 µM Reverse primer	0.5 µL
Template DNA	2.0 µL
Phusion DNA polymerase	0.5 µL
DMSO	1.5 µL
Nuclease free water	34 µL
Total volume	50 µL

Table 5: PCR conditions

Step	**Temperature, ° C**	**Time**	**Number of cycles**
Initial denaturation	95	2 min	1
Denaturation	95	30 s	25-33
Annealing	60(Tm-5)	30 s	25-33
Automated fluorescent extension	72	1 min/kb	25-33
Final extension	72	10 min	1
Hold		10° C	

2.10.3 Agarose gel electrophoresis

This technique is common for separating nucleic acids according to molecular weight. This is achieved by staining the acids with an intercalating and fluorescent dye such as SYBR safe DNA gel stain (Invitrogen). To this end, 1% agarose gels were prepared in 1 X TBE buffer (10 mM TRIS, 150 NaCl at pH 7.6) and 0.5 µg/mL ethidium bromide (Bio Labs) added. DNA product samples were then mixed with the loading dye (Thermo-scientific) and loaded onto the gel. The samples were separated with current set to 70 volts for 45 mins in 1 X TBE as the

buffer. A 10-kb ladder (gene ruler from Thermo-scientific) was also run alongside the samples as a control for the obtained sample band size estimates. Fragments were detected by UV trans-illumination using ChemiDoc™ MP imaging system (Bio-Rad). Gel electrophoresis was performed on PCR products from at least 3 biological repeats.

2.11 Chemistry experiments

2.11.1 Chloroform extraction of the supernatants

Medium including sfDMEM, VK, HL5 and fungal supernatants were prepared as described in (Sections 2.2, 2.4). These were then exhaustively extracted 3x with chloroform in a 2:1 v/v supernatant/control to chloroform ratio in a separation funnel, and the combined organic phases were dried over anhydrous sodium sulphate, filtered and fully evaporated under reduced pressure. The dried product was re-constituted in 500 µL of dimethyl sulfoxide (DMSO) or sfDMEM for high performance liquid chromatography (HPLC) analysis or phagocytic assaying as described in (Section 2.7.5), respectively. Chloroform extraction was performed on supernatants from at least three biological repeats harvested on different days.

2.11.2 HPLC analysis

Extract products and control samples including commercial cytochalasin D and cytochalasin E (0.2 mg/mL in DMEM) (standards) were separated through a Kinetex® C18-EVO column (Phenomenex®: 5 µm, 100 Å, 250 x 4.60 mm) maintained at 35ºC, and chromatograms were recorded by UV-Vis detection at 210 nm. HPLC analysis was performed using mixtures of solvent 'A' (water + 0.1% v/v trifluoro-acetic acid) and solvent 'B' (acetonitrile + 0.1% v/v trifluoro-acetic acid) as mobile phase with the following method: 3% B isocratic for 10 min, 3-50% B gradient for 30 min, 50% B isocratic for 5 min, 50-3% B gradient for 5 min, and 3% isocratic for 10 min, at a constant flow rate of 1 mL/min.

For **HPLC fractionation and peak isolation experiments,** 10 min HPLC fractions and individual HPLC peaks were collected in the parent elution buffer, concentrated under vacuum at 45ºC for 20 min to eliminate acetonitrile/trifluoro-acetic acid and freeze dried overnight for phagocytic assay. The freeze-dried products were reconstituted into 200 µL of DMSO, then diluted with DMEM to a final volume of 20 mL, and filter sterilised through a 0.45 µm filter for phagocytic assay, as described in (Section 2.7.5).

HPLC chromatograms were analysed and drawn using X chromatogram soft tool.

2.11.3 Mass Spectrometry

Reproducible supernatant HPLC peaks were isolated in an HPLC elution buffer and submitted for high-resolution mass spectrometry (HRMS) analysis. This was performed in the Chemistry School, University of Birmingham by Chi Tsang. Samples were also sent to Dyanne Brewer from the University of Guelph, Canada for Liquid chromatography mass spectrometry (LC-MS MS) and nuclear magnetic resonance (NMR) on the basis of further analysis by more sensitive techniques.

2.12 Metabolomics study of the supernatant

These experiments were performed and analysed by Dunn Warwick, Bioscience, University of Birmingham on supernatant and control samples collected and processed as described in (sections 2.3 and 2.4) above.

2.13 In vivo work

2.13.1 Zebrafish experiments

2.13.1.1 Cultivation and maintenance

We employed the wild type AB zebrafish as well as the transgenic zebrafish Tg (mpx: GFP) ^{i114} expressing green fluorescent protein (GFP) in neutrophils (Renshaw et al., 2006). The

Tg(mpeg1:Gal4-FF)^{gl25}crossed with Tg(UAS-E1b:NfsB.mCherry)^{c264} [herein referred to as Tg(mpeg1:G/U:NfsB-mCherry)] with macrophage-specific expression of red fluorescent protein mCherry (Ellett et al., 2011). Fish lines. The fish were cultivated and maintained in a recirculating system at the University of Birmingham Zebrafish Facility. They were kept under a 14 h-10 h light-dark cycle with water temperature maintained at 28°C. The eggs were obtained by natural mating, collected and incubated at ~40 per plate in 25-50 mL of E3 medium plus 0.00003% methylene blue and 26.6 µg/mL 1-phenyl-2-thiourea (PTU) (Sigma Aldrich). PTU at that concentration prevents melanisation of the fish, thus permitting microscopic investigation without impacting larval viability and development post fertilization (d.p.f). The medium was changed every 2 days (Inglesfield et al., 2018, Ellett *et al*., 2011, Renshaw *et al*., 2006, Voelz *et al*., 2015). All media and reagents used were purchased from Sigma-Aldrich unless otherwise indicated, and all husbandry and care procedures performed by the University Biomedical Service Unit (BMSU) staff.

2.13.1.2 Spore preparations for injections

Spores were collected in 10 mL of PBS, washed 3x, and countered using a haemocytometer (Section 2.3). For localization following injection, spores were stained with fluorescent brighter 28 in 0.1 M of $NaHCO_3$ or PBS (Sigma Aldrich) for 30 min. Stained spores were washed 3 times in PBS, counted and adjusted to 10^8 spores/mL in 10% (w/v) polyvinylpyrrolidone-40 (PVP) (Sigma Aldrich) in PBS with 0.05% phenol red (Sigma Aldrich) in ddH_2O. PVP was used as a carrier medium with increased density to prevent clogging of micro-injection needles.

2.13.1.3 Hindbrain injection

The fish were injected in accordance to a protocol by Brothers *et al*., 2011 and zebra fish development was assessed in accordance to Kimmel *et al*., 1995 (Brothers et al., 2011, Kimmel

et al., 1995, Voelz et al., 2015). The fish were injected at prim-25 stage following manual dechorionation and anaesthesia with 160 µg/mL of Tricaine (Sigma Aldrich) in ddH_2O. The fish were then micro-injected with 2 nL of 10% (PVP) in PBS or a high dose (10^8 spores/mL) of *R. microsporus* spore suspension in PVP through the otic vesicle (**Figure 11**) into the hind brain to achieve an inoculum dose of approximately 50-100 spores / larva. Following injection, the larvae were anesthetized with 160 µg/mL Tricaine in E3 media in a 96 well plate and screened by fluorescence microscopy, using the Zeiss Axiobserver Zi microscope equipped with Apotome system for the presence of spores (Section 2.9.3). Only larvae with approximate correct inoculum were selected and transferred to individual wells of 48 or 24 well plates containing E3 media (plus 0.00003% methylene blue). The fish were monitored over a period of 96 hours post infection (96 h.p.i) for survival or 72 h.p.i and at 96 or 72 h.p.i, the fish were killed with 1600 µg/mL Tricaine overdose.

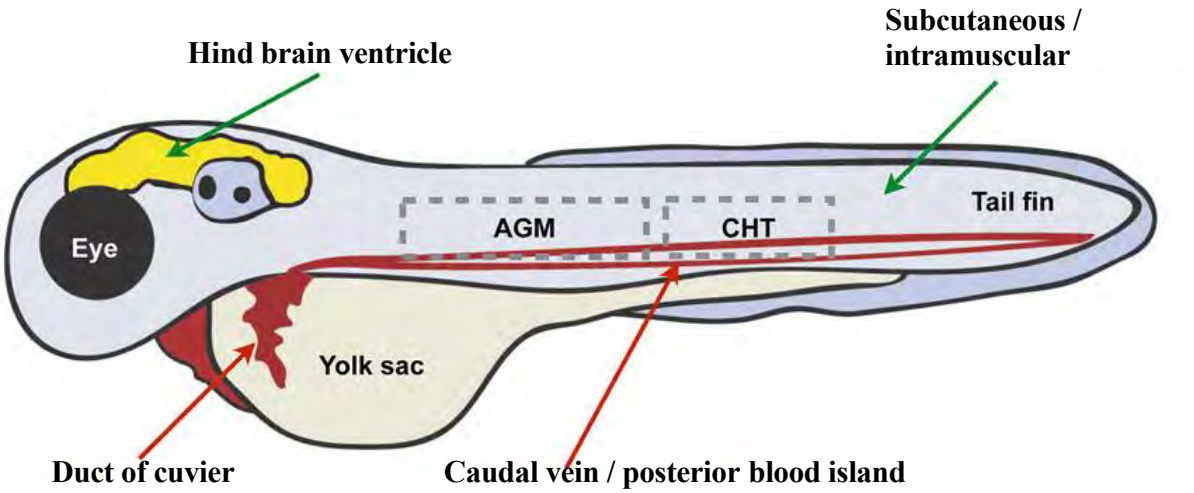


Figure 11: Injection sites of zebrafish larvae.

Anatomical representations of the different injection sites used to study fungal or bacterial infections of zebrafish larvae. Different sites are used to achieve specific objectives when using this model. Hindbrain injections like in our case were used to study directed leukocyte response to fungal whilst caudal vein and duct of cuvier can be used to study systemic infection and the dashed lines indicate where aorta gonad mesonephric (AGM) and caudal hematopoietic tissue (CHT) where emergency granulopoiesis takes place. The image was adopted from Duggan and Mostowy, 2018.

2.13.1.4 Evaluation of spore viability

Following injection, the larvae were euthanized with 1600 µg/mL Tricaine for 2-5 min. The fish were then homogenized individually in an Eppendorf containing 100 µL of E3 media supplemented with penicillin-streptomycin (5000 U/mL-5 mg/mL) (Sigma Aldrich) and gentamicin (10 mg/mL) (Sigma Aldrich) using pellet pestles. The homogenate was then plated out on SDA plates containing 100 U/mL-100 µg/mL penicillin-streptomycin and 30 µg/mL gentamicin. The antibiotics prevent growth of fish normal flora. The plates were then incubated at room temperature for between 24 and 48 h and colony forming units (CFUs) counted. At least 3 biological repeats were performed with 5 fish per condition (total of 15 fish) per condition. CFUs for each fish were recorded as individual data points and tested for normality.

2.13.1.5 Assessment of phagocyte recruitment

To quantify phagocyte recruitment, transgenic zebrafish larvae Tg (mpx: GFP) ^{i114} expressing green fluorescent protein in neutrophils or Tg (mpeg1: G/U: NfsB-mCherry) with macrophage-specific expression of red fluorescent protein mCherry were injected with high dose viable spores stained with calcofluor white and observed after 6 h.p.i up to 72 h.p.i (Renshaw et al., 2006, Ellett et al., 2011). Positive phagocyte recruitment was defined by accumulation of >10 neutrophils or macrophages to the site of infection. At least 3 biological repeats were performed with 5 fish per condition to give a total of 15 fish per group. Recruitment was analysed in Image-J using the macros plugin script written with the help of Dr Joao Correia. The principle behind this analysis is that the software creates 3D image of the infection site and enumerates the number of cells basing on the fluorescence signal. Number of recruited phagocytes per fish were recorded as individual's data points and tested for normality using D'Agostino & Pearson omnibus normality to determine appropriate statistical tests.

2.13.2 Galleria mellonella experiment

R. microsporus spores with or without the bacterial endosymbiont were harvested, washed, metabolically activated and quantified using a haemocytometer as described before (2.2.4), and spores' concentration adjusted to $1x10^8$ /10 μL for high dose or $1x10^4$/10 μL for low dose. The *Galleria mellonella* larvae were then injected with the respective doses, incubated at 25^o C, and both melanisation and survival scores recorded every 24 h over a period of 140 h. At least 3 biological repeats were performed with 5 larvae per condition to give a total of 15 larva per experimental condition.

2.13.3 Mice experiment

This experiment was performed by Ashraf Ibrahim's lab at the University of California Los Angeles (UCLA) on strains sent from University of Birmingham.

Immunocompetent CD-1 male mice were infected with 10^6/25 μL resting or pre-germinated spores of *R. microsporus* with or without the bacterial endosymbiont as shown in **Table 6**. The spores were pre-germinated in sfDMEM for 4 has described before (section 2.2.4). Infection was carried out intratracheally, with 5 mice per infection category. Lungs were harvested at two different time points: 4 h post infection and at day 2. At each time point, 5 mice per group were sacrificed, and right after infection two mice were sacrificed for inoculum verification. Following harvest, the organs were homogenized in 2 mL of PBS and 200 μL of both direct and diluted homogenate plated out on PDA + 0.1% Triton 100X plates; and incubated at 37 C.

Table 6: Inoculum plan of mice

Fungal strain	Time and number of mice used				
	4 h		**48 h**		
	Resting spores	Swollen spores	Resting spores	Swollen spores	Total
Parent strain of (*R. microsporus*)	N=5	N=5	N=5	N=5	20
Endosymbiont free (*R. microsporus*)	N=5	N=5	N=5	N=5	20

2.14 Bioinformatics

Genome sequencing of the respective bacterial strains was done by Microbes NG, at University of Birmingham. Following sequencing, a series of bioinformatics tools were used to analyse the genomes which included; Anti-smash for secondary metabolites and basic local search alignment (BLAST) tools from databases such as the National Centre for Biotechnology Information (NCBI), Ensembl, FungiDB and EuPathDB. Most of this work was done with help from Poppy Sephton-Clark.

2.15 Statistical analysis

At least 3 independent experimental biological repeats were performed for each assay on different days. For some experiments as respectively indicated subsets of 3 technical repeats were included.

For **phagocytic assays**, the data shown herein are for mean percentage phagocytosis computed from at least 3 biological repeats with subsets of 3 technical repeats. Up to about 1000 cells were scored from each technical repeat (Section 2.7.5).

For **spore, actin, β-tubulin,** and **phagosome maturation staining**, the data shown represent the mean staining intensity with the respective dyes as detailed in the individual sections. On average scoring was performed on 300 cells from 3 biological repeats.

For **chemotaxis,** the data shown represents tracks from 100 cells of a single biological repeat as detailed in (Section 2.8.4).

The raw data was processed in Microsoft excel before data summaries were transferred to Graph Pad prism for further analysis. The data were tested for normality using and normally distributed data was analysed by one-way anova. Multi-comparisons were also performed to identify statistically significant pairs considering a p value (< 0.05) as statistically significant.

CHAPTER THREE: Spore-Macrophage Interactions

3.1 Background

Mucormycosis is an opportunistic fungal infection targeting a range of at-risk individuals. The infection can be self-limiting and latent in health individuals. The significance of phagocytes in controlling filamentous fungal infections is becoming better appreciated, yet our understanding of the underlying molecular and cellular mechanisms is still very limited.

This is proven clinically when patients presenting with defective phagocytic effector functions including neutropenia show increased susceptibility to mucormycotic infections (Roden et al., 2005, Ribes et al., 2000).

Studies on *Aspergillus* show that both macrophages and neutrophils are essential components of innate antifungal immunity (Brakhage et al., 2010, Aimanianda and Latge, 2010, Dagenais et al., 2010, Luther et al., 2007, Herbst et al., 2015). Similarly, recent *ex* and *in vivo* studies also show the importance of innate immunity against mucormycete infections (Inglesfield et al., 2018, Kraibooj et al., 2014, Li et al., 2011, Voelz et al., 2015).

For instance, studies by Voelz *et al.* (2015), Inglesfield *et al*. (2018); and Chamilos et al (2008) have utilized zebrafish or fly models, respectively, to study specific innate immune responses during mucormycotic pathophysiology.

Overall these works report that phagocytes are recruited early to the site of infection and are important in protecting against disease onset. In zebrafish, the role of pro-inflammatory response in recruitment is highlighted with subsequent prevention of spore germination following recruitment, but lack of spore killing and reactive oxygen burst which may result to latency are also shown in zebrafish (Inglesfield et al., 2018, Voelz et al., 2015). In the fly, although readily recruited phagocytic cell display reduces phagocytic uptake of zygomycetes spores and sequencing reveals down-regulation of genes involved in pathogen recognition, immune defence, stress response and detoxification (Chamilos et al., 2008a).

Gebremariam *et al* (2014) and Ibrahim *et al.* (2005) also highlight the role of endothelial cells in mucormycete antifungal immunity and pathogenesis, specifically showing that endothelial cells are required for onset of angio-invasion. They demonstrate that *R. oryzae* spores adhere to, are phagocytosed by and damage endothelial cells offsetting a cascade of angioinvasive events, whilst Andrianaki *et al.* (2018), Li *et al* (2011) and Kraibooj *et al.* (2014) use *in vitro* systems to highlight the role and importance of innate immunity against mucormycetes These three works contribute an improved understanding of macrophage–fungal interactions. All showing that macrophage recognise and phagocytose mucormycete spores *in vitro* (Gebremariam et al., 2014, Ibrahim et al., 2005, Inglesfield et al., 2018, Voelz et al., 2015, Chamilos et al., 2008a, Andrianaki et al., 2018b, Li et al., 2011, Kraibooj et al., 2014).

From this stance, the role of innate immunity against mucormycete infections seems vital but our understanding of the principles driving these mechanisms is still poor. Several evidences highlight that fungal infecting particles are capable of evading phagocyte effector functions. This is particularly happens with *Aspergillus* species where dormant conidia elude recognition by macrophages, yet evasion is lost when spores are metabolically activated(Aimanianda and Latge, 2010). This is attributed to the presence of the hydrophobin protein coat which masks the spores (Aimanianda and Latge, 2010, Brakhage et al., 2010, Gow et al., 2017). Whether mucoralean fungi possess similar mechanisms remains a question that perhaps will be addressed in this work.

However, as regards mucoralean fungi, so far Li *et al* (2011) showed that, although readily phagocytosed by macrophages, *M. circinelloides* spores are not killed and can germinate inside macrophages. This is in addition to Chamilos *et al* (2008)'s demonstration of a pattern of down-regulation in the expression of phagocytic-related genes following zygomycotic infections; and recently, Inglesfield *et al* (2018)'s showing of the development of a latent mucormycete infection in a zebrafish model as mentioned before, where spores are maintained

and controlled in granulomas, possibly reactivating during immunosuppressive conditions (Li et al., 2011, Inglesfield et al., 2018, Chamilos et al., 2008a).

This background indicates how invaluable innate immunity can be against mucormycete infections, importantly highlights the possibility of immune evasion by the infecting particles. This is rather medically challenging given that mucormycete spores are ubiquitous, with an increasing range of at-risk individuals. Thus, it is apparent we navigate the molecular interactions involved in what appears to be a transition from saprophytic to opportunistic pathogens. In this chapter therefore, we first describe some *in vitro* experimental approaches developed and applied during this project to investigate spore macrophage interactions. We shall later phenotypically demonstrate spore-macrophages interactions.

3.2 Developing a Phagocytosis assay for Mucoralean Fungi

We still do not fully understand how mucormycete spores interact with phagocytes such as macrophages. Therefore, there are limited operating protocols to demonstrate *in vitro* spore-macrophage infection biology. In this project, we mainly focused on a clinical isolate of *R. 83icrospores* (Rm FP469-12) as the model organism, employing both murine and human derived PBMCs. Other mucormycete species were also employed for comparison.

In an infection assay, a phagocyte should recognise and subsequently engulf phagocytic targets such as fungal spores. However, to efficiently demonstrate and quantify phagocytic uptake, an appropriate infection dosage also known as multiplicity of infection (MOI) must be formulated. The MOI is the number of macrophages to the number of spores. For instance, if you have 10^5 macrophages and $5x10^5$ spores then the MOI would be 1:5. However, this does not reflect actual uptake. The higher the MOI, the more likely uptake events will occur. Too high MOI can lead to stuffing of macrophages, and too low MOI may lead to low or no uptake events, both of which subsequently affect data analysis.

Quantification of phagocytic uptake also depends on the ability to differentiate engulfed from an engulfed or associated targets. This is achieved through differential staining. The target is labelled prior to and post phagocytosis. This can be done with commonly used empirical fluorophores such as Fluorescein isothiocynate (FITC), tetramethylrhodamine (TRITC) and calcofluor white/fluorescent brightener (FB) (Card et al., 2013, Stewart and Deacon, 1995, Kraibooj et al., 2014). Common examples include but are not limited to Concanavalin A (Con-A), wheat germ agglutinin (WGA) and calcofluor white (CFW).

The principle of application depends on the fact that dyes bind specific fungal ligands such as amines, sulfhydryl and phenolate ions of tyrosine (FITC); non-reducing alpha D- mannosyl and alpha D-glucosyl groups of glycoproteins or glycolipids (Con-A); cellulose and chitin (CFW) and N-acetyl-D- glucosamine and sialic acid N- acetyl-D-glucosamine found naturally in the chitin of insects, cell membranes of yeast and bacteria, and in cartilages and cornea of mammals (WGA) (Podhradsky et al., 1979, Sumner et al., 1938, Marfurt, 1988, Hoch et al., 2005, Stewart and Deacon, 1995).

Below, we demonstrate appropriate MOI and staining conditions that can be used to study *in vitro* macrophage-spore interactions

3.2.1 Multiplicity of infection (MOI)

To determine the appropriate MOI, both J774 and PBMC-derived macrophages were co-incubated with a range of spore concentrations. Macrophages of each type were seeded at concentrations of 10^5 and $2x10^5$, respectively, and co-incubated with appropriate spore concentrations to give infection ratios of 1:1, 1:2, 1:3, 1:4 or 1:5. These were then incubated for 1 h, 1.5 h or 2 h to establish an optimal contact time required to engulf a spore (Section 2.7).

Here, we defined uptake rate as the number of macrophages containing at least one fungal spore. It is important to note that increasing the MOI significantly increased phagocytic uptake

by J774 macrophages, but no significant effect on uptake was seen with increasing incubation time. In this regard, the average uptake of 20 % at MOI 1:1 and significantly increased to 40 % at MOI 1:2 ($p < 0.01$), 45 % at MOI 1:3 ($p < 0.01$), 60% at MOI 1:4 ($p < 0.001$) and 85% at MOI 1:5 ($p < 0.0001$) was seen across all the incubation periods ($p = 0.8194$) (**Figure 12a**). With the highest uptake being obtained at MOI 1:5.

On the other hand, both increasing the MOI and incubation time seemed to affect phagocytic uptake by PBMCs with an increasing uptake from 10 % at 1 h to 15 % at 1.5 h ($p > 0.05$) and 20 % at 2 h ($p < 0.05$) for MOI 1:1; compared with 15% at 1 h to 20% at 1.5 h ($p > 0.05$) and 22% at 2 h ($p < 0.05$) for MOI 1:4; and finally, 40% at 1 h to 44% at 1.5 h ($p < 0.05$) and 65% an 2 h ($p < 0.001$) at an MOI of 1:5 (**Figure 12b**). The highest uptake was obtained at 2 h for MOI 1:5.

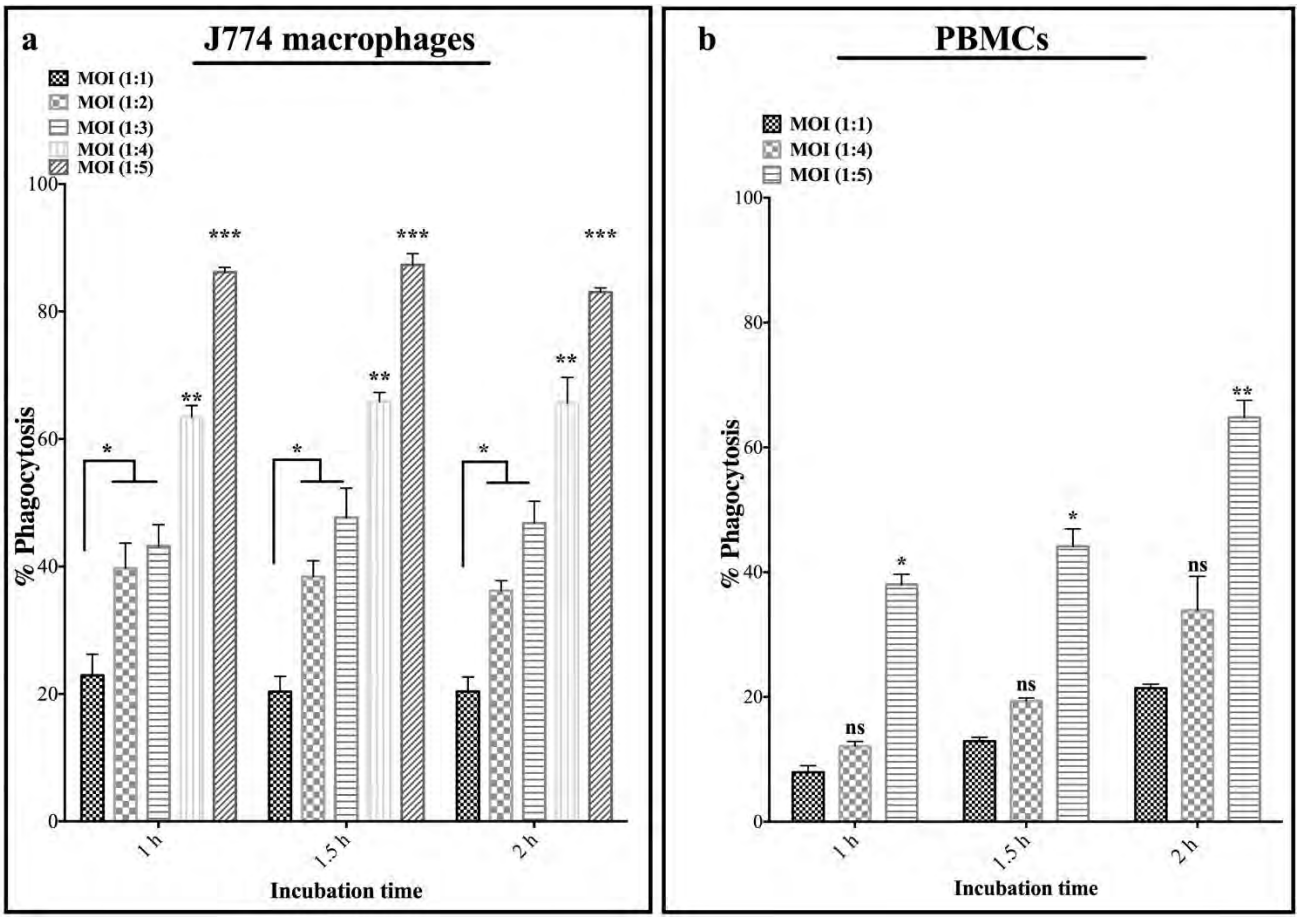


Figure 12: Multiplicity of Infection.

Resting spores of *R. microsporus* (FP 469-12) were harvested, washed, stained and counted. These were then co-incubated with (**a**) murine (J774) or (**b**) human (PBMCs) phagocytes at indicated infection ratios and time points at 37ºC before phagocytic uptake was assessed. For all assays, the number of phagocytes containing at least one spore were counted at the indicated time points (n=9000, One way ANOVA with Tukey's correction for multiple comparisons). In each graph, three biological repeats were examined and error bars represent s.e.m. (*=$p<0.05$, **=$p<0.001$, *** = $p<0.0001$ and ns=$p>0.05$).

Based on the results here, we chose MOI of 1:5 for both cell lines and incubation periods of 1h or 2h for J774 and PBMCs, respectively. On average J774 macrophages engulfed 45 % more spores than PBMCs within the first hour, 40 % after 1.5 h and 25 % more after 2 h of incubation. This clearly, demonstrated that J774 macrophages phagocytosed spores better than PBMCs could.

The difference in uptake here can be attributed to the difference in the level of activity by the cell lines. Where J774 macrophages which usually express interferon gamma even at baseline are more active than PBMCs which may require prior stimulation. Although, *in vitro* activation with specific bacterial lipopolysaccharides (LPS), IFN-gamma or Phorbol 12-myristate 13-acetate (PMA) (Crosby et al., 2010) is a possibility, we choose not to stimulate PBMCs to eliminate experimental comparison bias.

It should also be noted that PBMCs used in this study were obtained from buffy coats, and more studies are needed to establish this as a viable source for these cells. Given the experimental demand, majority of the infection assays performed with J774 macrophages with PBMCs only used to validate or compare findings with J774 murine macrophages

3.2.2 Mucoralean spores staining and its effect on phagocytic uptake

Differential staining is key for identifying phagocytosed from non-phagocytosed or associated particles during infection assays. However, it was imperative that the ability of spores to take up specific dyes, and the subsequent impact of labelling with specific dyes on phagocytic uptake is evaluated. Therefore, here spore labelling with dyes such as Con-A, FITC, CFW and WGA was evaluated to determine optimal staining conditions as described in (Section 2.9).

As shown in **Table 7**, several optimal conditions including appropriate concentrations, buffers and exposure time were optimised for the other mentioned dyes. Generally, spores were ably stained by FITC at an optimal concentration of 100 µg mL^{-1}, Con-A at 25-50 µg mL^{-1}, CFW at 250 µg mL^{-1} and WGA at 50 µg mL^{-1} for an average incubation time of 30 min.

It was interesting to see that although spores generally picked up the stains, some dyes such as Con-A and CFW demonstrated heterogeneity in their ability to label mucormycete spores (**Figure 13)**. Visual evaluation and quantitative scoring for stained intensity spores showed that over 95% of spores are ably stained with a range of concentrations for Con-A or CFW and 100% with FITC. Additionally, incubation time may influence staining by Con-A or CFW, especially at lower concentrations, but FITC was not affected (**Figure 13a**).

Table 7: Required conditions for mucormycete spore labelling with the indicated dyes.

	Conc. (µg mL^{-1})	Buffer (pH)	Incubation time (min)	Fluorescence filter set (excitation /Emission)
FITC	100	0.1 M Na_2CO_3(7.4)	30	490/525
Con A-TRITC	25-50	PBS (7.4)	30	557/576
Calcofluor white	250	PBS (7.4)	30	440/500
Wheat germ agglutinin (WGA)	50	PBS	30	475/509-520

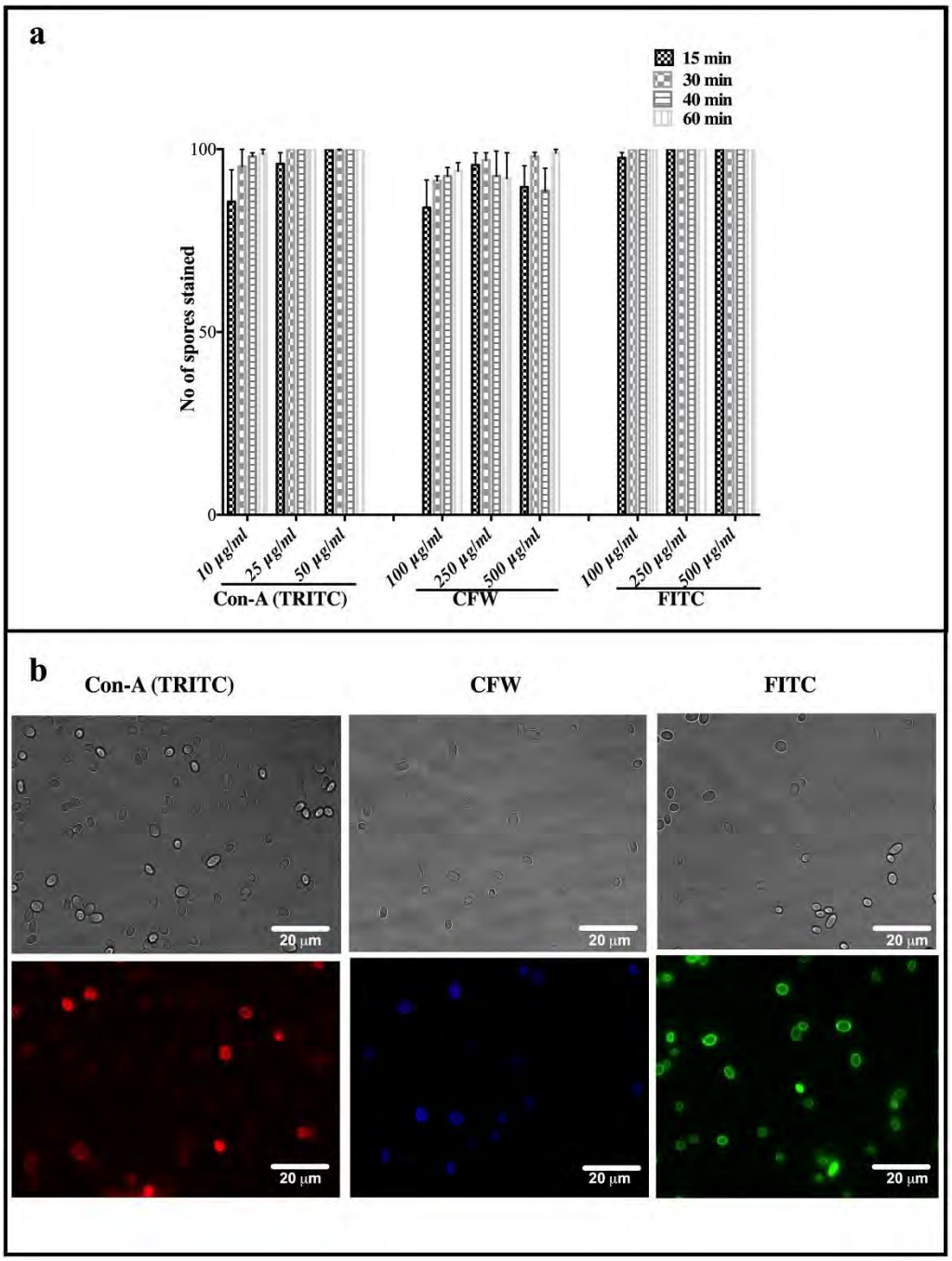


Figure 13: Mucormycete spores stain but demonstrate staining heterogeneity.

Resting spores of *R. microsporus* were harvested, washed, stained with the indicated fluorophores and images taken using fluorescence microscopy. (a) Stain uptake was quantified by visually counting the number of stained spore cells. The data are presented as standard error of means from 300 spore cells per indicated stained from at least three individual biological repeats. (**b**) Representative micrographs showing stain uptake for Concanavalin-A (Con-A), Calcofluor White (CFW) and Fluorescein isothiocynate (FITC).

To counter this effect, we hypothesised that pre-germination of the spores might improve dye uptake and indeed, it is interesting to note that pre-germination significantly improved stain uptake with CFW ($p < 0.0001$), but not Con-A or FITC ($p > 0.05$) (**Figure 14a**). However, even with improvement, the heterogeneous pattern was not eliminated (**Figure 14b**). Thus, we concluded that heterogeneous staining may be a characteristic for mucormycete spores, but more studies are still needed to fully understand the origin of this staining pattern.

However, despite the staining variations demonstrated, the dyes could still be used to quantify phagocytic uptake. Here, we employed FITC (green) as the primary stain, and either CFW (blue) or Con-A (red) were interchangeably used as post phagocytosis dyes (**Figure 15)**. CFW appeared to demonstrate the highest level of heterogeneity among the three dyes and for this reason it was mainly used as an alternative option, especially where microscopic differentiation of fluorescence channels was required.

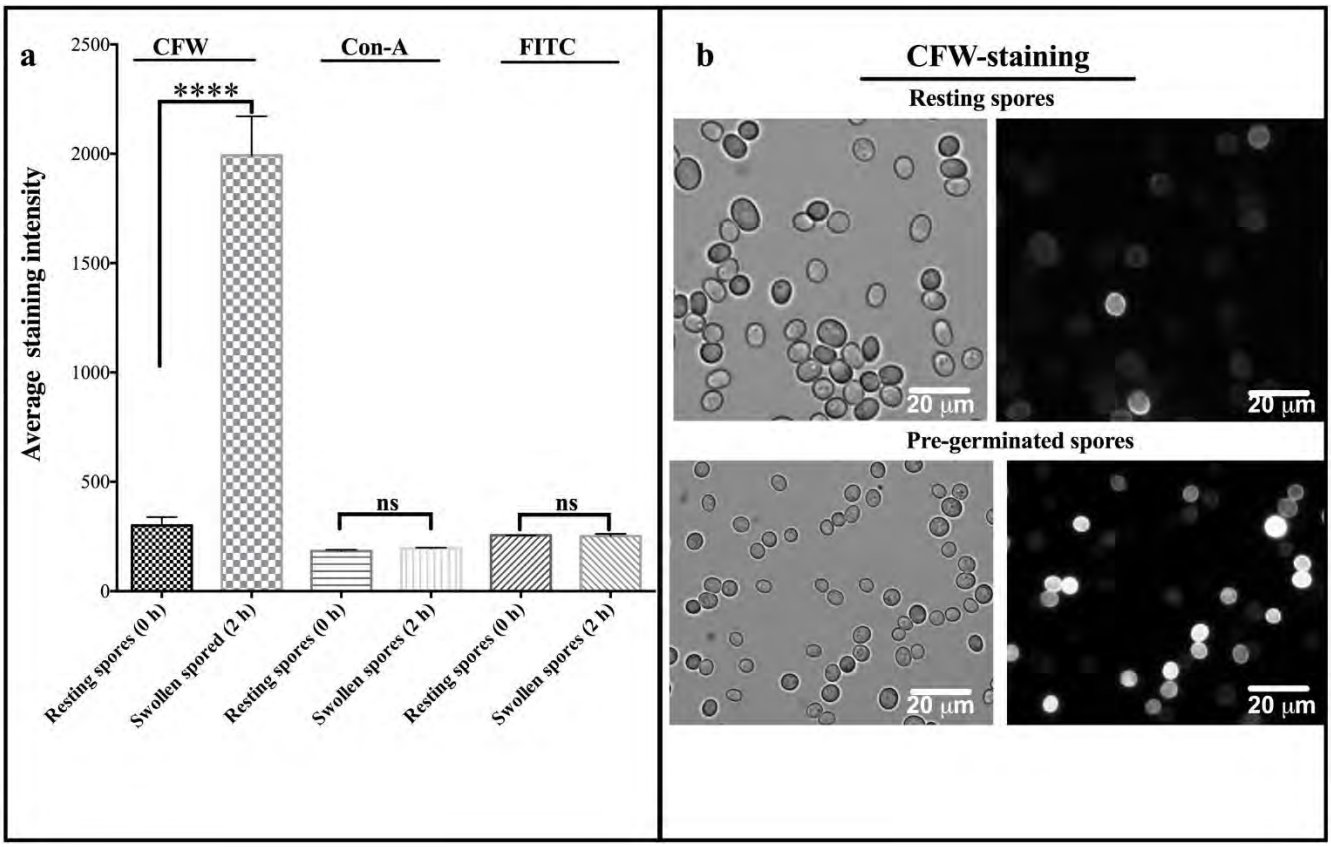


Figure 14: Spore pre-germination improves staining ability with CFW but doesn't eliminate heterogeneity.

Resting and pre-germinated spores of *R. microsporus* were washed and stained with the indicated fluorophores. Images were taken using fluorescence microscopy and stain uptake was quantified by scoring the average staining intensity. **(a)** At least three biological repeats were examined and error bars represent standard deviation (s.d) from 300 spore cells per condition. Data was assessed for normality using D'Agostino and Pearson omnibus normality test and significance assessed via un paired T test for normally distributed or 2Way ANOVA for non-normally distributed data. Statistically significant differences are indicated by asterisks where (****=$p< 0.00001$ and **ns**= $p>0.05$). (**b**) Representative micrographs demonstrate changes in the staining pattern for Calcofluor White (CFW) in resting vs. pre-germinated spores.

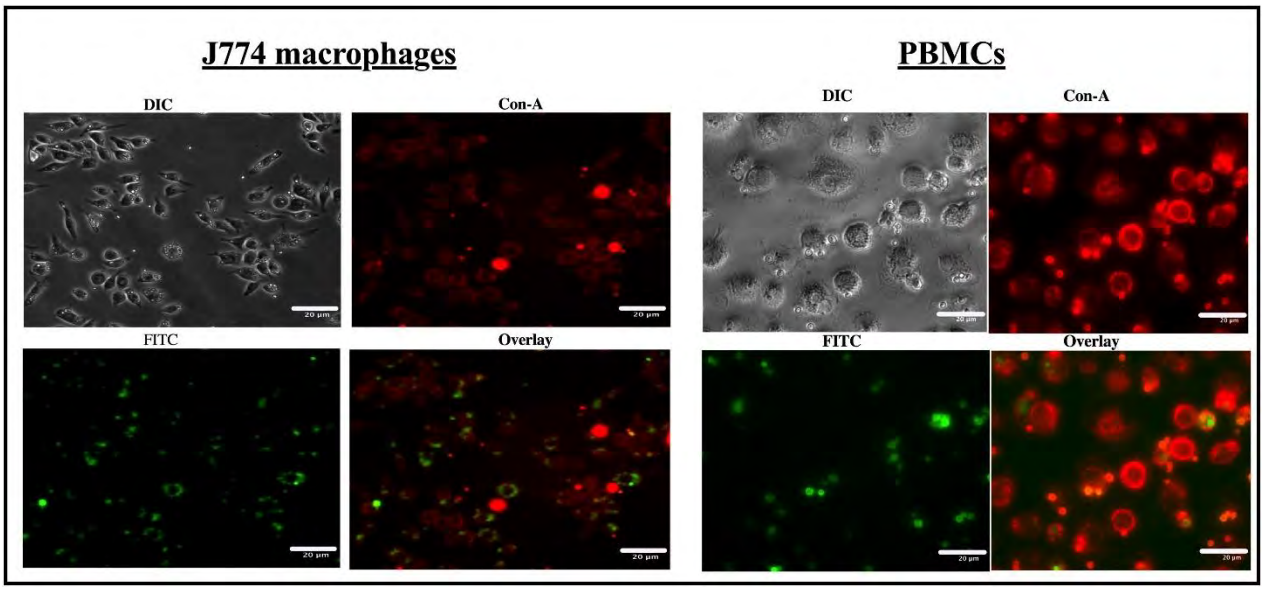


Figure 15: Primary and post staining during phagocytosis assay.

Resting spores of *R. microsporus* were harvested, washed, stained with FITC (green) and counted. These were then co-incubated with J774 and PBMC phagocytes for 1 h, fixed with 4% PFA, and counterstained with Con-A (red). Cells were imaged and analysed for single and double stained cells representing engulfed (green) and un engulfed (green and red) spores. Representative micrographs from one of at least three biological repeats are shown.

3.2.3 Spore staining has no effect, but pre-germination inhibits phagocytic uptake by macrophages

In addition, the effect of both staining and germination on phagocytic uptake of spores was determined. Accordingly, the spores were pre-germinated for 0 or 1 h, stained with FITC, Con-A or CFW, and phagocytic uptake by J774 macrophages was evaluated as described before. It is important to note that the addition of the dye did not affect phagocytosis of stained spores of *R. microsporus* where a percentage phagocytic rate of about 75% ($p > 0.05$) by J774 macrophages was realised compared to the un stained control (**Figure 16a**). However, it was rather intriguing to find that pre-germinated spores showed reduced phagocytic uptake of 55 % ($p = 0.015$) by J774 and 40 % ($p=0.00293$) by PBMCs (**Figure 16b**). This reduction in phagocytic uptake of pre-germinated spores probed further investigations as detailed below.

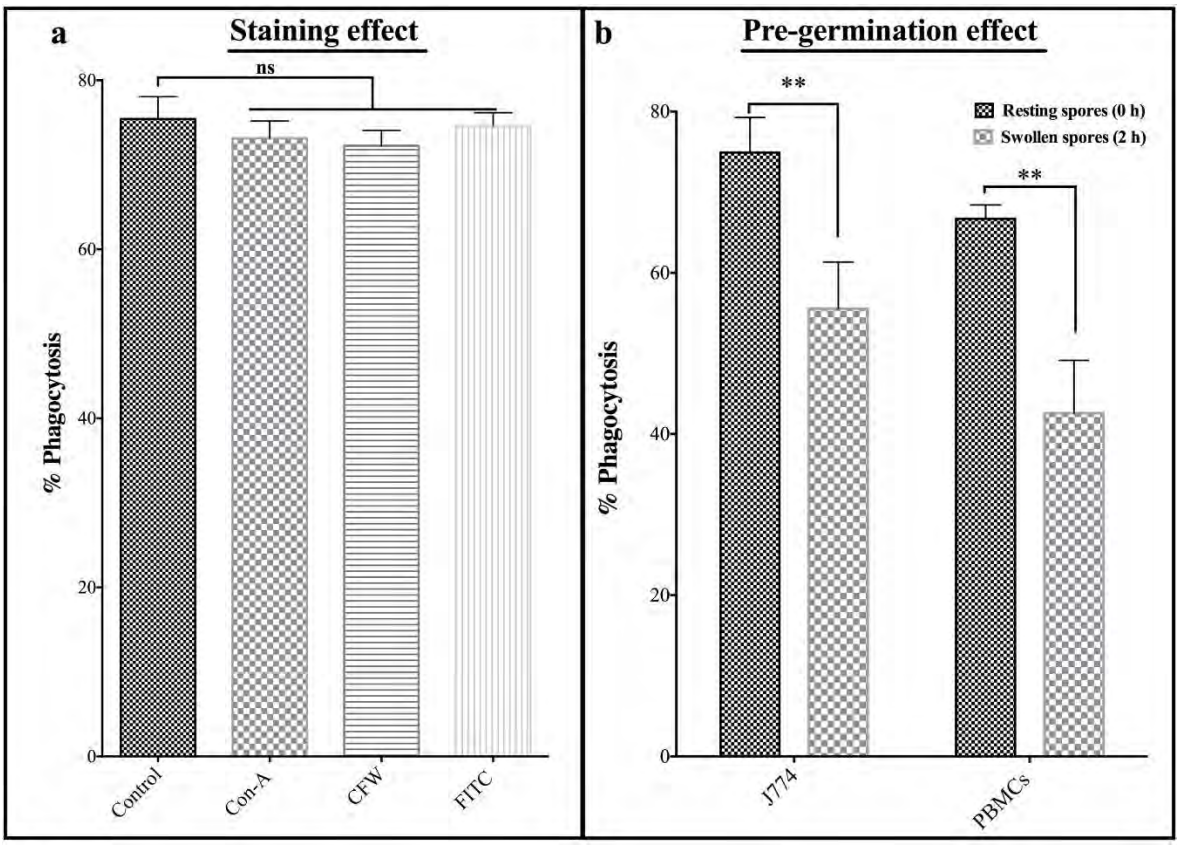


Figure 16: Addition of cell wall dyes does not affect phagocytic rate of *R. microsporus.*

(**a**) Harvested resting spores of *R. microsporus* were stained with the indicated dyes, washed and counted. These were then co-incubated with J774 phagocytes for 1 h and phagocytic uptake assed. (**b**) Spores were then pre-germinated, stained with FITC and co-incubated with J774 and PBMC phagocytes for 1 h and 2 h respectively and uptake assessed. For all assays, the number of phagocytes containing at least one spore were counted after 1 hour or 2 h (n=9000, One way ANOVA with Tukey's correction for multiple comparisons). In each graph, three biological replicates were examined and error bars represent s.e.m. (ns = $p>0.05$, ** $p<0.001$).

3.3 Spore-macrophage interactions in detail

3.3.1 Pre-germination effects are not only exhibited by *R. microsporus* but also other mucoralean fungi

The impact of pre-germination of *Aspergillus* conidia on macrophage recognition is fairly characterised (Aimanianda and Latge, 2010, Brakhage et al., 2010). These reports show that germination enhances macrophage recognition and uptake of conidia. However, these observations are opposite to what we observe here with *R. microsporus*.

Indeed, whilst resting spores of *R. microsporus* spores are readily phagocytosed by both J774 (75 %) or PBMCs (65 %) (**Figure 16b**), pre-germination significantly reduced their uptake. This characteristic was not only exhibited by *R. microsporus* but also other mucoralean fungal species. For instance, using pre-germination time points of 0, 2 and 4 h, we observed a general decrease in percentage phagocytic uptake of swollen spores of four other mucoralean species from an average uptake of 75 % at 0 h; to 30 % at 2 h or 15 % at 4 h for *R. microsporus* ($p < 0.00001$); to 20 % at 2 h or 30 % at 4 h for *M. circinelloides* (CBS 227490) ($p < 0.00001$); to an average of 30 % at 2 h and 4 h for *M. circinelloides* (NRRL 3631) ($p < 0.00001$); to 43 % at 2 h or 35 % at 4 h for *C. bertholletiae* ($p < 0.0001$); and finally from 55 % to 35 % at 2 h or 30 % at 4 h for *L. corymbifera* ($p < 0.001$) (**Figure 17**).

This data demonstrates that metabolic spores are capable of evading macrophage effector functions, particularly phagocytosis. However, whether the mechanism of this evasion is similar to what has been characterised with other filamentous fungi such *A. fumigatus* remains to be explored.

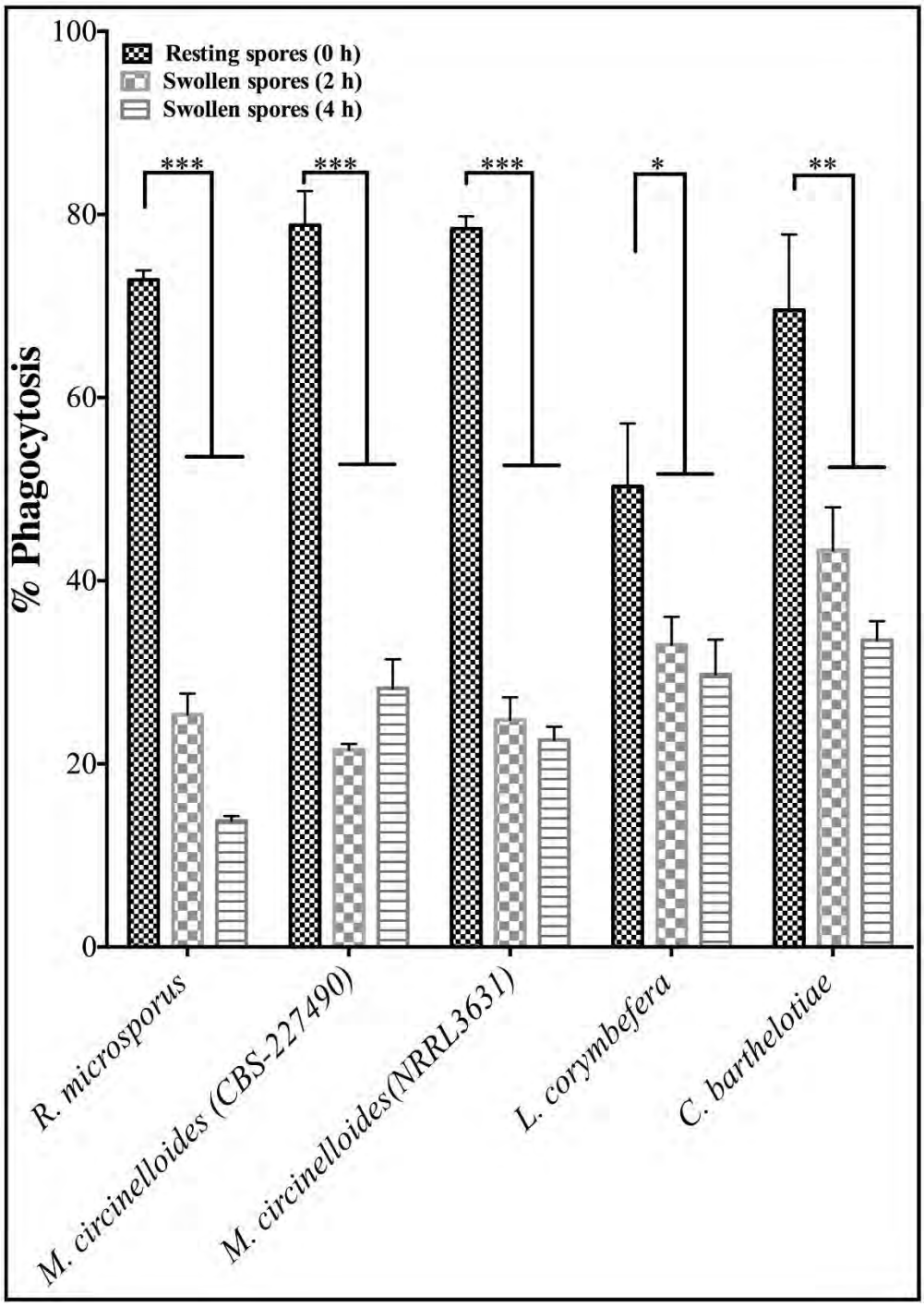


Figure 17: Pre-germinated spores of mucoralean fungi show reduced phagocytic uptake by J774 macrophages.

Harvested and pre-stained resting and pre-germinated spores of *R. microsporus* (FP469-12) and other mucoralean fungi as indicated were co-incubated with J774 phagocytes for 1 h and phagocytic uptake assessed. For phagocytosis assay, the number of phagocytes containing at least one spore were counted after 1 h (n=9000, One way ANOVA with Tukey's correction for multiple comparisons). In each graph, three biological replicates were examined and error bars represent s.e.m. (* = $p<0.01$, ** $p<0.001$ and ***=$p<0.0001$).

3.3.2 Resting spores of *R. microsporus* are readily phagocytosed but are not killed and do not germinate inside macrophages

Li *et al* (2014) has previously showed that *M. circinelloides* spores are phagocytosed but are not killed and can germinate inside macrophages. Here, the ability of *Rhizopus* spores to evade macrophage killing was also determined. To answer this question, both resting and pre-germinated spores were co-incubated with macrophages and fungal survival evaluated.

Accordingly, macrophages were co incubated with spores for 0, 6 and 24 h. Un-engulfed spores were washed away, then macrophages were lysed and the lysate sub-cultured for colony forming units (CFUs) on SDA plates as described (Section 2.3 and 2.7).

It is interesting to note that both resting and pre-germinated spores remained viable, with no statistically significant differences in fungal survival at 0 h and 6 h or 24 h ($p > 0.05$) (**Figure 18**).

Live cell imaging (Section 2.9) also showed similar findings; resting spores are well contained for the first 6 h of incubation. However, after 24 h, they begin to germinate inside macrophages (**Figure 18**). A similar trend is seen with swollen spores, although germination begins much earlier, at 6 h of incubation (**Figure 18**) and overall uptake rates are lower as already shown in **Figure 17** above. In addition, at 6 h, macrophages exhibit morphological differences including rounding (**Figure 18).**

The difference in macrophage containment of both spore types is intriguing and can be linked to several factors. Firstly, spore metabolism: Metabolically active spores are more likely to respond to and interfere with macrophage functions faster than resting can. Secondly, that macrophage viability deteriorates with increasing co-incubation time. This is more pronounced in the case of resting than swollen spores. The switch from a well spread out phenotype at 0 h to a rounded and deflated morphology of macrophages at both 6 and 24 h can be indicative of a declining viability and thus functionality (**Figure 18**).

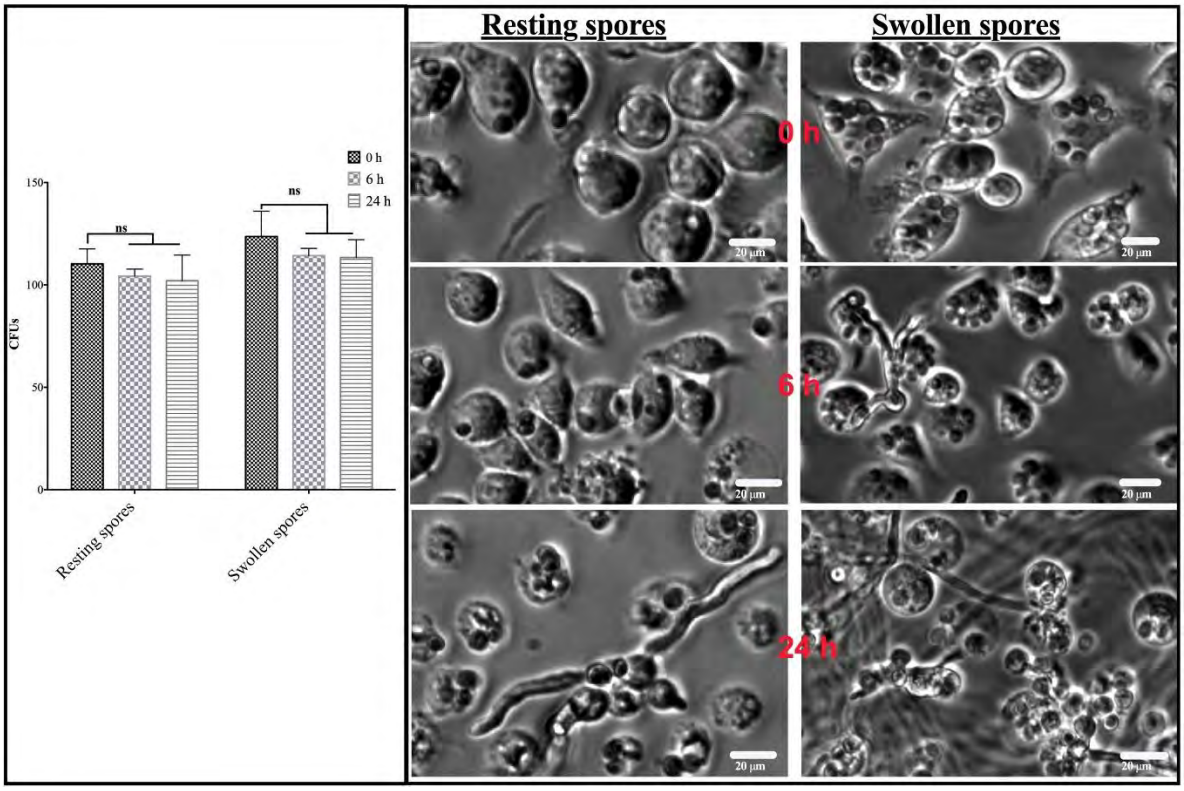


Figure 18: Macrophages inhibit germination but don't kill mucoralean spores.

Resting and swollen spores of *R. microsporus* were co-incubated with J774 macrophages for the indicated time points. The macrophages were then lysed and plated for CFUs for fungal survival. Data are plotted as s.e.m for fungal survival from 3 biological repeats and statistical differences are determined using One way ANOVA with Tukey's correction for multiple comparisons (n=3 biological replicates) where (ns=$p>0.05$). Representative micrographs are from time lapse microscopy.

3.3.3 Spores show minimal altered response to stress

The results above show that *R. microsporus* spores are resistant to macrophage killing. But since mucormycete fungi are environmentally ubiquitous, it is befitting to hypothesise that resistance towards macrophage killing is mediated in response to the various stresses in the wider environment. Indeed, fungal spores can withstand extreme conditions of desiccation, heat, ultraviolet light and high pressure (Setlow, 2014, Sephton-Clark et al., 2018). Thus, spore susceptibility to a range of environmental stresses was also considered and evaluated.

Accordingly, *Rhizopus* spores were incubated under different concentrations of oxidative, cell wall, antifungal, osmotic and nitrosative stress conditions as described (Section 2.6).

Growth of swollen spores was the most affected and mainly altered in their response to oxidative, osmotic, and antifungal stresses ($p<0.05$) when compared to cell wall (SDS) and nitrosative ($NaNO_2$) stress. However, for all treatments responses seemed concentration dependent since high concentrations such as (0.05%) SDS and (5 mM) H_2O_2, 0.3 M NaCl and 5 μg / mL AmB of either stress factors altered growth of both resting and swollen spores ($p>0.05$) (**Figure 19).** Generally, resting spores were more resistant to the stresses tested here than swollen spores. For all the stresses, susceptibility was concentration dependent.

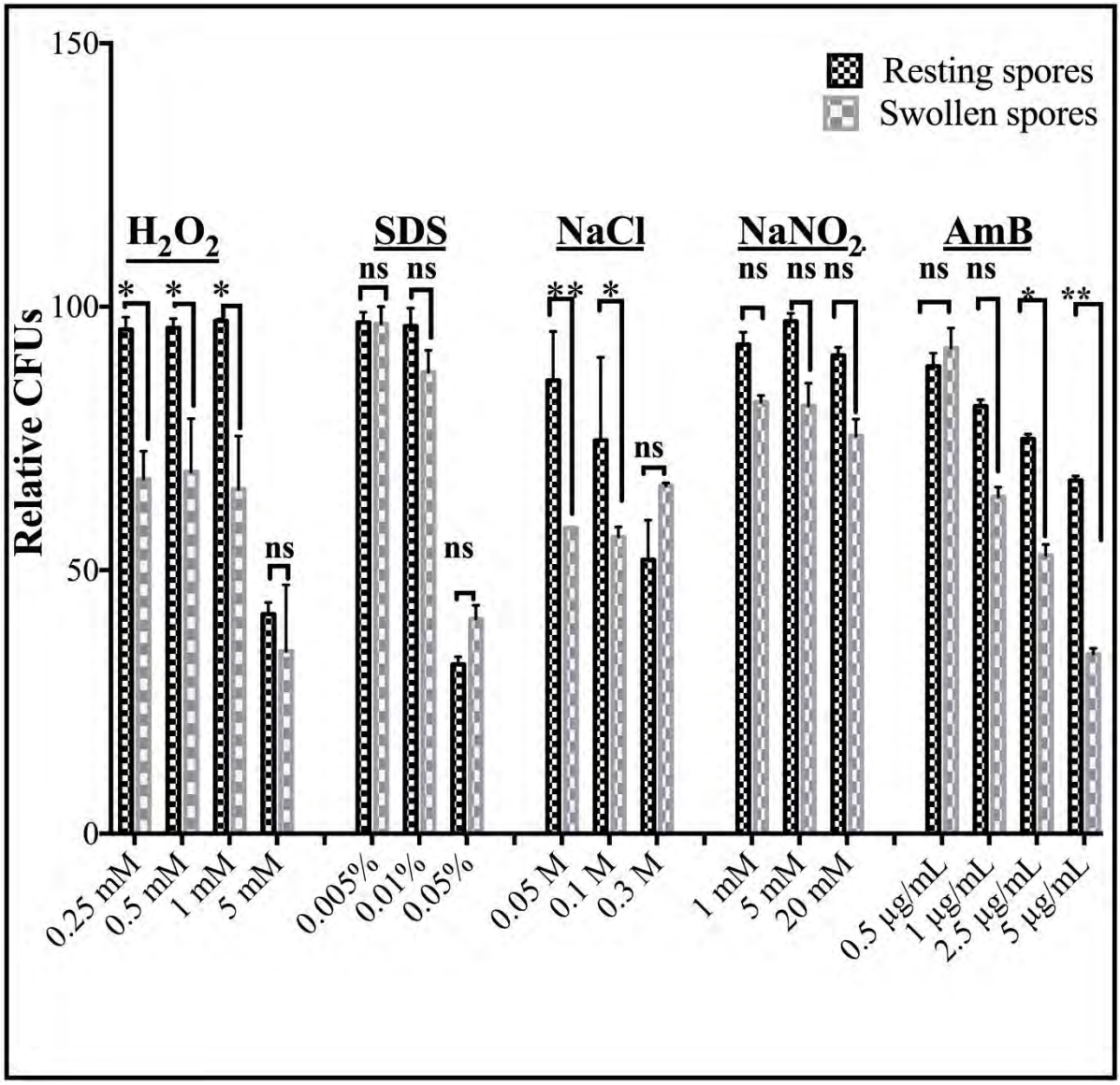


Figure 19: Resting and swollen spores show varying growth alterations under different stress conditions.

Harvested resting and swollen spores of *R. microsporus* were suspended in sfDMEM with varying concentrations of different indicated stress conditions and incubated for 24 h at 37ºC. Fungal survival was then evaluated by plating out for colony forming units on SDA at 37ºC at room temperature. Three biological repeats were examined and error bars represent s.e.m. Statistical significance was assessed via One way ANOVA with Tukey's correction for multiple comparisons (*=p<0.01, **=p=0.001, **ns**=p>0.05).

The swollen spore-stress responses here can be linked to metabolism, where key metabolic processes can be targets by some of such stress conditions that would not otherwise be expressed in a dormant state. However, on this account you would also expect swollen spores to be susceptible to macrophage killing, contrary to our observations in (sections 3.2.3 and 3.3.2). Thus, the findings here may partly highlight why mucoralean spores are resistant to macrophage killing but do not specifically address why swollen spores are able to evade phagocytic uptake.

So, to answer this question, we derived three hypotheses. That reduction in phagocytic uptake is **First**, a mechanical effect; **Second**, a cell wall dynamic effect; or **Third**, a result of spore secretory mechanisms.

3.4 Reduced phagocytosis of pre-germinated spores is not a mechanistic effect

It is important to note that spore size and morphological dimorphism have been linked to virulence, particularly in the heterothallic fungus *M. circinelloides* (Li et al., 2011). We therefore hypothesized that pre–germinating spores might lead to an increase in size, which in turn impacts engulfment by macrophages. To answer this question, specifically, we initially pre-germinated spores from 5 different mucormycete species in SDA at 37^{o} C for 0, 2, 4 and 6 h and measured spore size before evaluating phagocytic uptake (Sections 2.3 and 2.7).

Notably, spore size significantly increased with pre-germination (**Table 8** and **Figure 20**). For instance, from 4.58 µm at 0 h to 7.30 µm at 6 h ($p = 0.003$) for *R. microsporus*; 9.18 µm at 0 h to 13. 63 at 6 h ($p = 0.001$) for *M. circinelloides* (CBS-277490); 5.11 µm at 0 h to 7.55 at 6 h ($p = 0.01$) for *M. circinelloides* (NRRL-3631); 5.09 µm at 0 h to 9.46 µm at 6 h for ($p < 0.0001$) *L. corymbifera*; and finally, 8.02 µm at 0 h to 11.81 µm at 6 h ($p < 0.01$) for *C. bertholletiae*

(**Table 8**). Although, generally increased, *R. microsporus* spores remained smaller than other mucoralean fungi.

Table 8: Pre-germination leads to increase in spore size

	Resting spores 0 h	Swollen spores 2 h	Swollen spores 4 h	Swollen spores 6 h	*p* value
R. microsporus	4.58	6.32	6.46	7.30	0.003
M. circinelloides (CBS-277490)	9.17	12.61	14.96	13.62	0.001
M. circinelloides (NRRL-3631)	5.12	6.58	7.06	7.55	0.01
L. corymbifera	5.09	6.48	8.93	9.46	<0.0001
C. bertholletiae	8.02	10.91	11.02	11.81	< 0.01

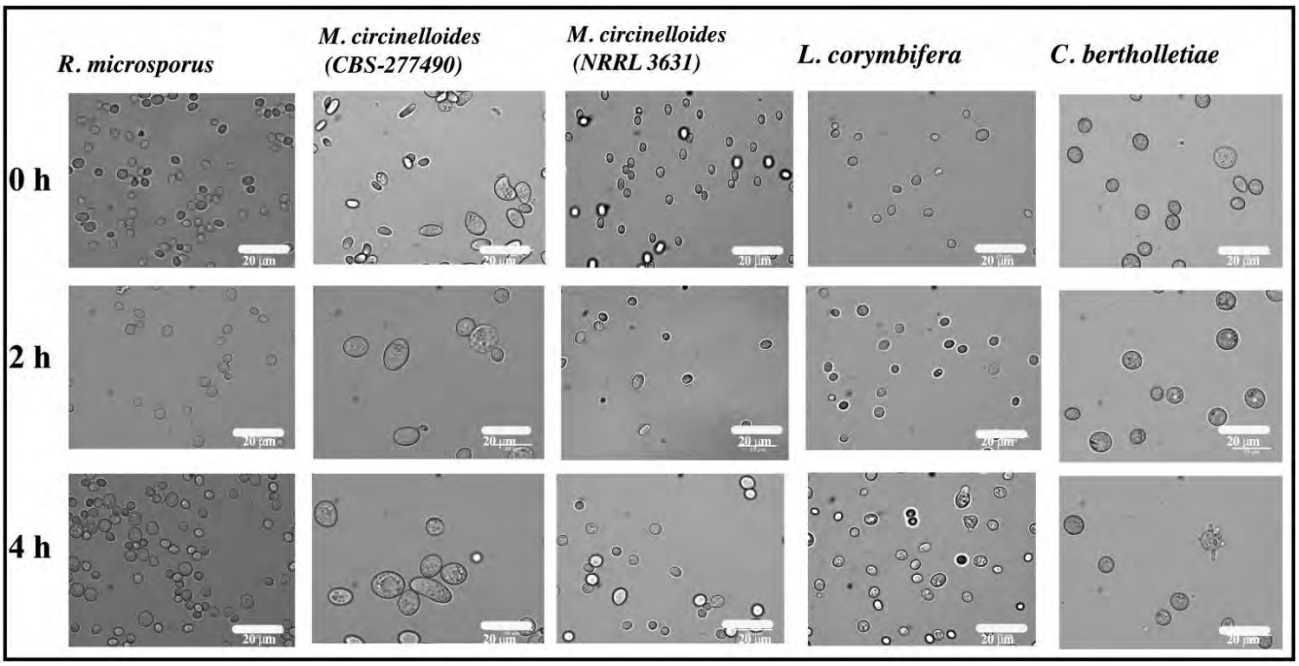


Figure 20: Pre-germination leads to increase in spore size.

Resting spores of mucoralean fungi including *R. microsporus* as indicated were harvested, washed, counted and pre-germinated in SDA at 37°C. At the indicated time points, images were taken and spore size scored. Representative micrographs from one of at least three individual biological repeats are shown.

We then compared uptake of the 4 h swollen spores of size 6.23 μm from *R. microsporus* with uptake of latex beads of 11.9 μm (**Figure 21**). Yet, still phagocytosis of the 4 h swollen spores was significantly ($p < 0.001$) lower than that of the latex beads (**Figure 21**).

These findings clearly showed that although spore size significantly increased during pre-germination, macrophages are able to phagocytose particles of comparable or larger size, suggesting that size alone does not explain the observed reduction in uptake.

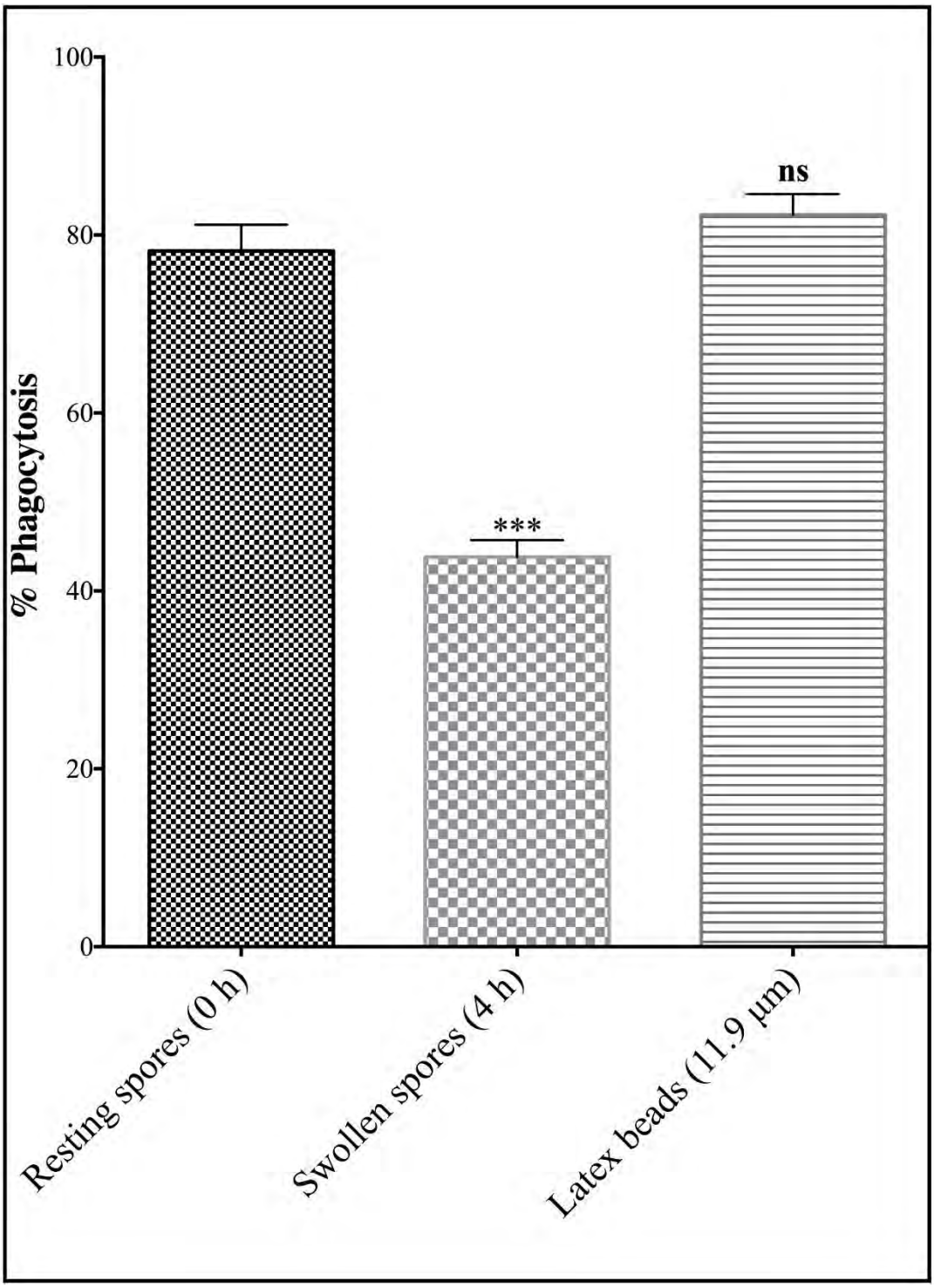


Figure 21: Phagocytosis of spores not affected by size.

Spores of *R. microsporus* were pre-germinated in SDA at 37ºC for 4 h and stained with FITC. These were then co-incubated with J774 phagocytes for 1 h and phagocytic uptake assessed. Controls with latex beads and dormant spore were both included as shown. The number of phagocytes containing at least one spore were counted at the indicated time points (n=9000, One way ANOVA with Tukey's correction for multiple comparisons) Three biological repeats were examined and error bars represent s.e.m. (***=$p<0.0001$ and **ns**= $p>0.05$).

3.5 Reduced phagocytosis of pre-germinated spores is not due to cell wall dynamism

The fungal cell wall plays an integral role between the cell and the surrounding environments. Under certain conditions, fungi can remodel their cell wall to meet demands of specific environmental niches (Lenardon et al., 2010, Dagenais et al., 2010, Melida et al., 2015, Wagener et al., 2014, Sherrington et al., 2017). Immunologically, cell wall remodelling can significantly modulate immune responses. Notably, conidial germination modulates immune recognition of *Aspergillus fumigatus* (Aimanianda and Latge, 2010, Dagenais et al., 2010). Observations with *A. fumigatus* are strongly linked to cell wall masking. Thus, we hypothesized that mucormycete spore pre-germination also enabled cell wall-based masking from recognition by macrophages.

To answer this question, we employed four approaches. Firstly, a bioinformatics search. With *A. fumigatus*, the influence of pre-germination on immune modulation has been linked to the presence of a hydrophobin protein layer (the rod let). Here, a BLAST search for its homologs among the mucormycetes was performed. However, no homologs among most of the mucoralean fungi were identified, except *L. corymbifera* where a hit with about 36% homology was found (**Figure 22**).

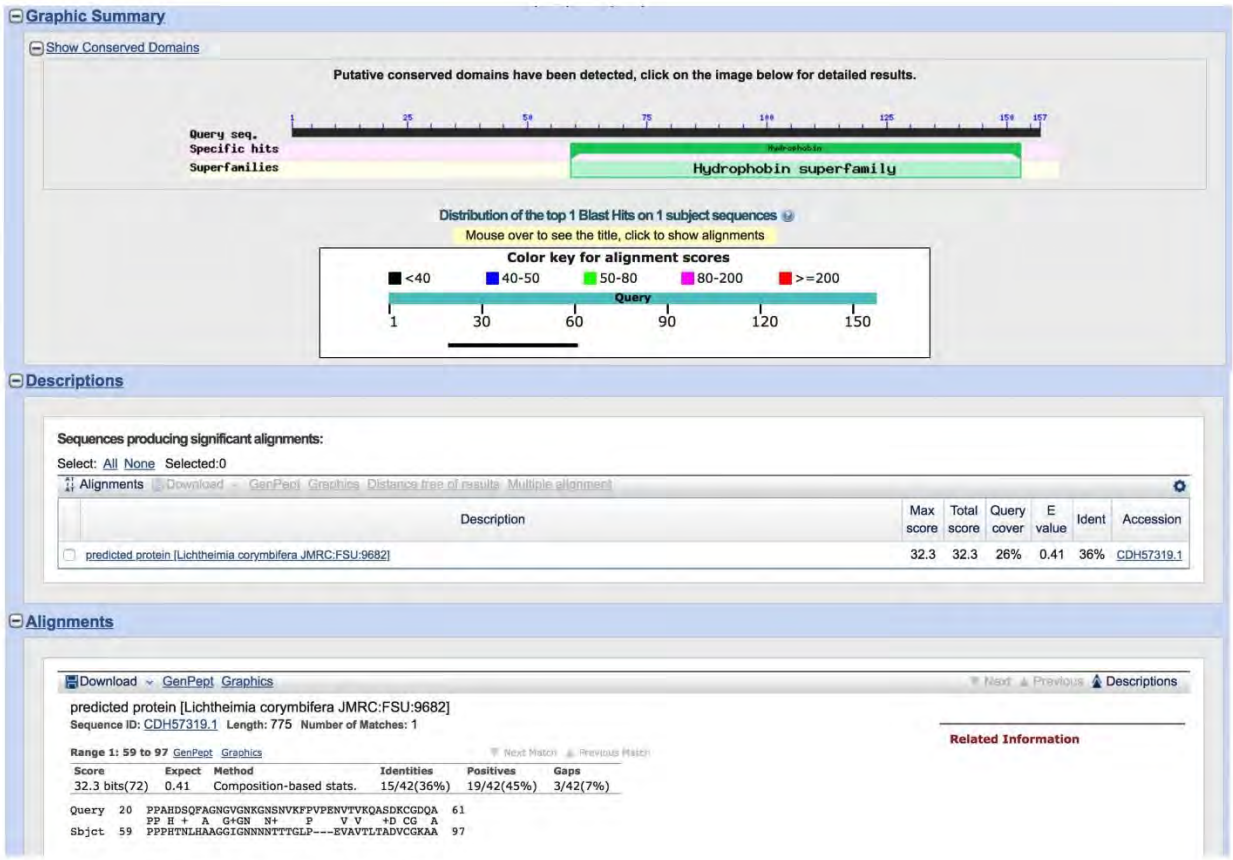


Figure 22: BLAST search reveals no homologs of hydrophobin protein layer in mucormycetes.

Basic local Alignment Search Tool (BLAST) for homologs of hydrophobin protein common to *Aspergillus* species. Using the nucleotide sequence of RodA gene a search was performed among the mucoralean fungi including *Rhizopus*, *Mucor*, *Lichtheimia* and *Cunninghamella* genera using the tools from National Centre for Biotechnology Information (NCBI) data base. Generated October, 2018.

Secondly, a staining approach where several fluorophores such as Con A-(mannans), CFW-(total chitin), WGA- (exposed chitin) and FITC-(glycoproteins) were used to monitor and measure change in availability of specific cell components during the course of pre-germination. Accordingly, spores were pre-germinated, stained and stain-uptake quantified as described before.

Interestingly the staining signal for total chitin by CFW significantly increased with swelling after 2 h and 4 h incubation ($p < 0.0001$); and exposed chitin by WGA staining drops after 2 h and 4 h but the difference is not statistically significant ($p > 0.05$). Mannan availability by Con-A staining also increased after both 2 h and 4 h incubation but the difference was not statistically significant ($p > 0.05$). While glycoproteins' availability by FITC barely changes after 2 h or 4 h incubation of germination (**Figure 23)**.

The increase in chitin availability here would be intriguing, but although chitin is a known immunomodulator, its effects are usually opposite to my observations here. Instead chitin is an activator of innate responses including innate cells such as eosinophils and macrophages with subsequent induction of cytokine production (Ghotloo et al., 2015, Elieh Ali Komi et al., 2018)

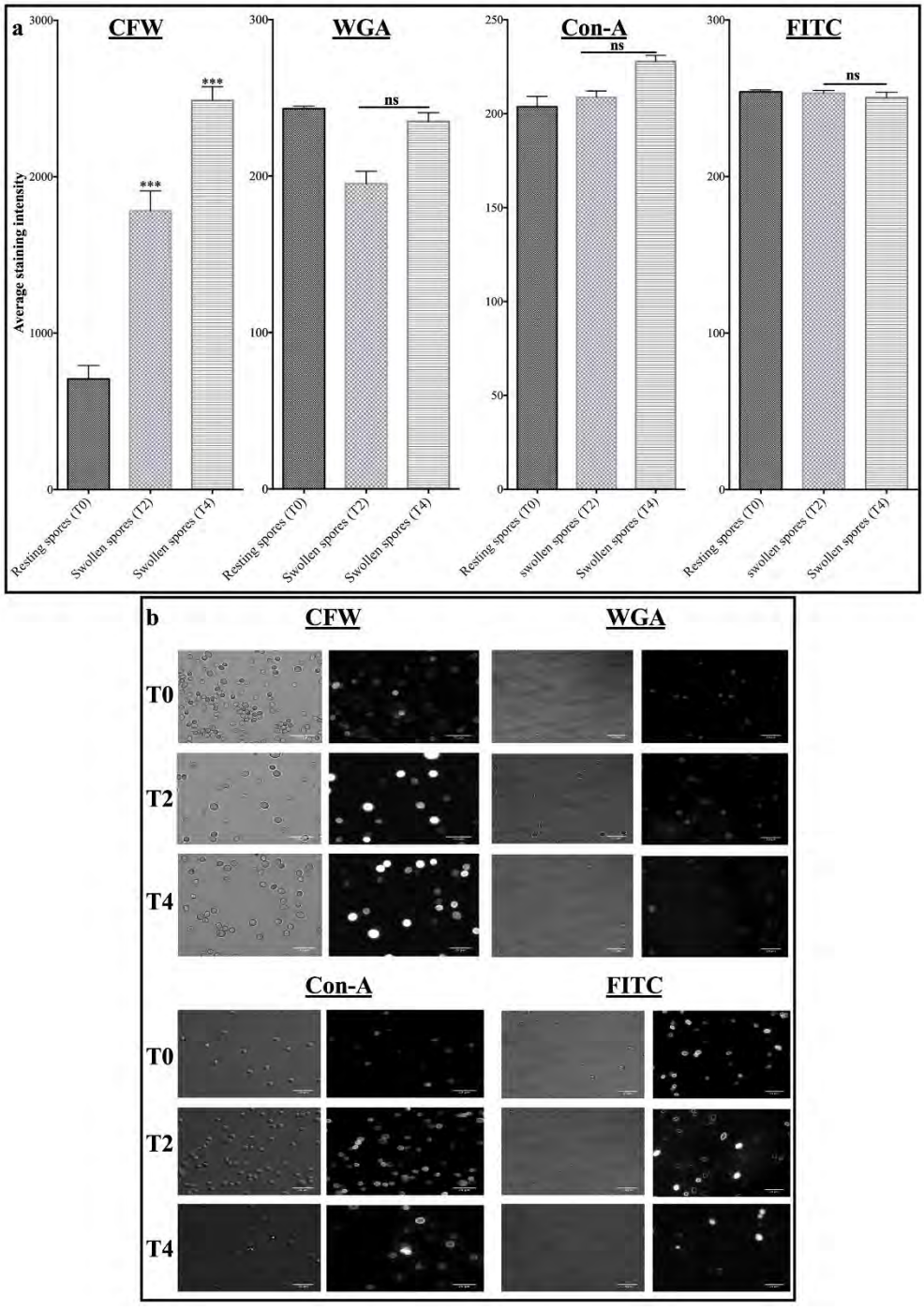


Figure 23: Cell wall staining of resting and swollen spores, CFW (total chitin), WGA (exposed chitin), Con-A(mannans) and FITC (total protein).

Resting *R. microsporus* FP 469-12 spores were pre-germinated at the indicated time points then with the indicated fluorophores, visualized by fluorescence microscopy and stain uptake for individual spores quantified. (n=300, One-Way ANOVA with Tukey's correction for multiple comparisons). Three biological repeats were examined and error bars represent s.e.m (ns = $p>0.05$, ***=$p<0.0001$). Micrographs are representative.

Thirdly, we tested the requirement for active cell wall remodelling by testing phagocytosis rates when spores were killed through UV or PFA-fixation treatments; For UV killing, spores were treated with 1200 joules/cm^2 or 4% PFA for 30 min and phagocytic uptake assessed as described before. This was aimed at inactivating metabolism with preservation of the cell wall integrity. Interestingly, whilst phagocytosis of live pre-germinated spores is significantly ($p < 0.0001$) reduced compared to resting spores, phagocytosis of the equivalent UV and PFA fixed spores was no different from fixed resting spores ($p > 0.05$) (**Figure 24**).

Notably though, there was a significant difference in uptake of resting spores fixed by either method compared to resting live spores ($p = 0.046$). Though not probed further, a reduction in phagocytosis of killed spores noted here is intriguing. Overall, these findings suggest that spore cell wall dynamism may not be involved in preventing uptake of pre-germinated spores but do suggest that spore metabolism could play a vital role.

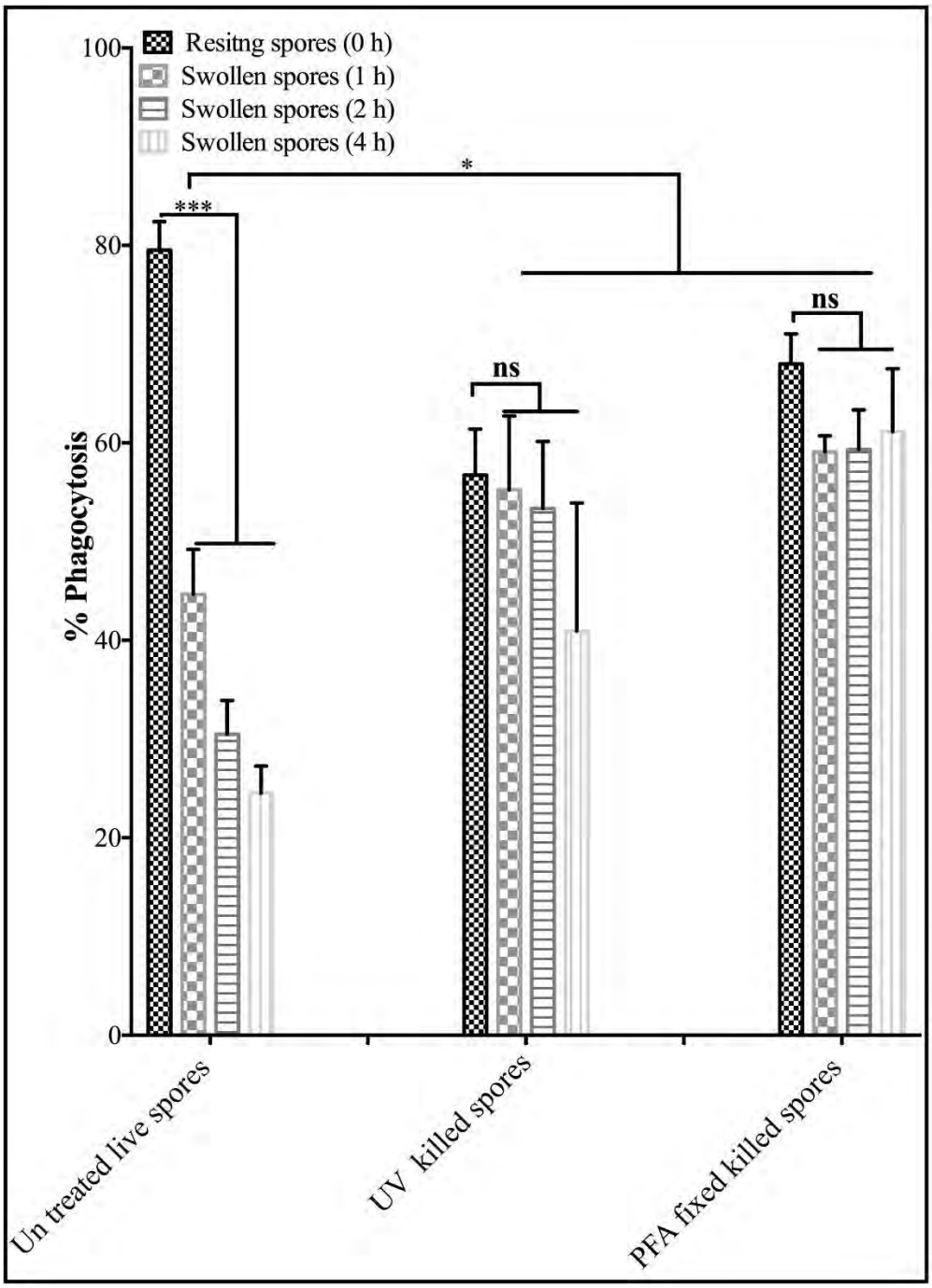


Figure 24: Phagocytosis of spores is restored when UV or PFA fixed killed.

R. microsporus spores were harvested, pre-germinated for the indicated time points, UV treated or PFA Fixed killed, washed and stained. These were then co-incubated with J774 phagocytes for 1 h and phagocytic uptake assessed. Live spores were also included as indicated. The number of phagocytes containing at least one spore were counted at the indicated time points (n=9000, One way ANOVA with Tukey's correction for multiple comparisons) Three biological repeats were examined and error bars represent s.e.m. (**ns** = $p>0.05$, *=$p<0.01$ and ***=$p<0.0001$).

Lastly, we tested the requirement for active cell wall remodelling by assessing phagosome maturation; this was also aimed at establishing the effect of cell wall dynamics on phagocytosis after germination. Accordingly, live or killed, resting or swollen spores were co-incubated with macrophages in *sf*DMEM containing 50 nM LysoTracker (DND-99), live cell imaging was performed, and phagosome maturation determined as described (Sections 2.3 and 2.9). Phagosome maturation was quantified by measuring the average LysoTracker staining intensity of phagosomes per macrophage using Image J software (Section 2.9).

As shown in **Figure 25**, we saw that both resting and swollen live spores inhibited phagosome maturation, with no statistical differences ($p>0.05$) noted between the two. However, significant phagosome staining intensity ($p < 0.0001$) was obtained with killed swollen spores. Meaning that if phagosome maturation inhibition by live spores was due to cell wall masking upon germination, then perhaps similar findings should have been revealed with killed spores. Generally, demonstrating that the differences seen here can eliminate the role of cell wall dynamism in inhibition of both phagocytosis of swollen spores and phagosome maturation. Most important, the role of spore metabolism is also highlight here as well.

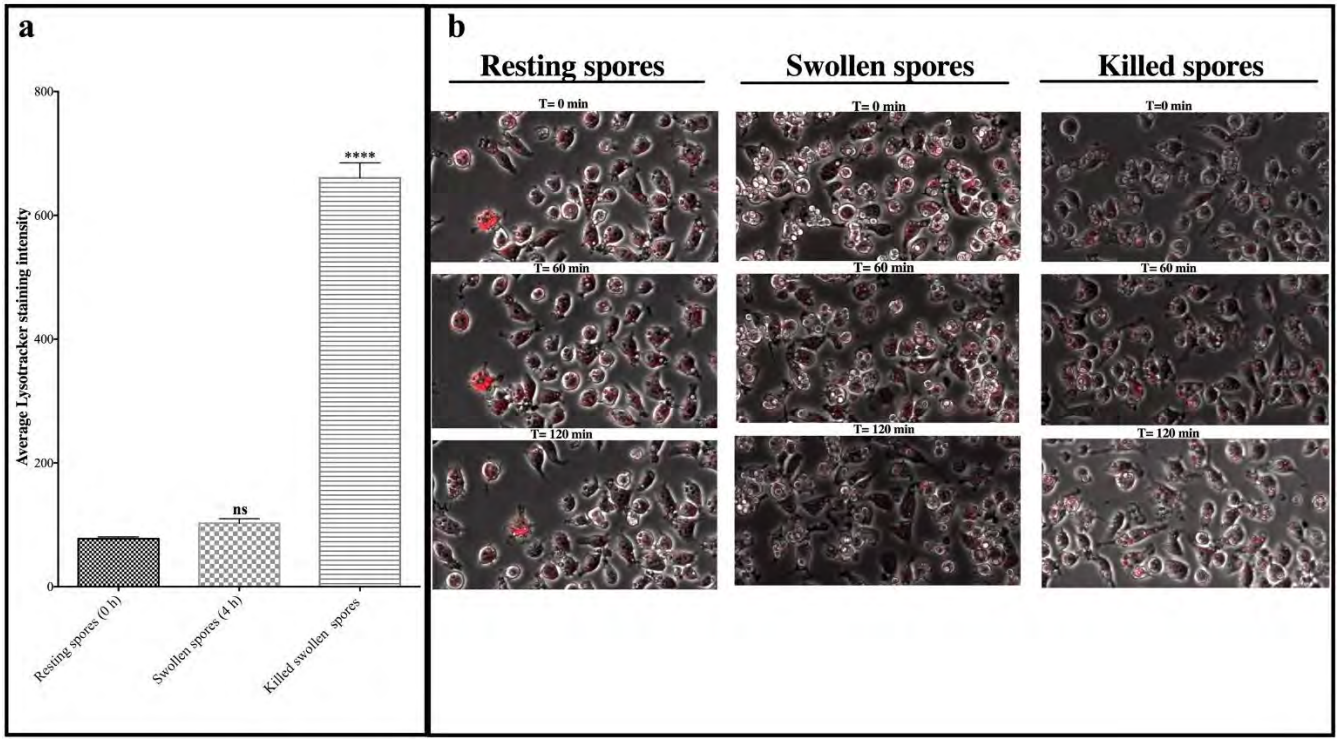


Figure 25: Live metabolic spores inhibit phagosome maturation.

(a) Live or killed resting and swollen spores of *R. microsporus* were co-incubated with J774 macrophages for 2 h and phagosome maturation determined with LysoTracker staining. The staining intensity per phagosome was quantified with Image J. The data are analysed using Student's two-tailed T-test with Welsh's correction for unequal variance (n=150 per replicate with three biological replicates). The data are shown as means with s.e.m (**ns** = ($p = 0.835$) and ****=$p<0.0001$). **(b)** Micrographs are representatives from time lapse microscopy.

3.6 Reduced phagocytosis of pre-germinated spores is due to spore metabolism

It is not uncommon for filamentous fungi to evade innate immunity through metabolic mechanisms. For instance, the most common example of such is via the production of mycotoxins e.g. aflatoxins or secondary metabolites such as cytochalasins (Qiao et al., 2011, Keller et al., 2005, Cox, 2007).

However, our understanding of such pathways is still limited. In regard to mucormycetes, identification of two key toxins, namely rhizoxin and rhizonin, has recently been made. These have so far been associated mainly with *R. microsporus*. Although implicated in plant pathology, there is no evidence of association with clinical mucormycosis. Most importantly, both toxins are produced by bacterial endosymbionts rather than the fungus (Gee et al., 2011, Lackner et al., 2009b, Ibrahim et al., 2008b, Partida-Martinez et al., 2008).

Thus, we hypothesized that pre-germinated spores produced a factor that antagonizes phagocytosis by macrophages, and that this might be a secreted factor. We hypothesized this factor might accumulate in supernatants of germinated spores. Therefore, accordingly spores were pre-germinated in sfDMEM for 4 h, supernatant collected, and its impact on macrophage functions evaluated (Sections 2.2 and 2.3).

The initial treatment was with 75% of the supernatant for 1 h, after which macrophages were washed with PBS and challenged with resting spores for 1 h. Treatment with *Rhizopus* supernatant significantly inhibited phagocytic uptake by both J774 macrophages, and PMBCs. Uptake by J774s decreased from 80% by untreated cells to about 38 % following supernatant treatment ($p < 0.00001$). For PBMCs, uptake decreased from 60% by untreated cells to about 37% following supernatant treatment ($p<0.0001$) (**Figure 26**). Additionally, supernatants collected from other species also exhibited similar but varying levels of inhibitory activity. For instance, their supernatants inhibited phagocytosis from about 78 % to 45 % by both *M.*

circinelloides species ($p<0.001$), 60% by *L. corymbifera* ($p=0.0375$) and 40% by *C. bertholletiae* ($p < 0.0001$) (**Figure 27**). Taken together, we can draw conclusions that the effect exhibited by swollen spores on phagocytosis is due to spore metabolism rather than cell wall or spore size.

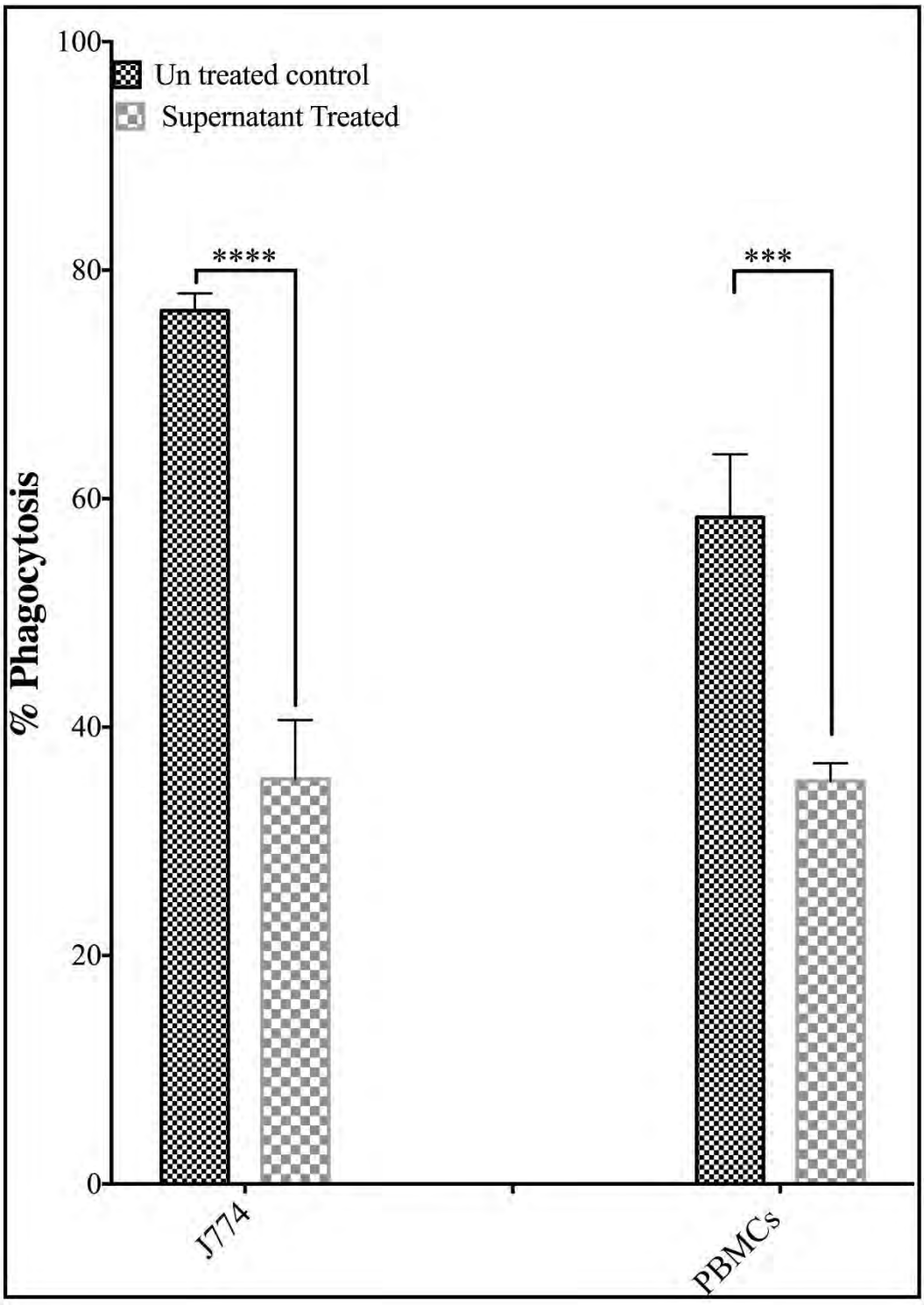


Figure 26: *R. microsporus* swollen spore supernatant can inhibit macrophage functions.

R. microsporus spores were pre-germinated in sfDMEM for 4 h and supernatant collected. This was then used to treat J774 and PBMC phagocytes for 1 h, which were then co-incubated with dormant spores of *R. microsporus* and phagocytic uptake assessed. For both assays, the number of phagocytes containing at least one spore were counted after 1 hour (n=9000, One way ANOVA with Tukey's correction for multiple comparisons). In each condition, three biological replicates were examined and error bars represent s.e.m. (**** $p<0.0001$, ***=$p<0.0001$).

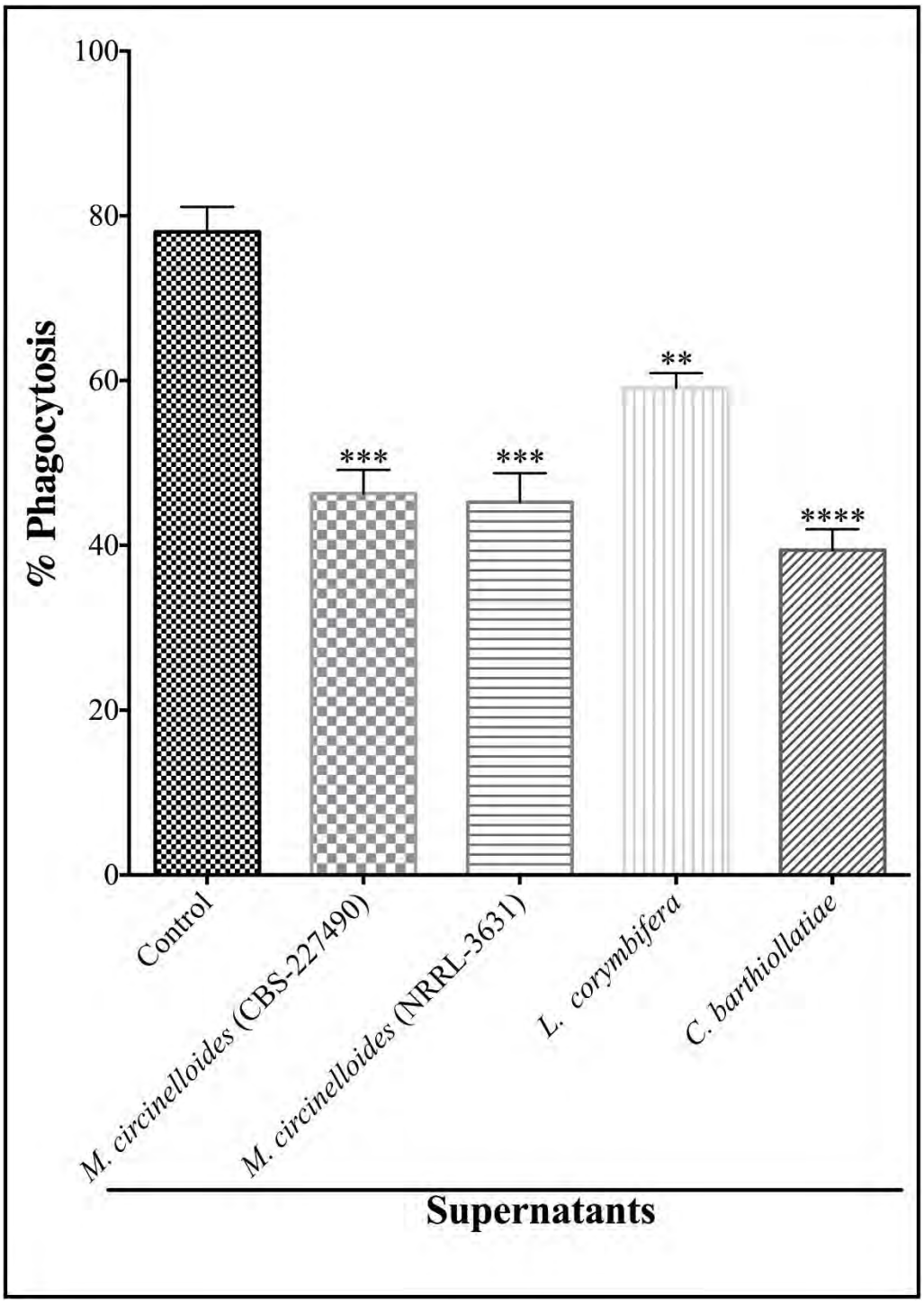


Figure 27: Supernatants from other mucoralean fungi inhibit phagocytic uptake by J774 macrophages.

Supernatants were collected from pre-germinated spores of other mucoralean fungi in sfDMEM. These were used to treat J774 macrophages for 1 h, then co-incubated with dormant spores of *R. microsporus* and phagocytic uptake assessed. For all conditions, the number of phagocytes containing at least one spore were counted after 1 hour (n=9000, One way ANOVA with Tukey's correction for multiple comparisons). Three biological replicates were examined and error bars represent s.e.m. (**=$p<0.001$, *** $p<0.0001$ and **** = $p<0.0001$).

3.7 Discussion

The pathobiology of mucormycosis has gained attention in the past decades specifically because of the nature of the disease. The disease is severely aggressive and targets individuals with key underlying conditions, especially those with defects in phagocyte effector functions. These conditions are the main drivers of what appears to be a transition from environmental saprophytes to now being one of the main emerging opportunistic filamentous fungal agents after *Aspergilli* species.

However, if we are to appropriately navigate the clinical aspects of mucormycosis, we ought to understand key defining molecular and cellular mechanisms governing interactions between the host immunity and infecting particles. We currently lack a clear understanding of how infection and eventually colonisation is initiated by mucormycetes spores.

The role of innate immunity and in particular phagocyte effector functions in combating filamentous fungal infection particles including mucoralean fungi has now been highlighted by several authors, but specific mechanisms to particular pathogens remain unclear (Horn et al., 2012, Brakhage et al., 2010, Inglesfield et al., 2018, Jahn et al., 2000, Kraibooj et al., 2014, Li et al., 2011, Voelz et al., 2015). Here, we employ a series of *in vitro* approaches to explore spore-macrophage interactions and show that infection due mucormycetes can be initiated through evasion of phagocyte effector functions.

Phagocytes such as macrophages represent the most prominent and professional component of innate immunity. Often resident in tissues, macrophage cells are constitutively primed to recognize, contain and eliminate infectious particles, in addition to activating other components of the host immune system, particularly adaptive immunity (Janeway, 2005, Roitt and Delves, 2001).

Here we report that both murine and human derived macrophages can be used *in vitro* to study mucormycete infection biology, and employing fluorescence dyes such Con-A, CFW, and FITC can be complementary to their application, similar with what has been established by others (Kraibooj et al., 2014, Li et al., 2011, Brakhage et al., 2010, Park and Voigt, 2014, Andrianaki et al., 2018b).

One difference between these mucormycetes and other fungi is that mucormycete spores may demonstrate staining heterogeneity. This can be linked to a number of factors, including difference in expression levels of certain cell wall components, as well as other factors such as melanin, spore size or metabolism. Indeed, large and metabolically active spores show improved staining ability (**Figure 14)**. It is not clear if melanin found in fungal spores impacts stain uptake, but has been reported to block mitochondrial staining in melanocytes, phagosome maturation and recently reported to be recognised by MelNec pattern recognition receptors (Ando et al., 2016, Stappers et al., 2018, Andrianaki et al., 2018b). On this account, staining protocols described here should be further evaluated and validated during application, since other specific parameters such as dye concentration can be affected under certain experimental conditions.

By applying the tools and conditions described here, we were able to explore several aspects of mechanisms involved during interactions between mucormycete spores and macrophages; particularly with a clinical strain of *Rhizopus*. *Rhizopus* is the most clinically relevant genus, although others included species from the *Mucor*, *Lichtheimia* and *Cunninghamella* genera to cater for the changing trends in aetiology.

We demonstrated here that dormant spores of *R. microsporus* are readily phagocytosed by macrophages. Yet, they do not germinate and are not killed inside the phagolysosome. These findings are similar to those by Kraibooj *et al* (2014), Park and Voight, 2014 and Li *et al* (2011) but differ from observations made with *Aspergilli* species, which show that dormant conidia

are poorly recognised by macrophages (Kraibooj et al., 2014, Park and Voigt, 2014, Aimanianda and Latge, 2010, Brakhage et al., 2010). These findings could provide an explanation for why we are protected from mucormycetes under normal health conditions but prone to the infection when phagocyte functions are compromised or with possible latent infection.

Profoundly though, we establish that whilst dormant spores are phagocytosed, pre-germinated spores of not only *R. microsporus* but also other mucoralean fungi showed reduced phagocytosis by macrophages. This is also similar to what Andrianaki et al (2018) has recently reported with species of *R. oryzae* and *R. delemar*. Interestingly, it is the reverse of what has been determined with *A. fumigatus* (Andrianaki et al., 2018b, Aimanianda and Latge, 2010, Brakhage et al., 2010).

While modulations by *Aspergillus* are linked with expression of a hydrophobin protein coat, mucormycetes, with the exception of *L. corymbifera* lack homologs of this protein. The homolog found in *L. corymbifera* shows homology of about 36 % to the *Aspergillus* hydrophobin. Indicating perhaps that this may not be a functional rod let in *L. corymbifera* and thus, could explain why resting spores of this strain are also phagocytosed as opposed to *Aspergillus* conidia. However, our observations most certainly point to spore's metabolism rather than cell wall masking as a major modulator of phagocytosis. Further details pertaining this observation are to follow in the subsequent chapters.

The data presented here describes several tools for assessing phagocytosis of the mucormycetes using both murine and human macrophage cell lines. The multiplicity of infection demonstrated here for both cell lines is applicable and can be adapted elsewhere. However, the staining tools used here for scoring phagocytosis can be hard to reproduce with other mucoralean fungal spores and thus, advisable that the protocols employed here are re-evaluated

on every occasion of application. Additionally, other techniques such as flow cytometry or immunostaining can be employed. On the other hand, the buffy coats can be used as a source for PBMCs but more studies are still required to establish it as reliable source of this cell line. Finally, we are unable to optimise a phagocytic assay with neutrophils, because are a tough cell line to work with in addition to lack of a reliable source for them.

CHAPTER FOUR: Impact of Spore Metabolism on Macrophage Functions

4.1 Chapter Overview

Part of the data presented in this chapter, was performed in collaboration with the chemistry department. Particularly, Lipidomics experiments were performed by Warwick Dunn, from metabolomics unit, school of Biosciences. Chloroform extractions were performed by myself with the help of Ignacio Insua in the Trillo Lab, school of Chemistry. HPLC work was done by myself with help from Ignacio Insua, Oliva Creese and Allen Bowden in the HPLC facility, school of chemistry and Mass Spectrometry analysis was performed by myself with technical help from Chi Tsang in the Mass spectrometry facility School of Chemistry, University of Birmingham.

4.2 Background

Data from chapter 3 suggests that mucormycete spores may secrete factors that negatively impact on macrophage effector functions. Indeed, it is not unheard of for filamentous fungi to evade host immunity via metabolic processes. For instance, *Aspergillus*, *Penicillium* and *Fusarium* species are all known producers of a diverse range of mycotoxins (Miller and McMullin, 2014, Felicio et al., 2011, Rousseaux et al., 2014).

These are bioactive compounds of low molecular weight. They include fungal secondary metabolites, and about 10,000 different kinds have been identified including but not limited to aflatoxins, ochra-toxin, T-2, HT-2, kojic acid, patulin, tremor genic toxins, zearalenone, ergot alkaloids, cytochalasins, trichothecenes, fumonism and deoxynirolenol toxins (Miller and McMullin, 2014, Pitt and Miller, 2017, Botha et al., 2014, Botha et al., 2018, Varga et al., 2003).

However, whilst some of these compounds are often implicated in plant pathology and agricultural produce contamination, association with human pathobiology may not be uncommon, though poorly characterized (Miller and McMullin, 2014, Duba et al., 2018, Liew and Mohd-Redzwan, 2018, Varga et al., 2003, Botha et al., 2018).

For instance, compounds such as the cytochalasins, aflatoxins, kojic acid and tremor genic mycotoxins are linked with angio-genic, carcinogenic or neuro-mycotoxin genic effects in both humans and ruminants (Sohrabi and Taghizadeh, 2018, Botha et al., 2014, Botha et al., 2018, Qiao et al., 2011).

On the other hand, our observations here with mucoralean fungi clearly indicate the possibility of a metabolically-based escape from phagocytic uptake and killing by spores. This directly implicates the role of metabolism in immune evasion.

Interestingly, mucormycetes such as *Rhizopus* are also associated with metabolic compounds like rhizoxin, a potent antimitotic toxin, and rhizonin, a potent hepatotoxic compound, but both are yet to be linked with virulence (Scherlach et al., 2006, Partida-Martinez et al., 2007c, Partida-Martinez and Hertweck, 2007, Partida-Martinez and Hertweck, 2005, Partida-Martinez et al., 2007a, Partida-Martinez et al., 2008, Ibrahim et al., 2008b).

What is clear so far is that both of these toxins are not directly produced by the fungus but rather by bacterial endosymbionts such as *Burkholderia rhizoxinica* (rhizoxin) or *Burkholderia endofungorum* (rhizonin). These maintain a mutualistic symbiotic relationship with the fungus (Partida-Martinez et al., 2007a, Partida-Martinez et al., 2007b). Additionally, whilst rhizonin is mainly an agricultural produce contaminant, rhizoxin is involved in the pathogenesis of blight disease in rice seedlings via inhibition of mitotic processes by targeting beta tubulin (Partida-Martinez and Hertweck, 2005, Partida-Martinez et al., 2007a, Lackner et al., 2009b, Moebius et al., 2014a).

From this stand point, it is fair to say that our understanding of mucormycete metabolism and thus its impact on host-fungal interaction is limited. Yet, it appears that spores are capable of evading phagocytic effector functions via metabolic processes. Although, rhizo-toxins are not implicated in the pathogenesis of mucormycosis, our demonstration of supernatant factors produced by metabolically active spores is intriguing enough to probe further characterization

and identification. In this chapter therefore, we attempt to describe the effect of factors secreted by fungal spores on phagocyte function by characterizing the supernatants, evaluating their impact on macrophage function, and finally attempting identification of the compounds.

4.3 The supernatant factor is relatively bio-stable at 4°C

If the supernatants were to be experimentally manipulated, characterised and identified, basic properties such as bio-stability of the factors at routine lab storage temperature of 4°C had to be established. Accordingly, supernatants were collected and the activity of different supernatant concentrations including 50, 75 and 100% in sfDMEM evaluated over a storage period of 4 weeks (Section 2.4).

As shown in **Figure 28**, all concentrations demonstrate significant activity at time of collection but then significantly declined with storage. For instance, average phagocytic uptake following exposure to supernatant increased from 52% when fresh to 63% after 1 week; 75% after 2 and 3 weeks; and to 80% after 4 weeks ($p < 0.05$) for all concentrations, indicating a loss of activity of the supernatant. These data show that at although relatively stable for the first 2 weeks of storage, the active factor begins to degrade with storage beyond 2 weeks, and thus freshly collected supernatants were used for all experiments. Where not possible the supernatant was stored at -80 ° C.

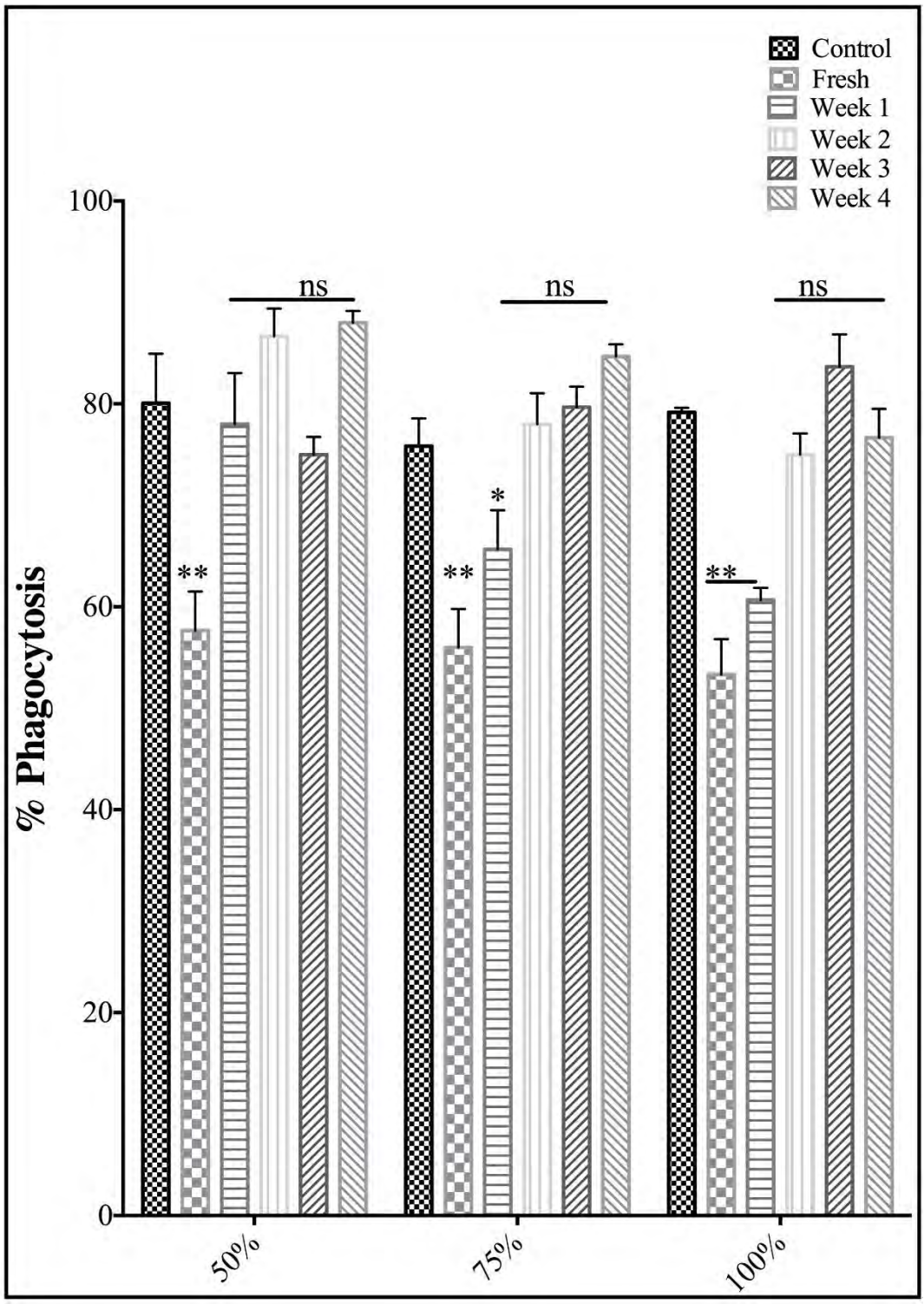


Figure 28: Supernatant factor is relatively bio degrades with storage.

Supernatants were collected, diluted to the indicated concentrations and tested for activity against phagocytosis of resting spores of *R. microsporus* by J774 macrophages after the indicated time points. For all experimental conditions, the number of phagocytes containing at least one spore were counted after 1 hour (n=9000, One way ANOVA with Tukey's correction for multiple comparisons). Three biological replicates were examined and error bars represent s.e.m. (ns=$p>0.05$, * $p<0.01$ and ** = $p<0.001$).

4.4 pH of the supernatant does increase but has no effect on macrophage function

Resting spores are in a state of low metabolic activity, a feat necessary for overwintering dormancy. However, under optimal conditions including moisture, oxygen, nutrients and ambient temperature, dormancy is broken to allow germination and growth. During infection and colonization by spores, substrates like pathological exudates can also promote germination and growth.

Inducing spore germination can also influence micro-environment pH by increasing extracellular bio-products. Undeniably, when associated with the host this may significantly affect physiological processes that are prone to pH change. For instance, low pH causes over expression of stablin-1 receptor involved in phosphatidylserine-dependent removal of apoptotic cells; and also causes reduced iron binding by ferrin carrier proteins increasing iron bio-availability (Park et al., 2012, Shirazi et al., 2015, Ibrahim et al., 2008c). Most importantly the optimal pH for phagocytosis is 6 - 7.5; and below pH 5 or above 8 phagocytosis is abolished and bactericidal activity by phagocytes is reduced (Gargan et al., 1993).

Here, the effect of supernatant pH on macrophage function was also considered. Accordingly, 4 h supernatants were collected from 5 mucormycete species of interest and pH measured as described in section 2.3.

Notably, there were no significant differences in the average measured values of sfDMEM control (pH 7.54) and individual supernatants from *R. microsporus* (pH 7.86), *M. circinelloides (CBS-227940*) (pH 8.46), *M. circinelloides (NRRL 3631*) (pH 8.41), *L. corymbifera* (pH 8.31), *C. bertholletiae (NB2)* (pH 8.47) ($p > 0.05$) (**Table 9**).

Table 9: Average measured pH values of medium control and supernatants.
The data are presented as standard error of means from at least three individual biological repeats, a t –test vs the control was performed and statistical differences are indicated; where **(**$p < 0.05$).

Supernatant source	Average pH +/- SEM	p value
Sf DMEM	7.54 ± 0.31	
R. microsporus	7.86 ± 0.17	0.5653
M. circinelloides (CBS-227940)	8.46 ± 0.22	0.1843
M. circinelloides (NRRL-3631)	8.41 ± 0.35	0.07234
L. corymbifera	8.31 ± 0.19	0.0971
C. bertholletiae (NB2)	8.47 ± 0.50	0.01862

Using the above findings as reference, a pH gradient of 8.00, 8.50 and 9.00 similar to the supernatants was created using sfDMEM to investigate the effect of this pH range on phagocytosis described in sections 2.4 and 2.7. As shown in **Figure 29,** untreated and adjusted-pH treated cells demonstrated percentage phagocytosis of 74% for a normal sfDMEM control, 79% for pH 8.00 adjusted sfDMEM, 75% for pH 8.50 adjusted sfDMEM and 60% for pH 9.00 adjusted sfDMEM. The differences observed in percentage uptake were not statistically significant ($p = 0.9231$). These data indicate that the inhibitory effects demonstrated previously are not due to increased pH of the supernatants, as there was no correlation between pH and bioactivity of the supernatants.

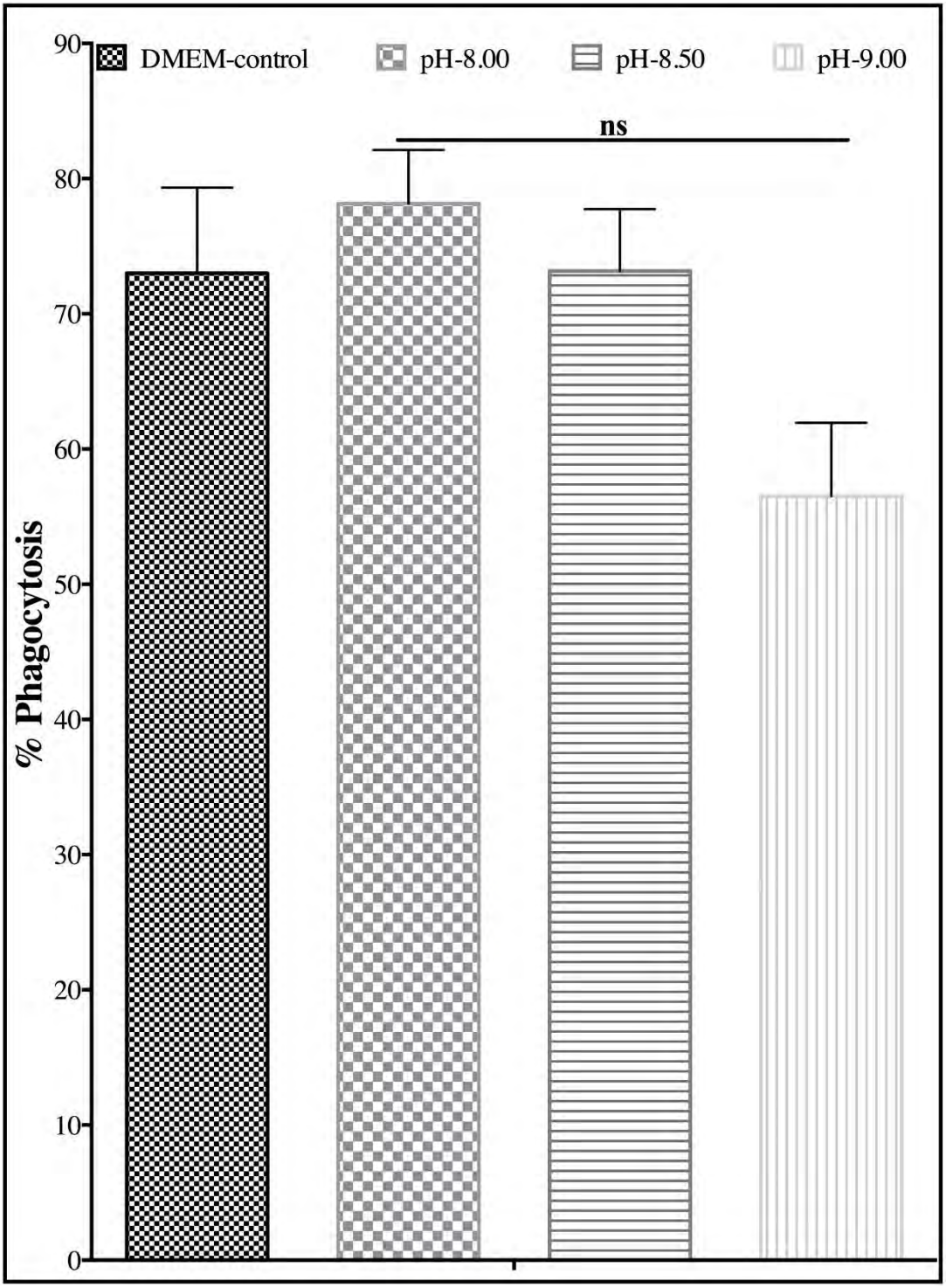


Figure 29: Supernatant pH does not affect macrophage functions.

The pH of sfDMEM was adjusted to the indicated ranges to match measured values for the collected supernatants. The adjusted medium was then used to treat J774 phagocytes, which were co-incubated with *R. microsporus* dormant spores for 1 h and uptake rate was assessed. For each adjusted pH conditions, the number of phagocytes containing at least one spore were counted after 1 hour (n=9000, One way ANOVA with Tukey's correction for multiple comparisons). Three biological replicates were examined and error bars represent s.e.m. (p=0.09321).

4.5 The supernatant factor also inhibits phagocytosis of particles other than resting spores

We have demonstrated in Chapter 3 that the supernatant inhibits phagocytosis of resting spores. Thus, activity against uptake of other particles such as latex beads and other fungal particles such as *Candida albicans* and *Saccharomyces cerevisiae* was also determined. Accordingly, macrophages were treated, challenged with the specified particles, and phagocytosis determined as in sections 2.4 and 2.7.

As shown in **Figure 30a**, treatment with *Rhizopus* supernatant significantly inhibited uptake of latex beads from an uptake of 70% to 37% ± 2 ($p < 0.001$) and *S. cerevisiae* from 80% to about 35% ± 4 ($p < 0.001$). Similarly, so, supernatants from other mucoralean fungi also showed varying levels of activity against uptake of *C. albicans* and *S. cerevisiae*. Particularly, supernatants of *M. circinelloides* (NRRL 3631) and *Cunninghamella* had a large significant impact against phagocytosis when compared with the untreated control (**Figure 30b**).

These findings continue to show that this is a cross-species characteristic, but most importantly, that inhibitory activity is universal against other particles, consistent with a metabolic, rather than intrinsic, property.

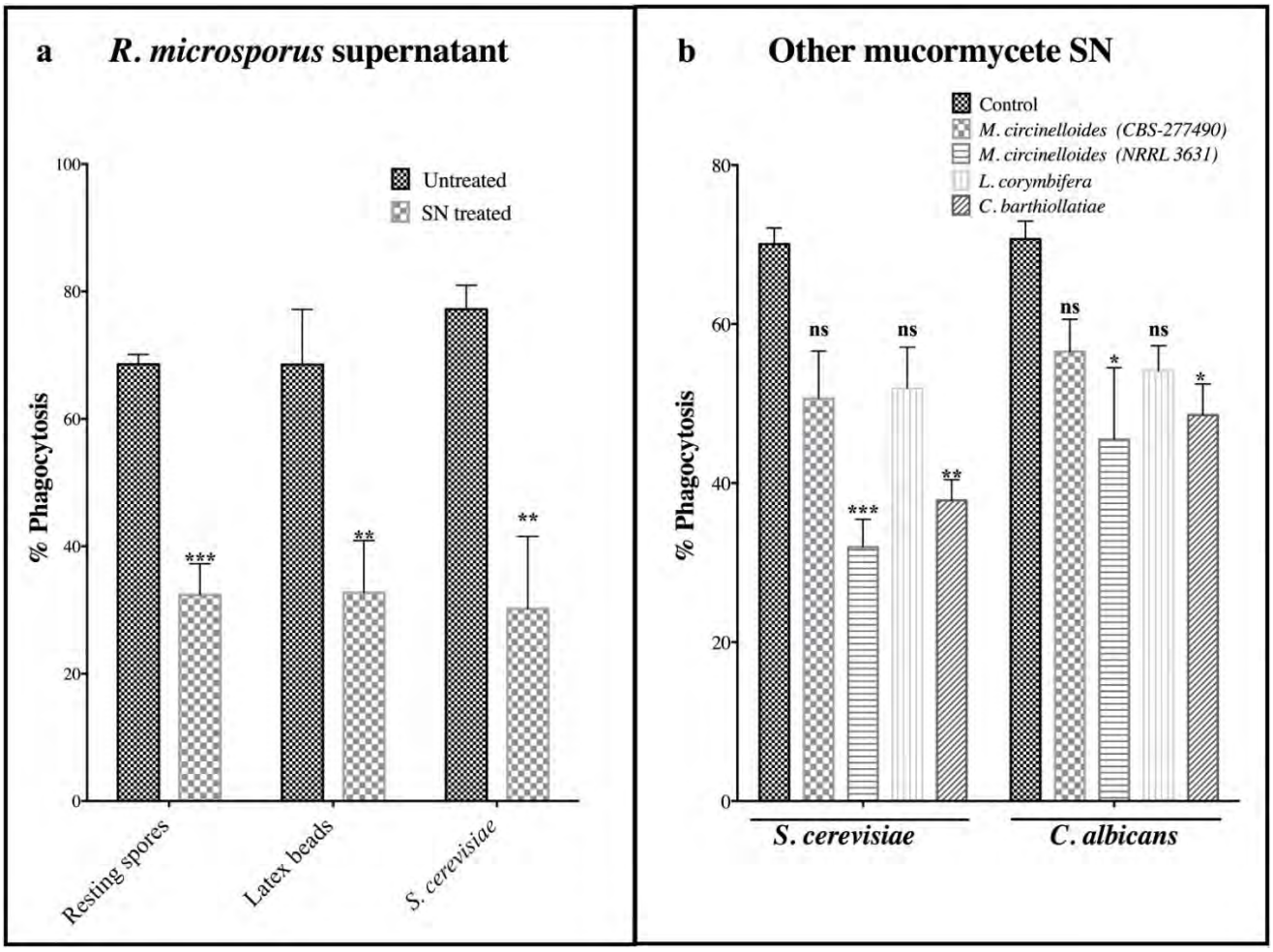


Figure 30: Mucormycete supernatant inhibits phagocytic uptake of other particles.

Supernatants from (**a**) *R. microsporus* and (**b**) other mucoralean fungi, as indicated, were collected and their activity against phagocytosis of particles other than dormant *R. microsporus* spores determined. The supernatants were used to treat J774 macrophages, which were then co-incubated with *R. microsporus* dormant spores or other phagocytic targets as indicated for 1 h and uptake rate was assessed. For both experiments, the number of phagocytes containing at least one spore were counted after 1 hour (n=9000, One way ANOVA with Tukey's correction for multiple comparisons). Three biological replicates were examined and error bars represent s.e.m. (*=p < 0.01, ** =P<0.001, ***p=<0.0001 and **ns**=p>0.05).

4.6 The supernatant factor also inhibits phagosome maturation

We already highlighted the importance of phagocytosis and phagosome maturation. Most importantly, we have also demonstrated the effect of spore metabolism on phagosome maturation. Thus, the effect of the supernatant factor on phagosome maturation was also considered here.

Accordingly, macrophages were treated with supernatant from pre-germinated *R. microsporus* spores, co-incubated with killed spores of the same species, and their ability to develop matured phagosomes determined through LysoTracker staining.

As demonstrated in (**Figure 31a**), supernatant treatment significantly reduces LysoTracker staining intensity ($p < 0.0001$). The differences are also depicted in representative images (**Figure 31b).** These findings comparable to those obtained when macrophages were co-incubated with live pre-germinated spores (Section 3.5), indicating that supernatant treatment of macrophages compromises their ability to undergo phagosome maturation.

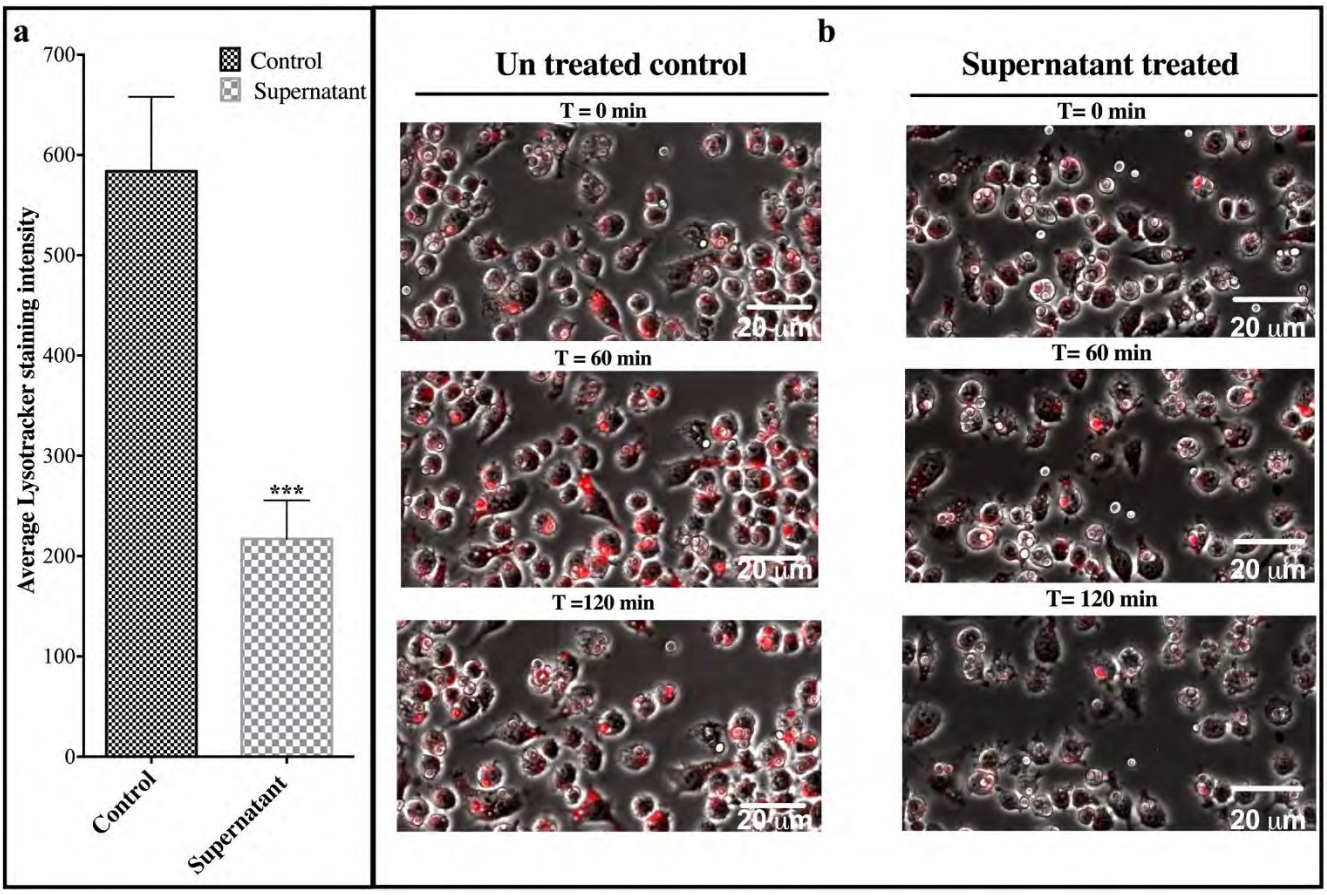


Figure 31: *R. microsporus* culture supernatant inhibits phagosome maturation.

Supernatants were collected and used to treat J774 phagocytes for 1 h, which were then co-incubated with fixed killed swollen spores for 2 h. (**a**) Phagosome staining following uptake was determined with LysoTracker and intensity quantified by Image J. (**b**) Effect of the treatment on phagosome maturation was also monitored by time lapse microscopy for the indicated time points. For the graph, 450 cells with phagocytosed spores from three biological repeats were examined; error bars represent s.d**.** where (***=$p<0.0001$). Micrographs are representative.

4.7 Cytotoxic effects by the supernatant factor on macrophages

4.7.1 Secreted factor is not as toxic at collected supernatant concentrations

The cytotoxic effects of some mycotoxins such as cytochalasin and rhizo-toxins are well known and thus we examined if the secreted factors exhibited similar properties (Qiao et al., 2011, Partida-Martinez and Hertweck, 2005, Partida-Martinez et al., 2007a).

Accordingly, macrophages were treated with the supernatant, and the effect on viability and cell injury was determined through trypan blue staining and LDH assay, respectively, as described (Section 2.8).

Trypan blue, stains dead cells blue leaving live ones unstained. An LDH assay calorimetrically measures the amount of LDH enzyme released due to cell injury. As shown in **Figure 32a,** treatment with either 75 or 100 % supernatant did not affect macrophage viability, there was no difference in the number of cells that took up the blue dye between treated and untreated controls meaning that the overall viability was 100% ($p = 0.1198$).

Similarly, the LDH assay showed supernatant treated cells to release significantly lower enzyme levels compared to the water treated positive control ($p < 0.0001$). When co-incubated with pre-germinated spores, the amount of LDH enzyme slightly increased compared to the supernatant treatment, but the increase was not statistically significant compared to the supernatant ($p>0.05$) (**Figure 32b**). The data showed that although the supernatant affects macrophage functions, it may not be through direct killing or damage.

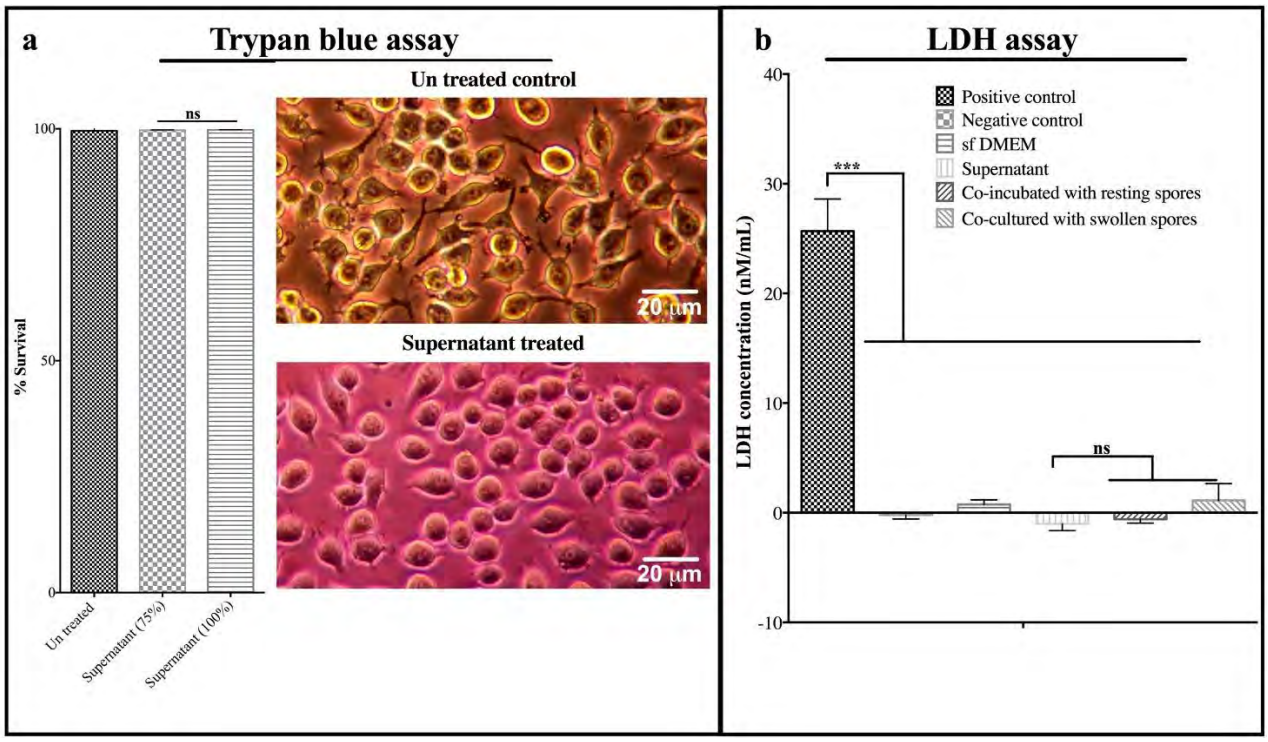


Figure 32: Supernatant factor has no effect cell viability.

Quantification of macrophage viability by **(a)** Trypan blue staining following treatment with swollen spore supernatant. **(b)** Quantification of cell viability by LDH release following treatment of J774A.1 macrophages with swollen spore supernatant. Cell lysis buffer (positive) and DMEM only (negative) treated cells serve as controls. For all graphs, 100 cells with phagocytosed spores from three biological repeats were examined; error bars represent s.d**.** where (***=p<0.0001). Micrographs are representative.

4.7.2 The supernatant factor affects cytoskeleton and morphology J774 phagocytes

The eukaryotic cytoskeleton is dominated with structures such as actin and tubulin. Macrophages require these for motility, phagocytosis, antigen presentation, and other morphological dynamics (Jonsson et al., 2012, Ting-Beall et al., 1995).

However, if pathogens are to successfully evade phagocyte effector functions, inhibiting polymerization of actin or tubulin can be a crucial step. In this regard, several pathogens are already known to secrete compounds that specifically inhibit polymerization of cytoskeletal components, including actin and tubulin (Hartwig and Yin, 1988).

Amongst these are two cytochalasins, chaetoglobosin and rhizoxin, that target F-actin and ß-tubulin respectively (Lackner et al., 2009b, Partida-Martinez and Hertweck, 2005, Qiao et al., 2011). Cytochalasin B can also cause defects in nitrogen oxide (NO) production (Kim et al., 2014). Undeniably, some pathogens have evolved mechanisms to evade macrophage functions including disruption of the cytoskeleton. Therefore, with evidence of metabolic spores evading phagocytosis here, the effect of spore metabolism on phagocytosis was also determined.

Accordingly, macrophages were treated, stained for F-actin or ß-tubulin, and staining analysed (Sections 2.4 and 2.9) Cells were examined for the presence of actin filaments and microtubules, indicative of an intact cytoskeleton (Prota et al., 2014, Takahashi et al., 1987, Sullivan et al., 1990, Shoji et al., 2012, Cooper, 1987). As demonstrated in (**Figure 33**), there was a significant decline in actin staining of treated cells compared to untreated ($p<0.0001$). additionally, there was significant differences between actin staining with supernatant and Cytochalasin D (an inhibitor of actin polymerisation) ($p<0.001$), used as a positive control. This is perhaps suggestive of similar target and mode of action between supernatant factor and the positive control. These differences are also depicted in the representative images (**Figure 33).**

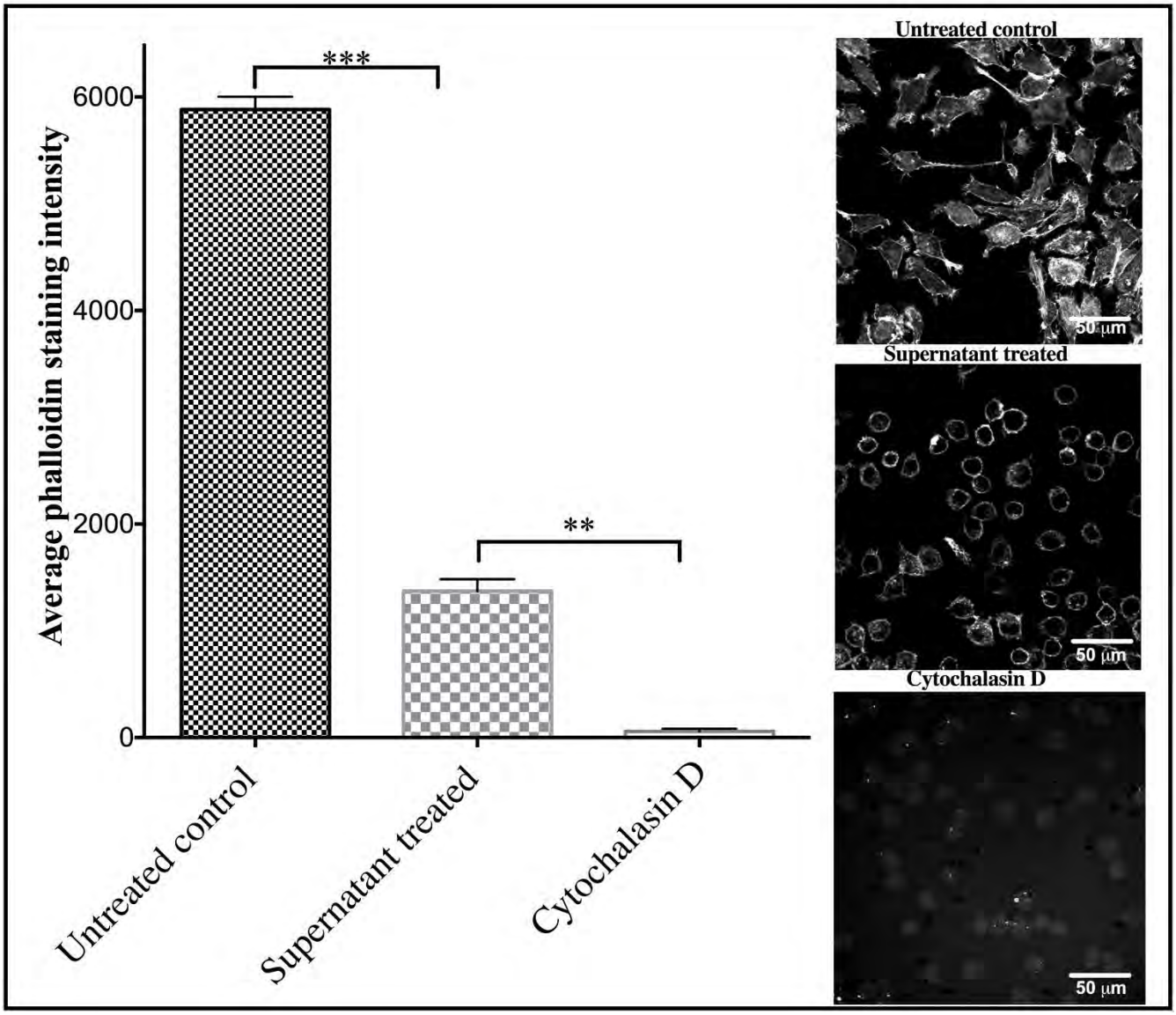


Figure 33: *R. microsporus* culture supernatant can inhibit macrophage cytoskeletal (actin) function.

J774 macrophages were treated with the collected *R. microsporus* culture supernatant or cytochalasin D as positive control for 1 h, stained for F-actin for 15 min with rhodamine-conjugated phalloidin, and staining intensity quantified with image J. Analysed using Student's two-tailed T-test with Welsh's correction for unequal variance (n=150 per replicate, with three biological replicates). For the graph, data shown are mean with s.e.m. (**$p<0.001$, *** $p < 0.0001$). Representative micrographs are shown.

Similarly, with beta tubulin immuno-staining, there were significantly marked differences between average staining intensity of untreated cells and those treated with supernatant culture (p <0.0001). In addition, similar findings as with the supernatant were obtained with Rhizoxin D, a known beta tubulin in inhibitor (p <0.01). Here, Rhizoxin was used as a positive control for microtubule destabilisation (**Figure 34**). The differences are also depicted in the representative images.

However, in both cytoskeletal experiments above, there was a significant change in morphology of the cells treated with supernatant and thus further investigated. This defect was further examined through evaluating cell shape such as scoring their circularity where; (0 is a perfect line and 1 a perfect circle).

As demonstrated in (**Figure 35**), significant changes in average cell shape occurred from 0.19 when untreated to 0.25 when treated (p<0.00001). Taken together, defects in cytoskeletal staining and changes in cell shape here all point to significant cytotoxic effects driven by spore metabolic processes.

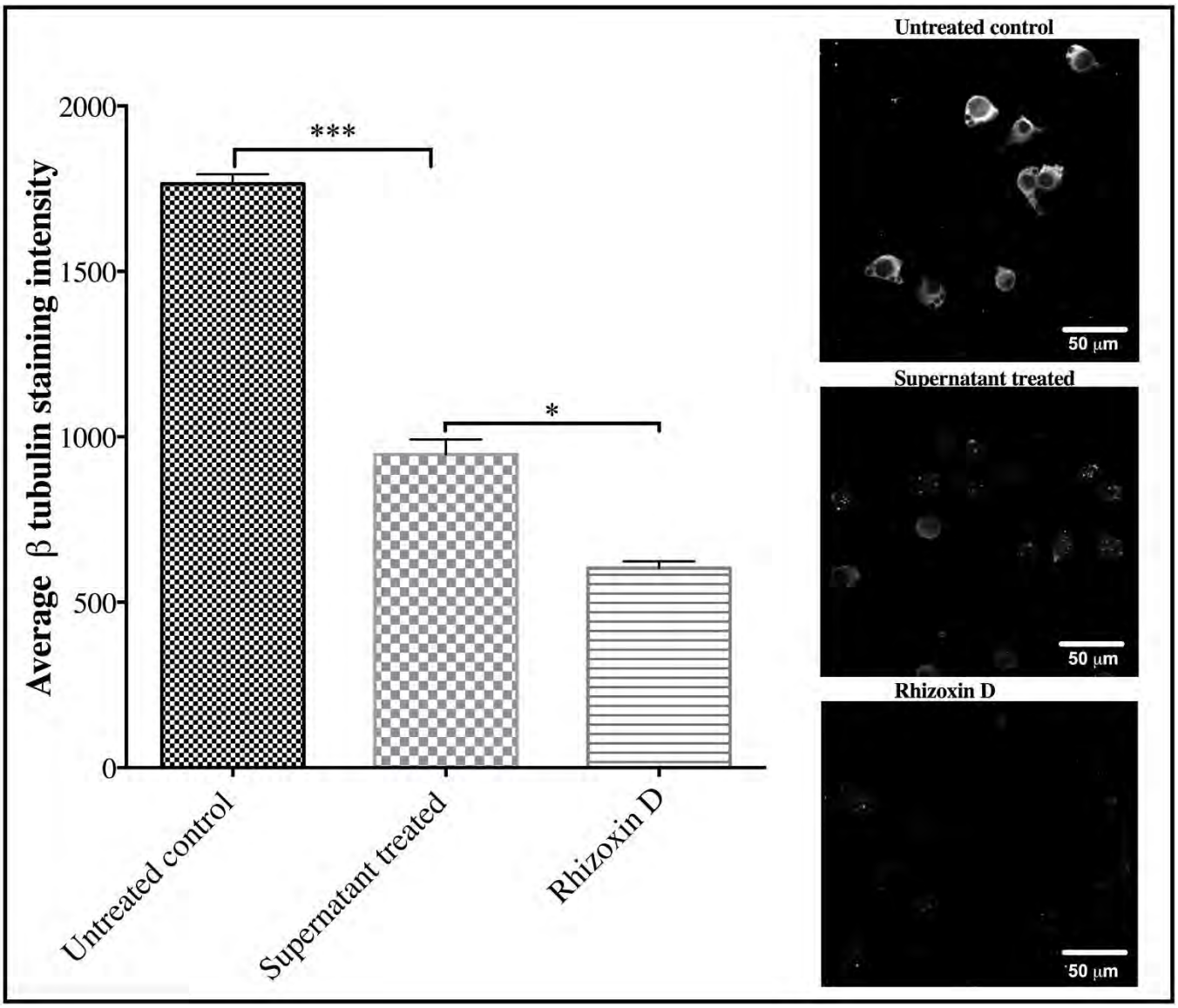


Figure 34: *R. microsporus* culture supernatant can inhibit macrophage cytoskeletal (beta tubulin) function.

J774 macrophages were treated with the collected *R. microsporus* supernatants or rhizoxin D as positive control for 1 h, immuno-stained for 1h with anti-beta tubulin antibody and counterstained with secondary antibody (goat anti-rabbit) and stain intensity quantified with image J. Analysed using Student's two-tailed T-test with Welsh's correction for unequal variance (n=150 per replicate, with three biological replicates). For the graph, data shown are mean with s.e.m. (**p<0.001, *** p < 0.0001). Representative micrographs are shown.

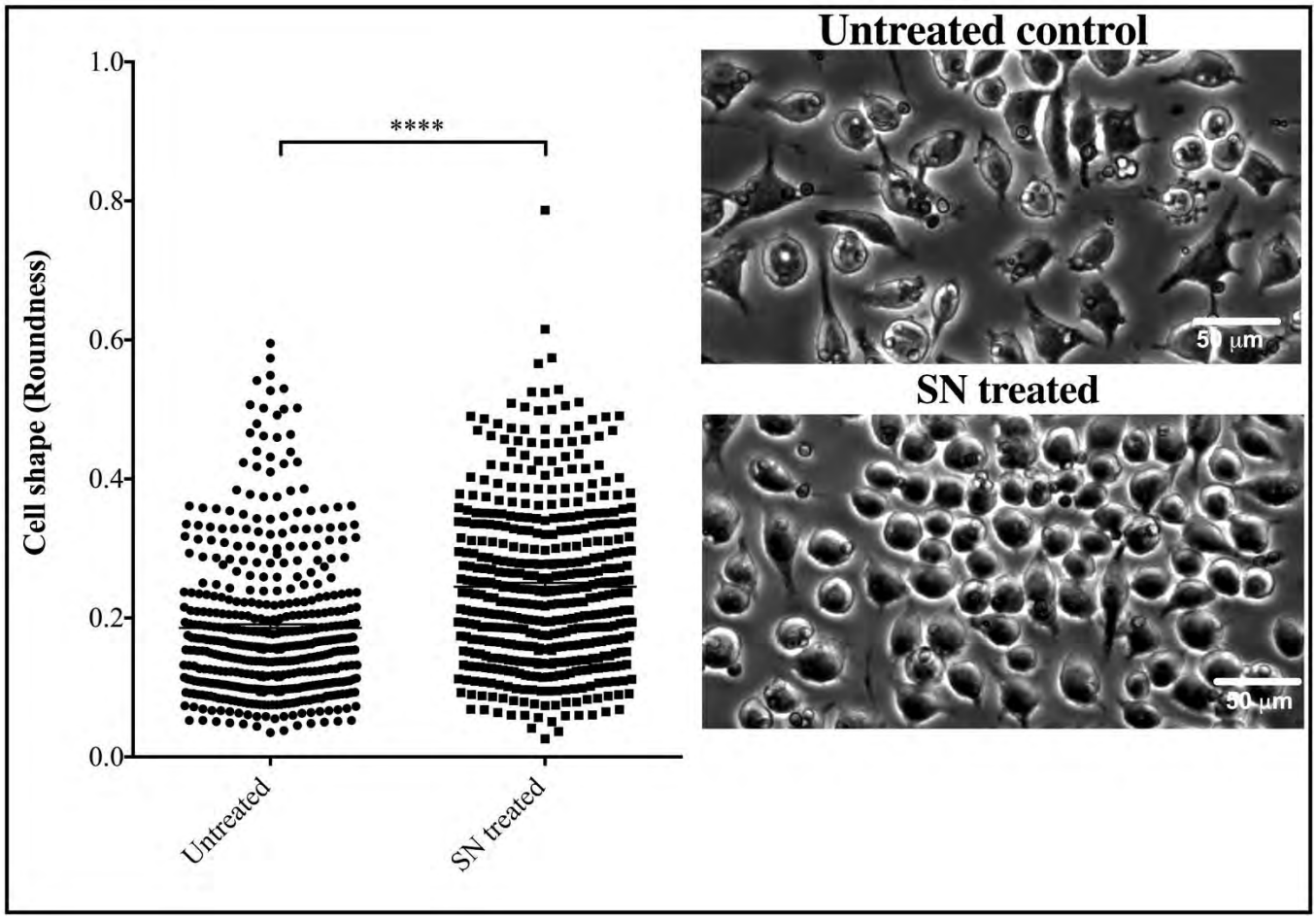


Figure 35: *R. microsporus* culture supernatant affects macrophage cell morphology.

J774 phagocytes were treated with collected supernatants for 1 h and cell shape (roundness) scored using image J. 1 represents a perfect circle while 0 a perfect line. Analysed using one-way ANOVA (Mann Whitney test) for non-parametric data (n=150 per replicate, with three biological replicates). For the graph, error bars shown are for s.d (**** $p < 0.00001$). Representative micrographs are shown.

4.8 Effect of the supernatant factor on other human cells

Hallmark symptoms of mucormycosis include severe angio-invasion, thrombosis and tissue death as already mentioned (Ghuman and Voelz, 2017, Ibrahim and Kontoyiannis, 2013, Ibrahim et al., 2012, Kontoyiannis and Lewis, 2006, Roden et al., 2005, Riley et al., 2016). Some of these manifestations demonstrate a pattern of affinity and association between the fungus and particular host components that perhaps facilitate pathogenesis of the disease. For instance, mucormycotic dissemination progresses via a strong association between infecting spores and endothelial cells and this is facilitated by the fungal surface CotH ligand that strongly binds the host GRP78 receptor (Gebremariam et al., 2014, Liu et al., 2015, Ibrahim, 2014, Gebremariam et al., 2016). This interaction allows adhesion of fungal spores to the host endothelial cells facilitating invasion by the fungus.

Also, recently, Ghuman *et al* (2018) showed that *M. circinelloides* spores induce platelet aggregation in whole human blood and platelet rich plasma (PRP) through interactions with surface receptors including the low affinity immunoglobulin G (IgG) receptor ($Fc_{\gamma}RIIA$) and the most abundant surface glycoprotein (platelet integrin $_{\alpha}IIb_{\beta}3$). Both of these are also heavily involved during platelet –bacterial interactions (Ghuman et al., 2018, Watson et al., 2016, Moriarty et al., 2016, Arman and Krauel, 2015, Hamzeh-Cognasse et al., 2015). Together, these interactions are thought to mediate platelet aggregation

Most importantly though, Ghuman *et al* also highlights the influence of spore developmental stages on platelet responses. They show that 3 h germinating spores significantly induced platelet aggregation more than dormant spores and germinated hypha (Ghuman et al., 2018). This difference in affinity for platelets suggests that metabolically active spores are capable of influencing platelet functions compared to when they are in a dormant state.

Therefore, given the important role that spore interaction with different cell types has on disease progression, the effects of spore metabolism on other human cells such as erythrocytes and neutrophils were also considered here.

4.8.1 Spore metabolism affects morphology but does not lyse erythrocytes

Emerging data suggest that spore metabolism can manipulate or create an imbalance in the host's hemodynamic during infection, and similar interactions with erythrocytes may not be an exception. Platelets, fibrin and erythrocytes are all-part of the clotting system (Janeway, 2005, Roitt and Delves, 2001). Intriguingly, there have been some preliminary reports from a different mucormycete research group that *R. oryzae* spore metabolic processes may promote erythrocyte lysis (Ibrahim et al., Personal communication). Thus, the effect of supernatant factors on erythrocytes, particularly lysis, was also investigated here.

Accordingly, erythrocytes were treated with the supernatant, co-incubated with both dormant or metabolically active spores; and haem release due to lysis determined microscopically and calorimetrically as described before (Sections 2.2, 2.3, 2.7 and 2.9).

Notably, all treatments caused significantly low levels of haemolysis ($p < 0.0001$) when compared with the positive control, indicating no lysis caused by the various indicated treatments (**Figure 36**). Interestingly, microscopically, treatment with both supernatant and co-incubation with metabolic spores caused a significant change in erythrocyte cell morphology from a smooth surface to a crenate phenotype (**Figure 36**).

This phenotype can be linked to hypertonic tension for the supernatant treatment but osmolality was not measured to rule this out. What is clear is that the supernatant did not lyse red cells and if we were to ignore osmotic pressure here, these findings show that spore effects may go beyond inhibiting only phagocyte functions to encompass other key physiological parameters during pathogenesis.

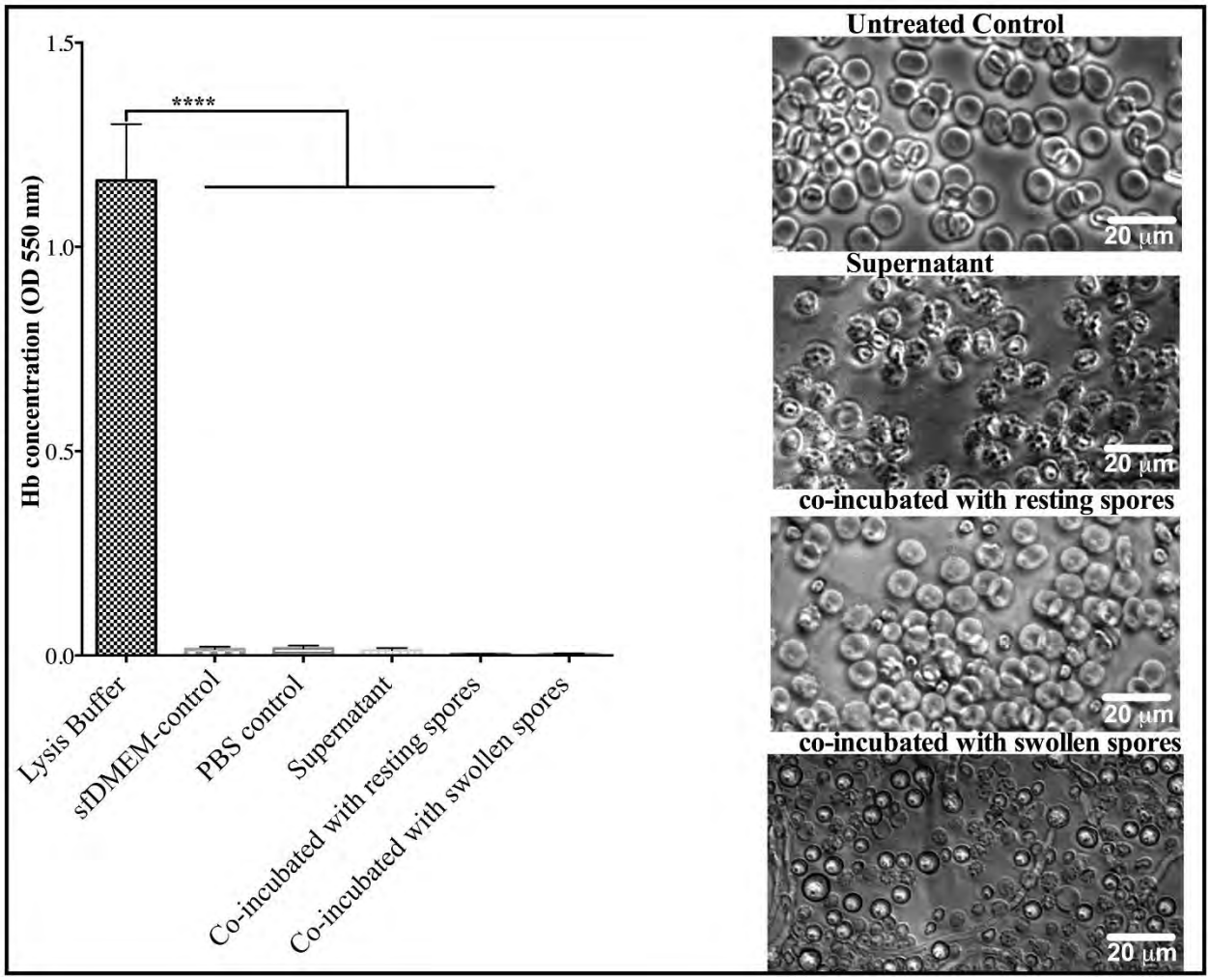


Figure 36: *R. microsporus* culture supernatant doesn't lyse but affects erythrocyte cell morphology.

Whole blood erythrocytes were washed in PBS and treated with collected supernatants, indicated controls, or co-incubated with spores as indicated. Co-cultures were assessed for cell lysis (Hg binding) calorimetrically or effect on cell morphology determined as represented by micrographs. For the graph, the data was analysed by ordinary One-way ANOVA (Brown Forsythe test) for non-parametric data. The data is from three biological repeats and errors bars represent s.e.m, where (***= ($p < 0.0001$)). Micrographs are representative.

4.8.2 Spore metabolism influences neutrophil migration

Neutrophils compose about 40-60% of peripheral blood cell count and are an integral part of the innate immune system (Summers et al., 2010, Miralda et al., 2017). Just like macrophages, neutrophils are primed prior to activation and their immunological response is dependent on the availability and exposure to a particular stimuli such as inflammatory cytokines, anaphylatoxins, chemokines or PAMPs (Miralda et al., 2017). However, when the interplay between all these is not well controlled, a diverse range of life-threating conditions including serious inflammatory responses may arise (Kannengiesser et al., 2008, van de Vijver et al., 2012, Miralda et al., 2017).

In regards to antifungal immunity, neutrophils also possess phagocytic attributes and are invaluable in controlling the damage of the hyphal form of filamentous fungi by the formation of neutrophils extracellular traps (NETs) (Brakhage et al., 2010). This subsequently limits dissemination and extent of damage by hyphae. Indeed, neutropenic patients are prone to not only bacteria but also filamentous fungi as already mentioned (Ibrahim et al., 2012, Kousha et al., 2011, Kontoyiannis and Lewis, 2006, Ibrahim and Voelz, 2017).

Currently, our understanding of neutrophil-mucormycete spore interactions is very limited. Given the evidence so far regarding the impact of spore metabolism on other human cell functions, spore metabolic influence on neutrophil function, particularly recruitment, was also considered and investigated here. The impact of supernatant factors on neutrophil migration was determined using an *in vitro* micro-slide migration assay, with appropriate gradient setups as described (Section 2.4 and 2.8). In this assay, cells loaded into a central well are analysed for attraction towards two gradients

As shown in (**Figure 37 and Table 10**), the ability of neutrophils to migrate between two chemotactic gradients was investigated using both sfRPMI and sfDMEM medium as negative and N-formyl-Methionyl-leucyl Phenylalanine (fmlp) as positive controls. In control

experiments, there was no significant difference in migration towards two sfRPMI only chambers, with mean accumulated distance of 70.1 μm, mean Euclidian distance of 8.3 μm and mean velocity of 1.17 μm/min (p = 0.154648) (**Figure 37a and 37b**); nor was there a difference in migration towards sfRPMI or sfDMEM gradients, with mean accumulated distance of 116.4 μm, mean Euclidian distance of 18.5 μm and mean velocity of 1.94 μm/min (p = 0.172552) (**Figure 37a and 37b),** although cells covered more distance towards sfDMEM than towards the sfRPMI gradient. Significant differences were noted with the positive control fmlp. Cells in the fmlp gradient demonstrated a mean accumulated distance of 445.4 μm, mean euclidian distance of 195.3 μm and mean velocity of 7.42 μm / min ($p < 0.05$) (**Figure 37a and 37b**);

When a spore supernatant gradient was examined, significant neutrophil migration was observed, with a mean accumulated distance of 415.8 μm, mean euclidian distance of 53.0 μm and mean velocity of 6.93 μm / min ($p < 0.05$) **(Figure 37a**). These data clearly show that the supernatant gradient significantly influences neutrophil migration, although not to same level as fmlp.

Interestingly, a combination of both supernatant and fmlp (**Figure 37a)** revealed reduced mean accumulated distance of 144.6 μm, mean euclidian distance of 34.05 μm and mean velocity of 2.41 μm / min ($p < 0.05$) almost similar with sfDMEM control gradient. Although statistically significant migration was observed, this may still indicate an inhibitory effect of supernatant on migration in the presence of fmlp. More studies are need to fully explore specific mechanisms of this interaction. Generally, we can to some extent conclude here that spore metabolism may influence neutrophil migration but remains to be fully explored by future studies.

Similar readings are seen with forward migration index (FMI), showing significant migration in the Y (FMI parallel) direction between supernatant and negative controls ($p<0.001$); between the supernatant and FMLP (positive control) ($p<0.00001$) and; a combination of supernatant + FMLP and FMLP (positive control) ($p<0.00001$) (**Figure 37b**). In the X (FMI perpendicular) direction, there is a difference in migration between supernatant and the negative controls ($p>0.05$). However, significant differences existed between supernatant and FMLP (positive control) ($p<0.0001$). Yet a significant migration in the X direction by the supernatant + FMLP combination was obtained as opposed to FMLP (positive control) ($p<0.01$) (**Figure 37b**)

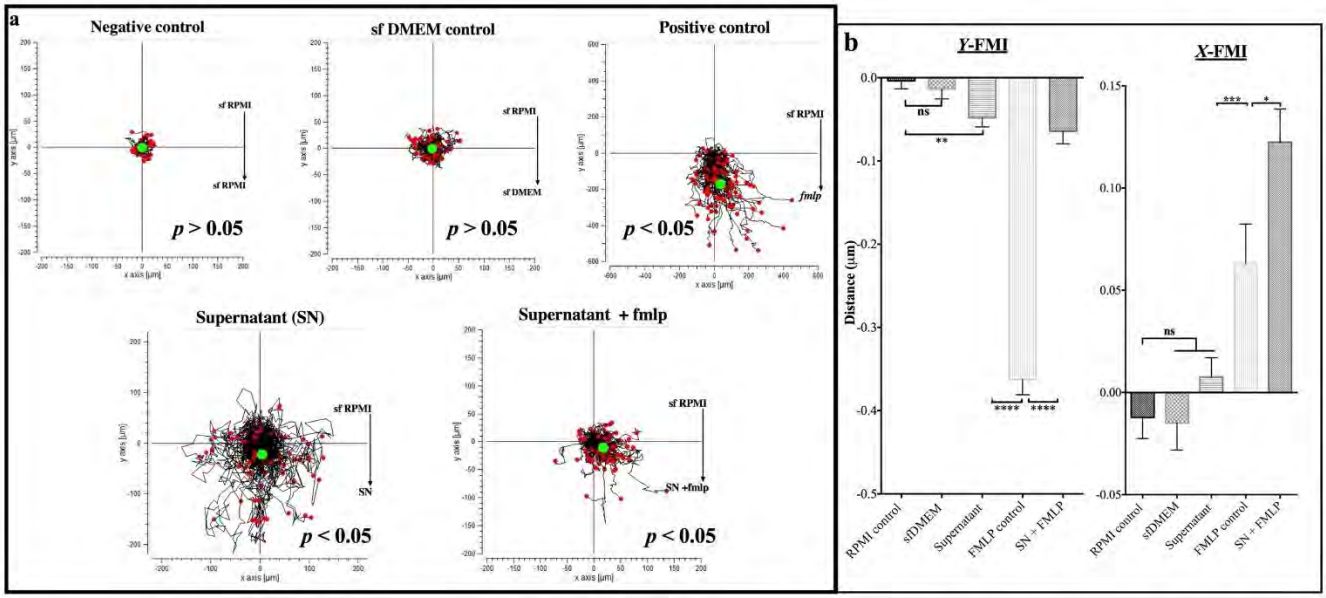


Figure 37: *R. microsporus* shows minimal effect on neutrophil migration.

Cell trajectory, using an Ibidi micro slide kit, **(a)** the ability of neutrophils to migrate towards supernatant and other indicated control gradients was determined. The gradients included; sfRPMI control, sfDMEM as a negative control, N-formyl-methionyl-leucyl-phenylalanine (FMLP) chemotactic peptide as a positive control, the supernatant and FMLP + supernatant test gradients. **(b)** Forward migration index (FMI) of cells (n=100) was scored and analysed using one way ANOVA (Mann Whitney test) for non-parametric data. The data presented here is from 1 biological repeat and error bars represent s.e.m. (ns=p>0.05, *=p<0.01, **=p<0.001, ***=p<0.0001, ****p<0.00001).

Table 10: *R. microsporus* shows minimal effect on neutrophil migration.

Sample ID	Mean accumulated distance (µm)	Mean Euclidean distance (µm)	Mean velocity (µm/min)	P value (using end points)
Negative control (sf RPMI)	70.1012	8.30383	1.16835	0.154648
Positive control (fmlp)	445.47	195.317	7.4245	1.2730e-34
Sf DMEM control	116.474	18.5146	1.94123	0.172552
Supernatant	415.834	53.019	6.93057	6.39179e-06
Supernatant + fmlp	144.662	34.0547	2.41104	8.1226e-12

4.9 Chemistry of the supernatant factor

My data thus far suggest that mucormycete spores metabolically secrete factors that facilitate evasion of innate antifungal immunity. Phenotypic characterization of the supernatant so far points to a range of bioactive attributes as highlighted previously. Interestingly, some of the attributes described, particularly cytotoxic effects, escape from phagocytosis and macrophage killing, reveal a pattern in mode of action similar to that demonstrated by some known metabolic compounds such as cytochalasins and rhizo-toxins. However, these observed effects could still be the result of a range of virulence factors including peptides, sugars, lipids or aromatic compounds that are yet to be identified and characterized (Londono-Hernandez et al., 2017, Abe et al., 2007).

In this regard, a phenotypic-comparison drawn between supernatant factors and some of the characterized compounds can mean one of three things here. **Firstly**, that the supernatant factors are the same in structure and function to some of those already characterized. **Secondly**, that supernatant factors are different in structure but similar in function. **Thirdly,** that supernatant factors are totally different in structure and perhaps function. Thus, to answer these questions we used known compounds as references and employed several approaches such as physical denaturation, chromatography, mass spectrometry and lipid profiling in an attempt to identify and characterize these metabolic factors produced by *R. microspores*.

4.9.1 Supernatant factor is a compound of small molecular size

The raw and crude collected supernatants from germinating spores can be a pool of secreted and shed bio-products of different molecular sizes and chemical conformation. Here we asked whether the causative compound is a small molecule (< 3kDa) or larger. To answer this question, supernatants were collected, respectively filtered through columns of size 100 kDa and 3 kDa by ultra-centrifugation and activity of both column fractions i.e. the flow-through and remaining solute, were determined as described before in (section 2.3 and 2.6). In both experiments an unfiltered supernatant control was also included.

As shown in (**Figure 38a**), for the 100 kDa column, both the unfiltered (mean uptake rate 54%) and flow-through (mean uptake rate 56%) supernatants demonstrated similar levels of activity against phagocytic uptake ($p > 0.05$) and were significantly different from the untreated control (mean uptake rate 75%) ($p < 0.001$). The activity of the remaining solute (mean uptake rate 63 %) was not statistically significantly different to the untreated control ($p = 0.3912$). This meant that most of the active compounds were able to pass through the column, suggesting the compounds in question are smaller than 100 kDa.

Upon further analysis, significant differences in activity levels were noted with the 3 kDa column fractions, where flow-through showed significantly higher activity against phagocytic uptake to about 40 % ($p < 0.0001$) than both the unfiltered supernatant (mean uptake rate 56%) and remaining solute (mean uptake rate 55%) ($p = 0.0452$) when compared to untreated control of (mean uptake rate 76%) (**Figure 38b**). This data suggests that the most active compound is less than 3 kDa in size, but active factors large than 3 kDa may also be secreted alongside, indicating that activity may not be orchestrated by a single compound.

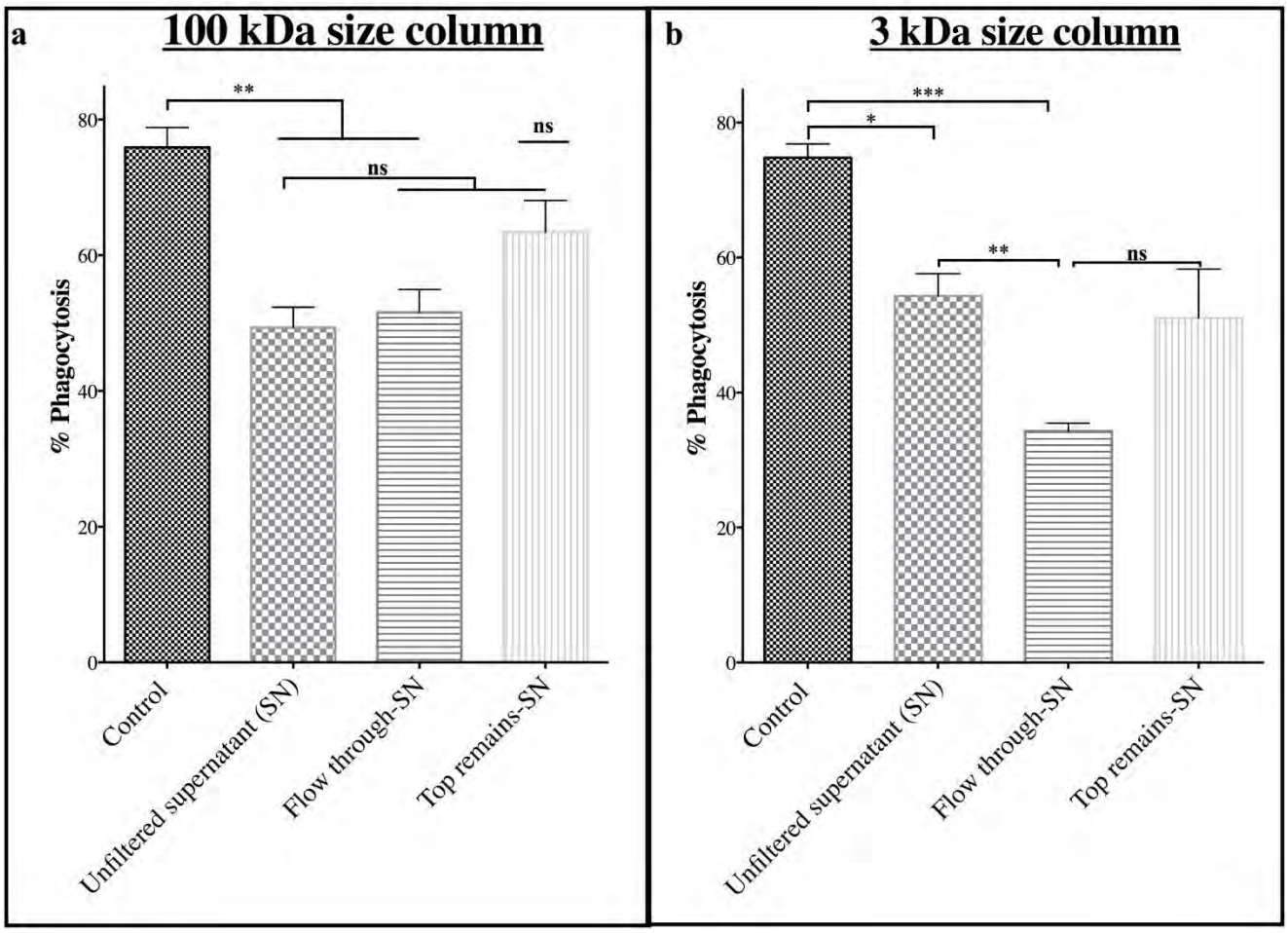


Figure 38: Effect of seclusion of molecular size on activity of the supernatant factor.

Collected supernatants were filtered through columns for (a) 100 kDa and (b) 3 kDa fractionation to collect both the column (Top remains) and filtered (Flow through) portions. The total unfiltered supernatant and column portions were then used to treat J774 phagocytes, which were co-incubated with *R. microsporus* dormant spores for 1 h and uptake rate assessed. For both assays, the number of phagocytes containing at least one spore were counted after 1 hour (n=9000, One way ANOVA with Tukey's correction for multiple comparisons). Three biological replicates were examined and error bars represent s.e.m. (*=$p < 0.01$, ** =$P<0.001$, ***$p=<0.0001$ and ns=$p>0.05$).

4.9.2 Supernatant activity withstands protein digestion, sugar oxidation or heat denaturation

We have already highlighted the possible candidates for the compounds in question including proteins such as hydrolytic enzymes and other peptides, sugars and lipids. To rule out if the active compound is any of these, the supernatant was digested, oxidized or profiled for lipids. Thus, the supernatant was physically denatured through boiling at 100 ºC for 1 h and treated with proteinase K (50 μg/ml) or sodium periodate (1mM/mL) for 60 min. Additionally, the supernatant was also analysed for lipids through a lipidomics spectrometry to establish a hydrophilic interaction liquid chromatography (HILIC) profile as highlighted in (Sections 2.4, 2.7 and 2.12).

The principle governing some of these approaches include; proteinase K being a serine protease with a broad range of cleavage specificity for peptide bonds while sodium periodate is an inorganic salt that oxidizes saccharide rings into aldehyde groups and was used here to denature compounds with such chemical conformation including carbohydrate sugars.

It was interesting to see that all treated supernatants still exhibited significant activity against phagocytosis. Percentage uptake significantly decreased from 80% by untreated cells to about 35% by cells treated with the unmodified supernatant, 40% by cells treated with boiled supernatant, 36% by cells treated with enzyme-digested supernatant and 42% by cells treated with oxidized supernatant ($p < 0.0001$) (**Figure 39**). Additionally, the differences in uptake noted between treatments with various supernatant types compared to unmodified supernatant were not statistically significant ($p = 0.2095$).

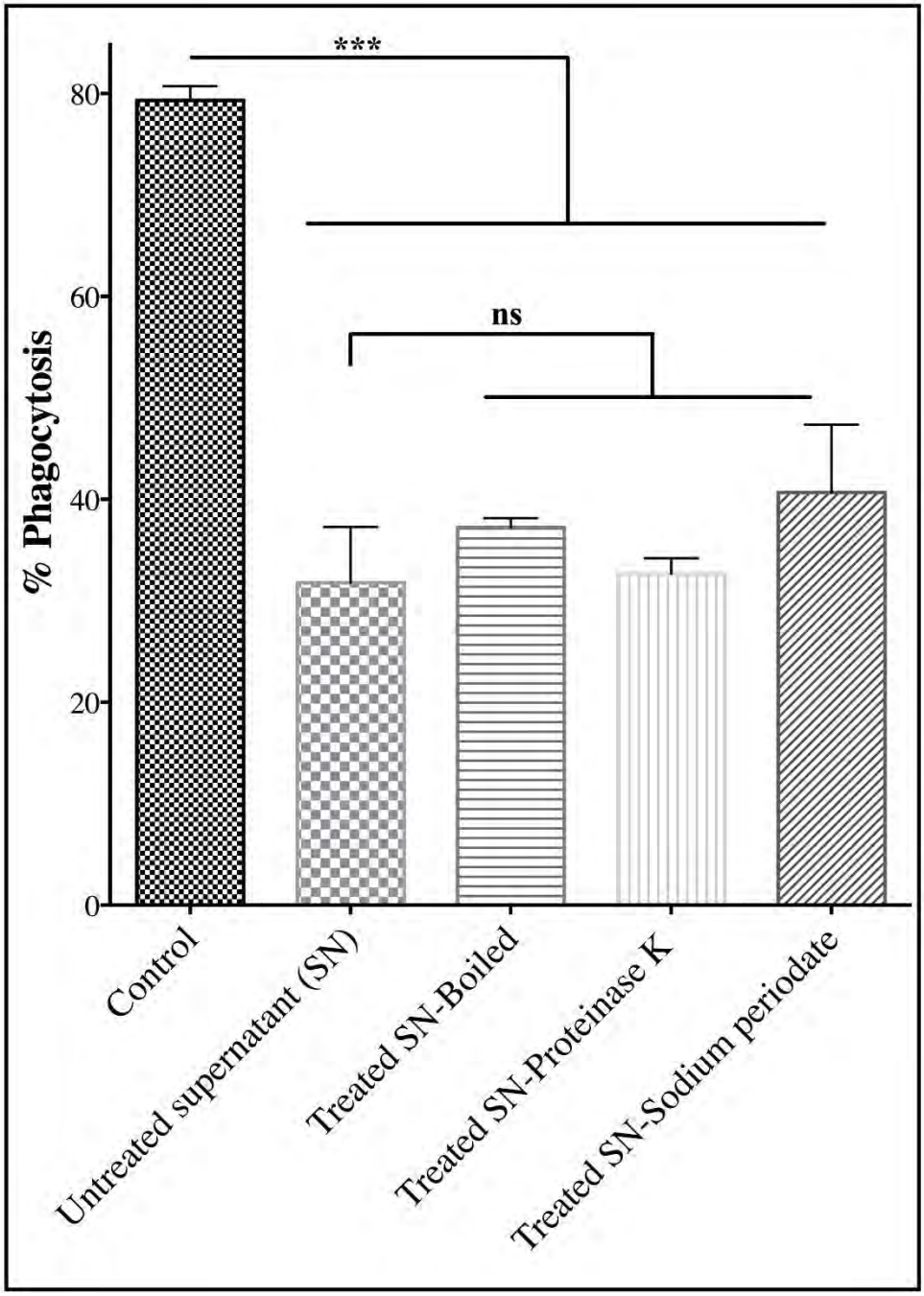


Figure 39: Protein digestion, oxidation and physical denaturation has no effect on the supernatant activity.

Collected supernatants were treated for protein denaturation with proteinase K digestion and boiling; and sugar oxidation with sodium periodate. Both the untreated and treated supernatants were then applied to J774 phagocytes, which were co-incubated with *R. microsporus* dormant spores for 1 h and uptake rate assessed. For all assays, the number of phagocytes containing at least one spore were counted after 1 hour (n=9000, One way ANOVA with Tukey's correction for multiple comparisons). Three biological replicates were examined and error bars represent s.e.m. (***p=<0.0001 and ns=p>0.05).

HILIC analysis revealed lipid candidate compounds including L-citramalyl-CoA a compound annotated in the KEGG database to be involved in a multitude of metabolic pathways; Phosphatidylinositol-3,4,5 trisphosphate (PIP_3), heavily involved in activation of downstream signalling components such as protein kinase that activate anabolic signalling pathways required for cell growth (Auger et al., 1989). In addition, a few other compounds that were HILIC positive are unidentified, making this finding inconclusive (**Table 11**). Taken together the findings here showed that the factor in question is unlikely to be a protein, carbohydrate but perhaps is a lipid or a secondary metabolite.

Table 11: HILIC profile of medium control and supernatant.

HILIC positive are the compounds that tested positive for lipids and verse vasa for the HILIC negative.

XCMS_ID	m/z	Matched molecular formula	Possible analytes	Lipid/HILIC profiles
M161T257	160.842065		Unidentified	HILIC negative
M377T466	377.085803	$C_{12}H_{22}O_{11}$	Trehalose, Turanose, Neotrehalose, mannobiose, Kojibiose and inulobiose	HILIC negative
M538T29	537.53525		Unidentified	HILIC positive
M269T410	269.08776	$C_{10}H_{14}N_4O_5$	Hydroxymelatonin, isoleucyl-thronine, Temurin, nananol and spermic acid	HILIC negative
M379T466	379.08267		Lactose, maltose, epimelibiose, Galabiose and Galactinol	HILIC negative
M300T473	299.91923	$C_3H_8NO_6P$	Phosphoserine	HILIC negative
M455T466	455.10214	$C_{16}H_{26}O_7$	Flavin mononucleotide, Epicatechin 3-0-(3-0-methygallate), 5-Hydroxyfenchone glucoside and Gericudranins B	HILIC negative
M405T466	405.10826	$C_{15}H_{20}O_{10}$	Edulane, N-Demethyleletriptan, Edulenol, Louisfieserone A, B; Gibberellin, 2,5-Fenchanediol 2-0-b-Dglucoside	HILIC negative
M898T307	898.14463	$C_{26}H_{42}N_7O_{20}P_3S$	L-Citramalyl-CoA	Lipids positive
M214T473	214.03339	$C_5H_9NO_4$	L-4-Hydroxyglutamine Semialdehyde; L-Glutamic acid, N-Acetyll serine; O-Acetyl serine	HILIC negative
M378T466	378.08923		Lactose, maltose, epimelibiose, Galabiose and Galactinol	HILIC negative
M236T35	236.14908		Unidentified	Lipids positive
M157T60	157.01419	$C_4H_8N_2O$	3-hydroxy-cis, cis-Muconic acid, Fumaryl acetic acid, Zymonic acid, Pentanesulfenothioic acid, Hypoxanthine, N-nitroso-pyrrolidine	HILIC negative
M187T412	187.07226	$C_{10}H_{14}O_2$	Agmatine, Valaraldehyde propyleneglycol acetal, Suspensolide methyl heptanoate, Perillic acid, Cyromazine, L-glycyl-L-hydroxyproline	HILIC negative
M248T81	248.00204	$C_5H_{11}NO_3S$	Methionine sulfoxide; "1,2,3,4-tetralydroisoquinoline"	HILIC negative
M214T53	213.89215		Unidentified	HILIC negative
M703T323	702.9380043	$C_{12}H_{24}O_{22}P_4$	Phosphatidylinositol-3,4,5 trisphosphate	HILIC positive

4.10 Chloroform extraction of the supernatant

Stemming from the above findings, we hypothesized that the factor in question is not a protein or carbohydrate but possibly a lipid or secondary metabolite. In other systems, compounds such as cytochalasins and rhizo-toxins (**Figure 40**) have been identified as having similar activity to supernatant factor here.

To determine whether the anti-phagocytic compound might be a secondary metabolite, the supernatant was subjected to secondary metabolite extraction methods. Specifically, a chloroform extraction was performed because the majority of secondary metabolites are alkaloids, which are readily soluble in organic solvents such as chloroform (Lucio et al., 2015, Tsuruo et al., 1986). Additionally, chloroform is denser than water with a low boiling temperature making it easier to extract and dry the aqueous supernatant under reduced pressure. Accordingly, the supernatant was exhaustively extracted in a volume ratio of 2:1 v/v (supernatant: chloroform), extract obtained under reduced pressure at 45º C (**Figure 40a**), reconstituted into dimethyl sulfoxide (DMSO) and subsequently into sfDMEM (final DMSO concentration of 1%), and activity determined as described in section 2.11.

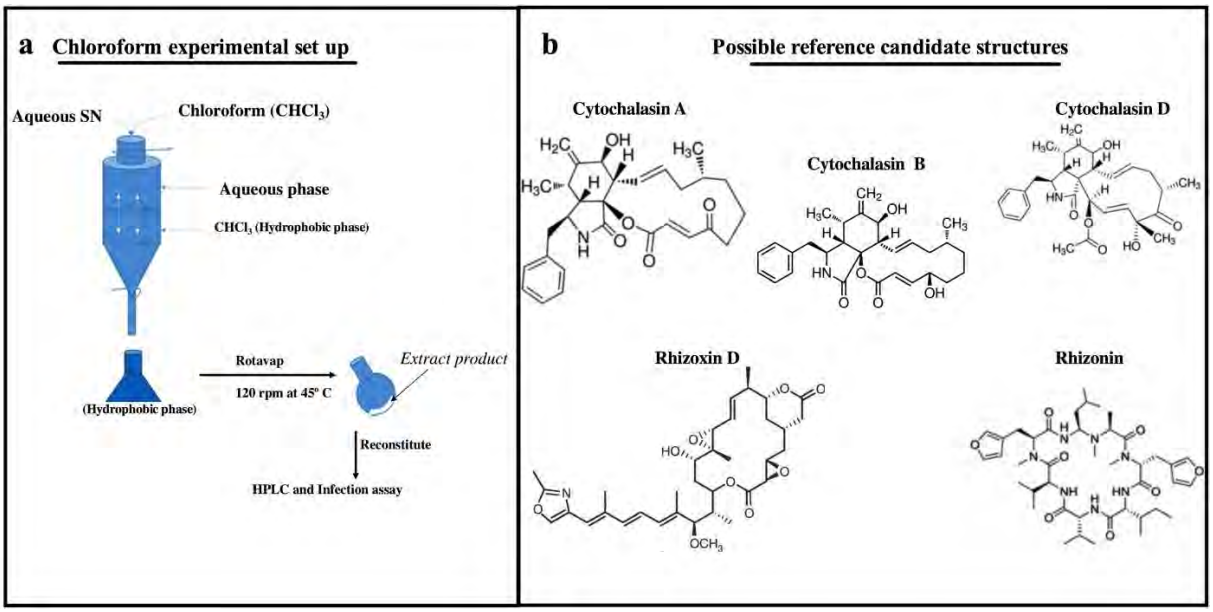


Figure 40: Chloroform extraction setup and reference compounds for the supernatant factors in question.

The collected supernatant was **(a)** exhaustively extracted with chloroform, dried under reduced pressure at 45 ^{o} C and extract reconstituted into DMSO (for HPLC) + SfDMEM (for the infection assay). Extractions were made from at least individual biological repeats. **(b)** Reference candidate compounds for the supernatant factor in question.

It was interesting to see significant differences in activity of both the unextracted and chloroform extracted supernatants. While the unextracted supernatant significantly decreased percentage uptake from 75% to 35% on average ($p < 0.0001$), the chloroform extract showed moderate activity, decreasing uptake from 70% to 50% on average. The difference in uptake in the extract was not statistically significant compared to unextracted ($p > 0.05$) (**Figure 41**). Because reconstitution of the extract products was initially made in DMSO then sfDMEM, we also sought to determine the effect of DMSO on phagocytic uptake. However, DMSO did not show any significant effect against uptake, with respective average uptake rates of 73% for unextracted sfDMEM + DMSO and 70% for extracted sfDMEM + DMSO (**Figure 41**).

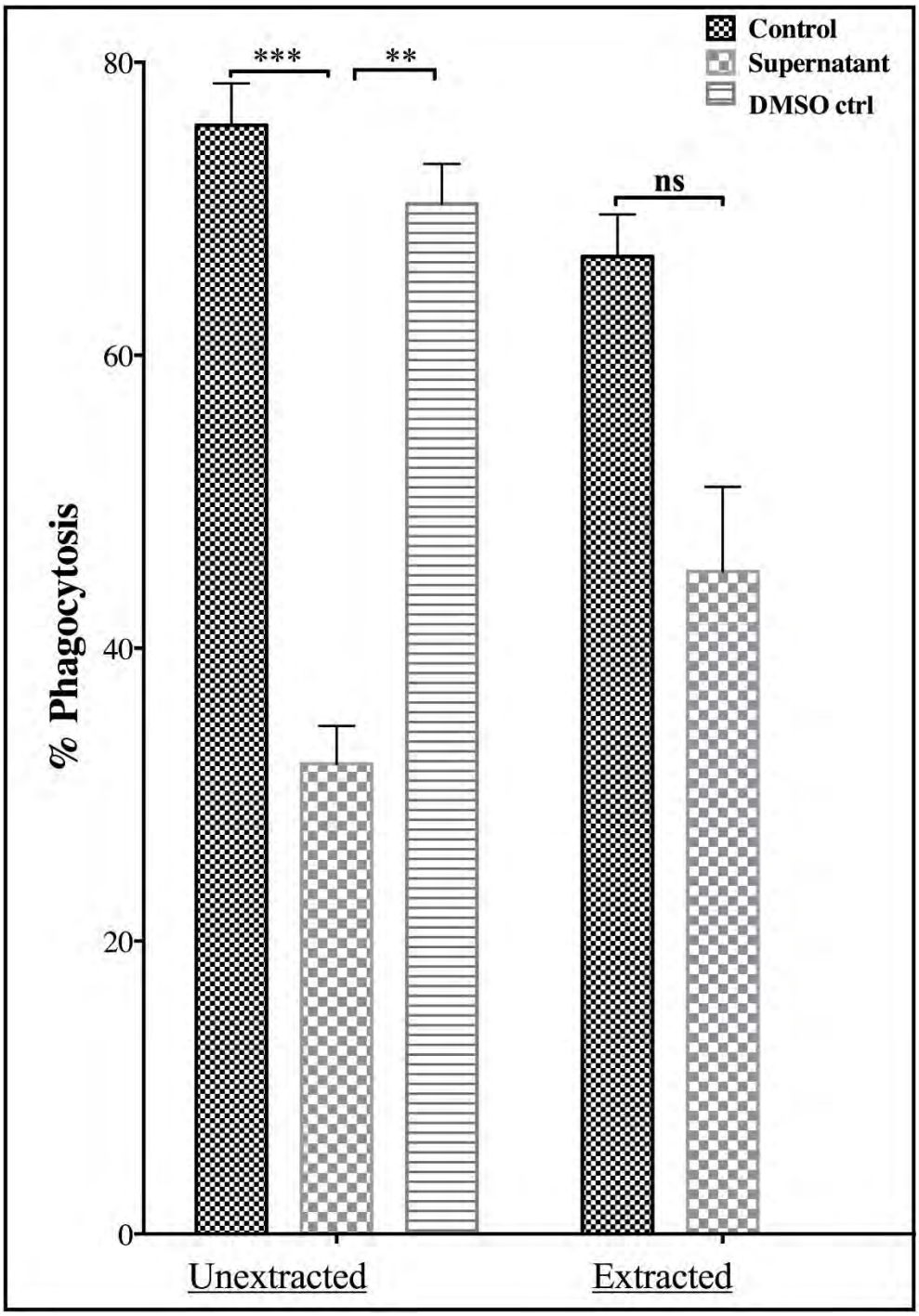


Figure 41: Chloroform extracted supernatant shows reduced anti-phagocytic effect on J774 macrophages.

The collected supernatant was exhaustively extracted with chloroform and reconstituted in DMSO and sfDMEM. Both the unextracted and extracted supernatants were then used to treat J774 phagocytes, which were co-incubated with *R. microsporus* dormant spores for 1 h and uptake rate assessed. For both assays, the number of phagocytes containing at least one spore were counted after 1 hour (n=9000, One way ANOVA with Tukey's correction for multiple comparisons). Three biological replicates were examined and error bars represent s.e.m. (**$p<0.001$, ***$p=<0.0001$ and ns=$p>0.05$).

However, the decline in activity of the extracted supernatant suggested that the extraction process might affect concentration, molecular conformation and interaction ability of the active compounds. We were unable to rule out the possibility that the extraction method destroys the compound of interest. However, the reduction in activity observed upon treatment with the extract supports the hypothesis that some active compound remains intact. To further investigate the identity of this compound. We sought to use more sensitive and specific techniques such as high-performance liquid chromatography (HPLC), mass spectrometry (MS) or nuclear magnetic resonance (NMR).

4.10.1 HPLC analysis reveals significant differences between extract products of supernatant and control

HPLC analysis is a common technique in analytical chemistry often employed to identify, quantify or separate a sample of mixed compounds under pressure/ (Carr et al., 2011, Vella et al., 2015, Horvath et al., 1967, Aturki et al., 2014). The technique is based on pumping a pressurized sample mixture through a column usually lined with granular adsorbent material such as silica or polymers in the presence of a buffer that supports binding. Separation of the sample mixture is dependent on how individual sample components interact with the column during elution with an increasing gradient of a second buffer (Vella et al., 2015, Sparkman, 2007).

The paradigm behind application of HPLC analysis here was based on the principle that polarity of the medium influenced individual molecular interactions (**Figure 42**). We draw three hypotheses for this; **First**, that in the hydrophilic phase molecules are far apart in charge and interacted freely with the column. **Second**, that following extraction the molecules tightly interact and that freedom is lost. **Third**, HPLC elution causes protonation of the molecules, changing molecular conformations, and subsequently frees molecules for interaction but other confounding factors such as synergy and antagonistic activity may not be ignored. Thus, HPLC

profiling here would quantitatively determine the relative abundance of the individual compounds in a mixed sample. With suspicion that the factor in question is an alkaloid, a reverse phase (RP) polarity-based chromatography was performed using the carbon chain (C18) column as described (Section 2.11).

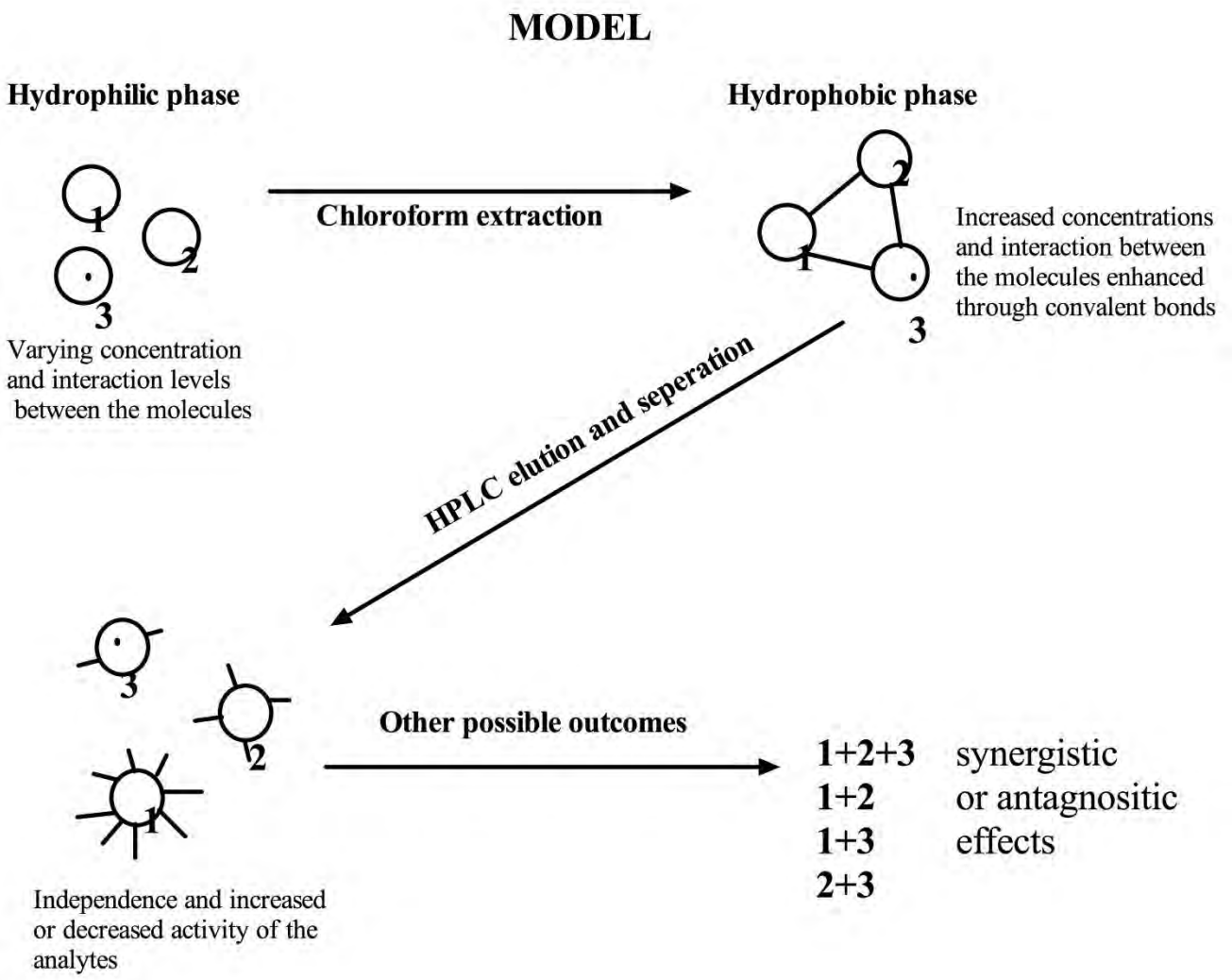


Figure 42: A paradigm for possible molecular interactions of a particular solute.

A paradigm demonstrating molecular interactions in both hydrophilic and hydrophobic phases extrapolated to explain activity of the obtained HPLC products.

HPLC profiling identified the peaks in the fungal supernatant that were absent in the media only control (**Figure 43**). When compared with the reference compounds rhizoxin D and cytochalasins (B and D), there were peaks with similar retention times in the supernatant profile (**Figure 43**). For instance, all major peaks in the reference compound controls come out late in the HPLC run i.e. between 34 - 42 min (**Figure 43**). This is comparable with 4 of the supernatant peaks (**Figure 43**). This can point to a similarity in molecular conformation such as polarity, and in this particular case would indicate that these compounds are mainly hydrophobic because they appear late in the HPLC profile.

To specifically identify the active compounds, the activity of individual HPLC analytes was investigated. Two approaches were employed here. **Firstly,** fractionation: 10-minute interval HPLC fractions were dried, and activity against phagocytic uptake determined (Section 2.11). In total, 6 fractions were collected (0-10 min (1), 10-20 min (2), 20-30 min (3), 30-40 min (4), 40-50 min (5) and 50-60 min (6)) (**Figure 44).** Interestingly, with the exception of fraction 1, all other fractions demonstrated significant activity against phagocytic uptake.

Uptake rate decreased from an average percentage uptake of 80 % of the control to 70 % by fraction 1 ($p > 0.05$); 60 % by fraction 2 ($p = 0.04987$); 55 % by fraction 3 ($p < 0.01$); 40 % by fraction 4 ($p < 0.0001$); 64 % by fraction 5 ($p < 0.05$); and 45 % by fraction 6 ($p < 0.001$) (**Figure 44**). Fraction 4 and 6 gave the largest reduction in uptake. We observed a broad distribution of activity in the supernatant fractions, with fraction 4 having the strongest effect. Therefore, in subsequent work we focus on fraction 4. When interpreting this data, we noted that some of the demonstrated activity may be due to a background impact by HPLC elution solvents such as acetonitrile (AcN), formic (FA) or trifluoro-acetic acid (TFA). However, to mitigate the possible impact of these contaminants on macrophage behaviour, the samples were thoroughly evaporated prior to re-suspension. When a media only control was passed through

the same workflow, no significant impact on phagocyte uptake rates were observed (**Figure 44**).

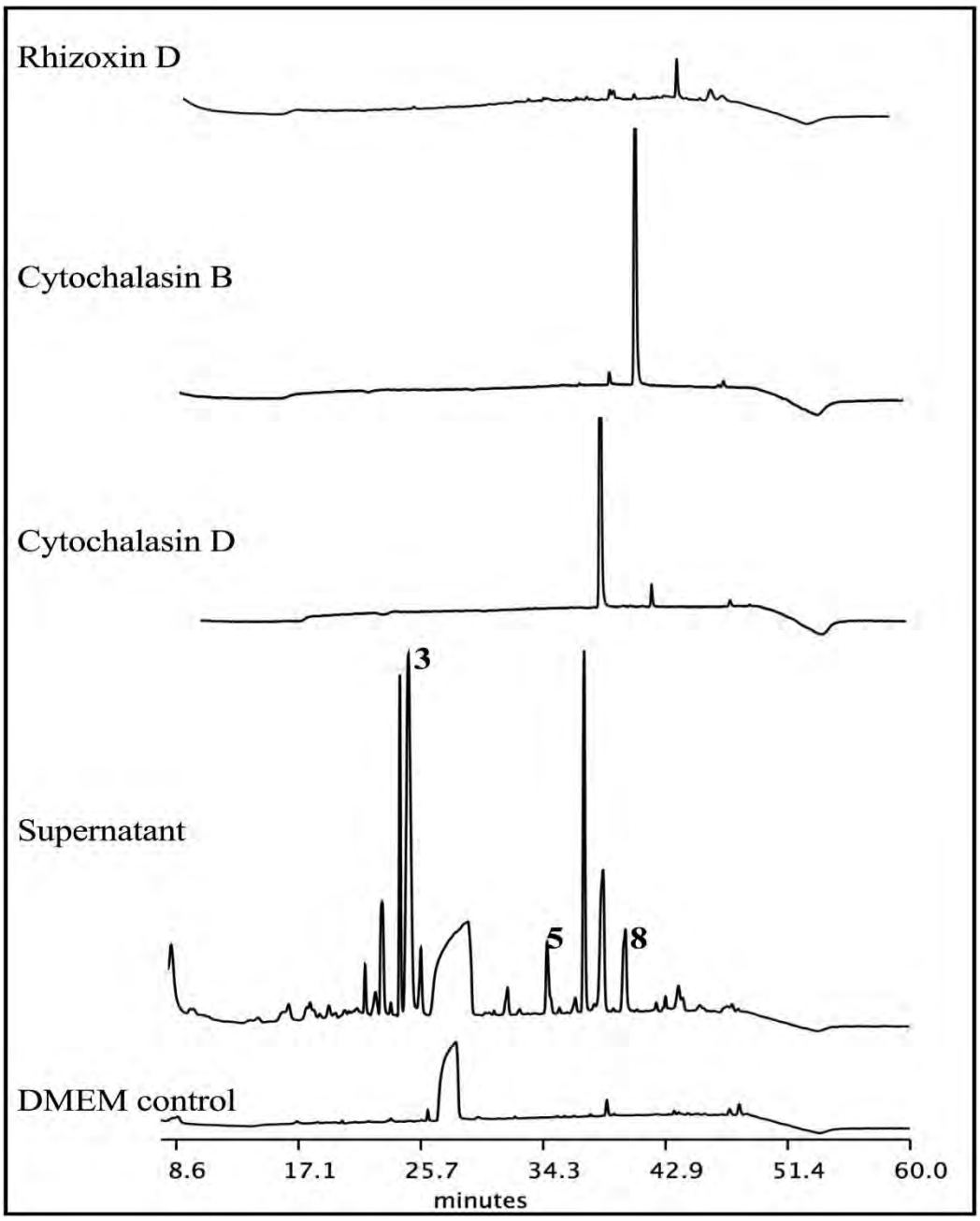


Figure 43: HPLC profiles of individual extracts and reference controls.

High performance liquid chromatography (HPLC) profile of extracted supernatant and sfDMEM control samples; and purified reference compounds including rhizoxin D, cytochalasin B and D. For the extracted samples, data are presented from at least three biological extraction repeats.

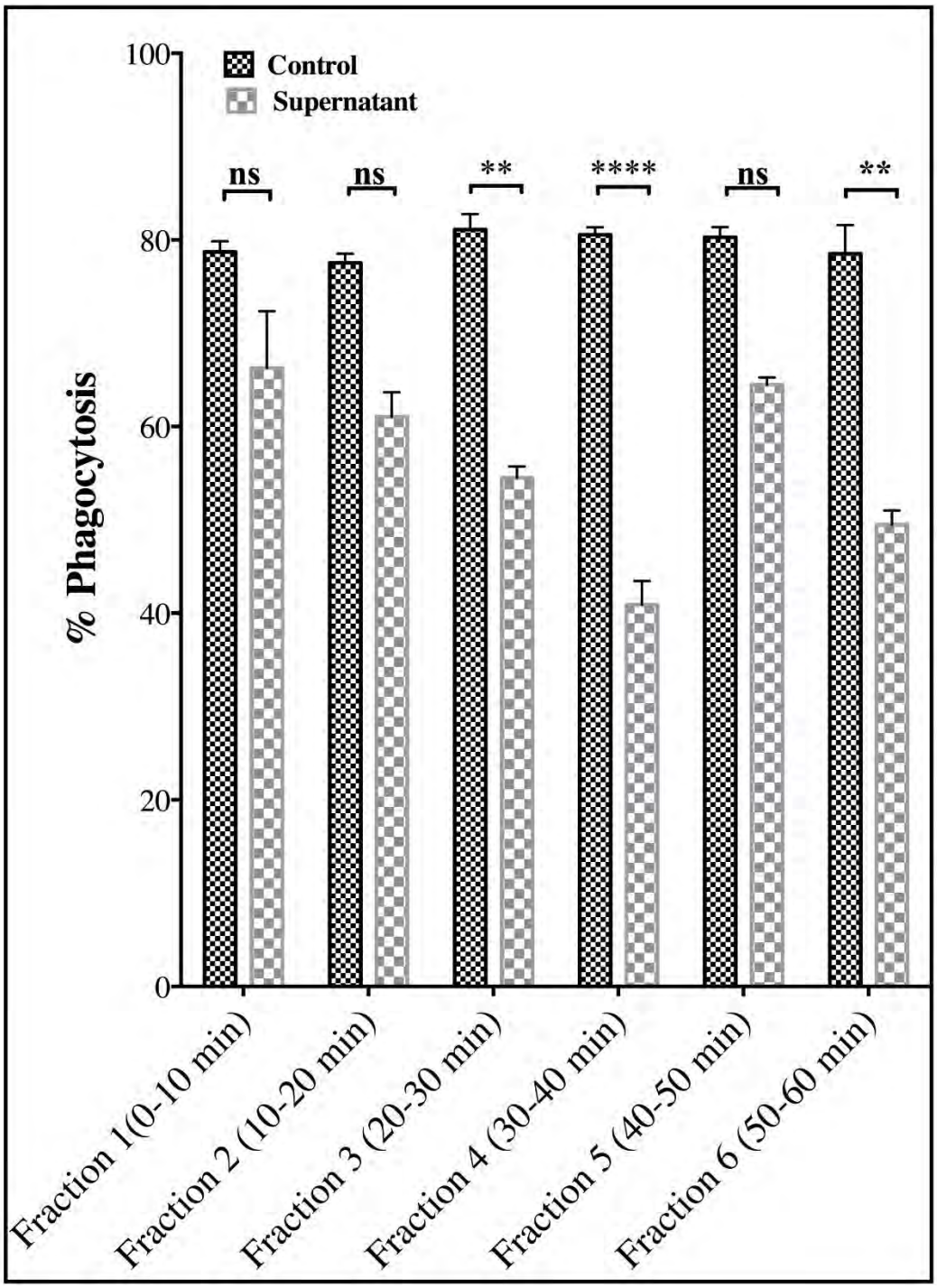


Figure 44: HPLC fractions of the *R. microsporus* culture supernatant exhibit anti-phagocytic activity.

HPLC products of both supernatant and control were fractionated as indicated, freeze dried and reconstituted in sfDMEM. The fractions were then used to treat J774 phagocytes, which were co-incubated with *R. microsporus* dormant spores for 1 h and uptake rate assessed. For all fraction assays, the number of phagocytes containing at least one spore were counted after 1 hour (n=9000, One way ANOVA with Tukey's correction for multiple comparisons). Three biological replicates were examined and error bars represent s.e.m. (**p<0.001, ****p=<0.00001 and ns=p>0.05).

Thus, the **second** approach was based on isolating individual HPLC peaks and determining their activity. This way, a total of 8 (1-8) peaks (**Figure 45a**) were collected from the pooled supernatant, dried and activity determined as described before (section 2.10). Interestingly, peaks 3, 5 and 8 demonstrated activity against phagocytosis from 80 % by the control to an average uptake of 60 % by peak 3 ($p < 0.05$); 40 % by peak 5 ($p < 0.0001$); and 50 % by peak 8 ($p < 0.001$) (**Figure 45b**), with peaks 5 and 8 giving the highest level of activity.-Both peaks 5 and 8 belonged to fraction 4 that had earlier shown the highest level of activity among the fractions, while peak 3 belonged to fraction 3 as shown in (**Figure 44**). Together, these data suggest that the active compound elutes in the 30-40-minute range and can be separated into at least two elution profiles (peaks 5 and 8).

With these data sets, we can conclude that the supernatant factor contains more than one active factor as depicted with peaks 3, 5 and 8, but most importantly, further characterization of these to specific compounds required more advanced characterization techniques such as Mass Spectrometry (MS).

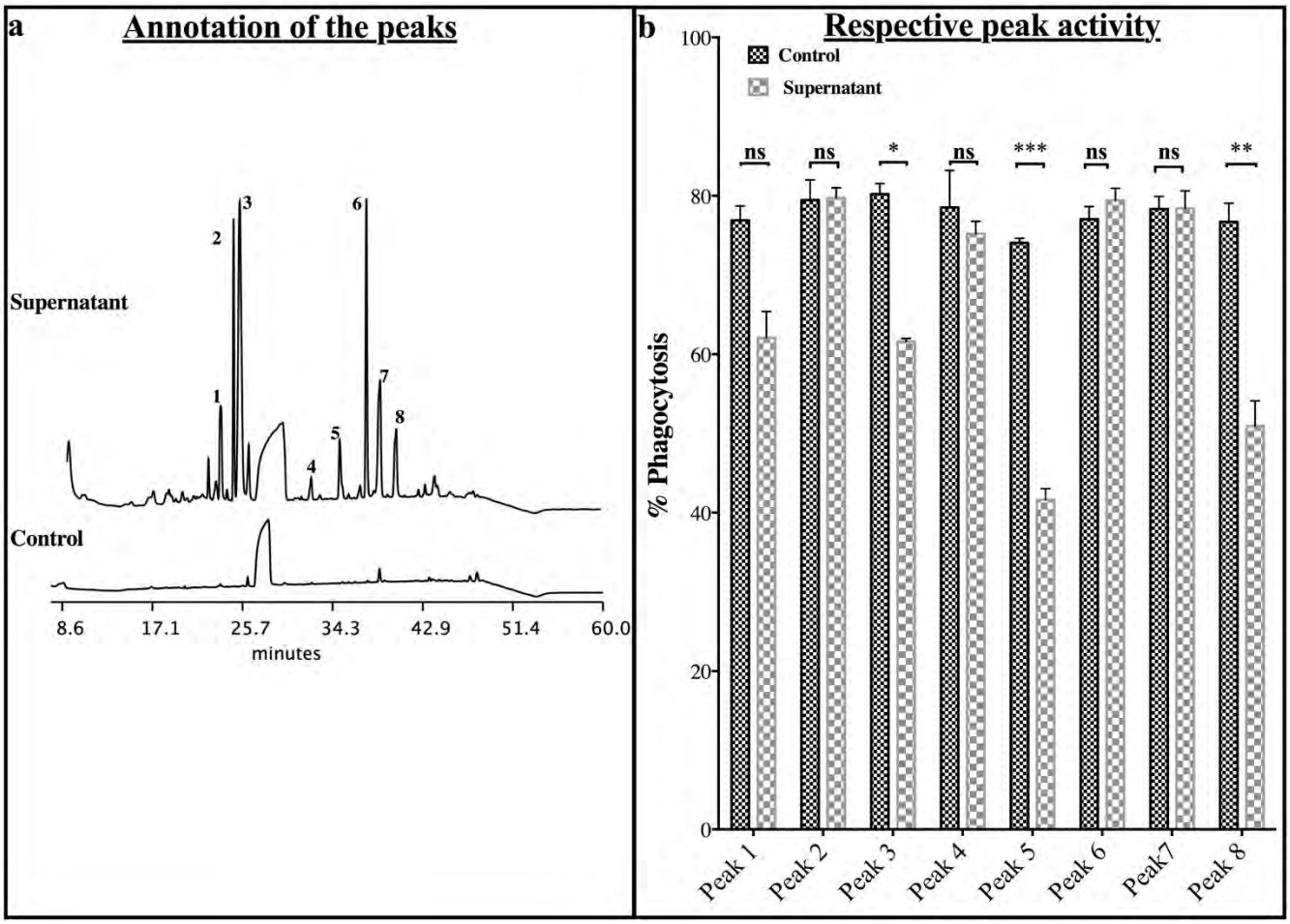


Figure 45: Individual supernatant HPLC peaks demonstrate similar anti-phagocytic activity.

Individual HPLC peaks (a) annotated were isolated, freeze dried and reconstituted in sfDMEM. (**b)** The peaks were then used to treat J774 phagocytes, which were co-incubated with *R. microsporus* dormant spores for 1 h and uptake rate assessed. For each peak, the number of phagocytes containing at least one spore were counted after 1 hour (n=9000, One way ANOVA with Tukey's correction for multiple comparisons). Three biological replicates were examined and error bars represent s.e.m. (*p<0.01, **p<0.001, ***p=<0.0001 and ns=p>0.05).

4.10.2 Mass spectrometry profiles of active HPLC peaks reveals masses consistent with rhizo-toxins

Mass spectrometry is an analytical technique often employed to characterize chemical species by mass to charge ratio (Sparkman, 2007). This way, a sample is ionized with electrons that cause the sample to break into smaller charged fragments. These can then be separated according to their charge to mass ratio when subjected to an electromagnetic field (Sparkman, 2007). Therefore, mass spectra are used to describe the chemical structure of molecules through elucidation of their isotropic signatures (Sparkman, 2007).

Using this method, some studies have already characterized rhizo-toxins and give mass ranges of between 300 and 812 *m/z* for rhizoxin and rhizonin derivatives (Scherlach et al., 2006, Partida-Martinez et al., 2007a, Partida-Martinez and Hertweck, 2007). Here, a similar approach was used to characterise the active peaks. Accordingly, peaks were isolated and high-resolution mass spectrometry characterization performed as described (Section 2.11).

As shown in **Figures 46-48**, peak 3 yielded masses of 144, 162, 163 and 194 *m/z* **(Figure 46);** peak 5: masses of 251, 319, 421, 698 and 700 *m/z* **(Figure 47);** and peak 8 yielded 130, 162, 203, 290 and 330 *m/z* **(Figure 48).** Whilst most of the peaks here are uncharacterised, special attention was given to masses 330 *m/z* (from peak8) (**Figure 48**), 698 *m/z* and 700 *m/z* (both from peak 5) (**Figure 47**). These masses are consistent with rhizonin and rhizoxin (**Figure 49**) (Scherlach et al., 2006, Partida-Martinez et al., 2007a). The questions at this stage would be whether these are also derivatives of rhizo-toxins or not. Nonetheless, if we are to assume that they are, we ought to recall that the rhizo-toxins are not secreted by the fungus but bacterial symbionts (Lackner et al., 2009b, Partida-Martinez et al., 2007a, Partida-Martinez et al., 2007b, Partida-Martinez and Hertweck, 2005) Yet bacterial symbionts are yet to be implicated in virulence of mucormycosis.

Peak 3 - mass spectra profile

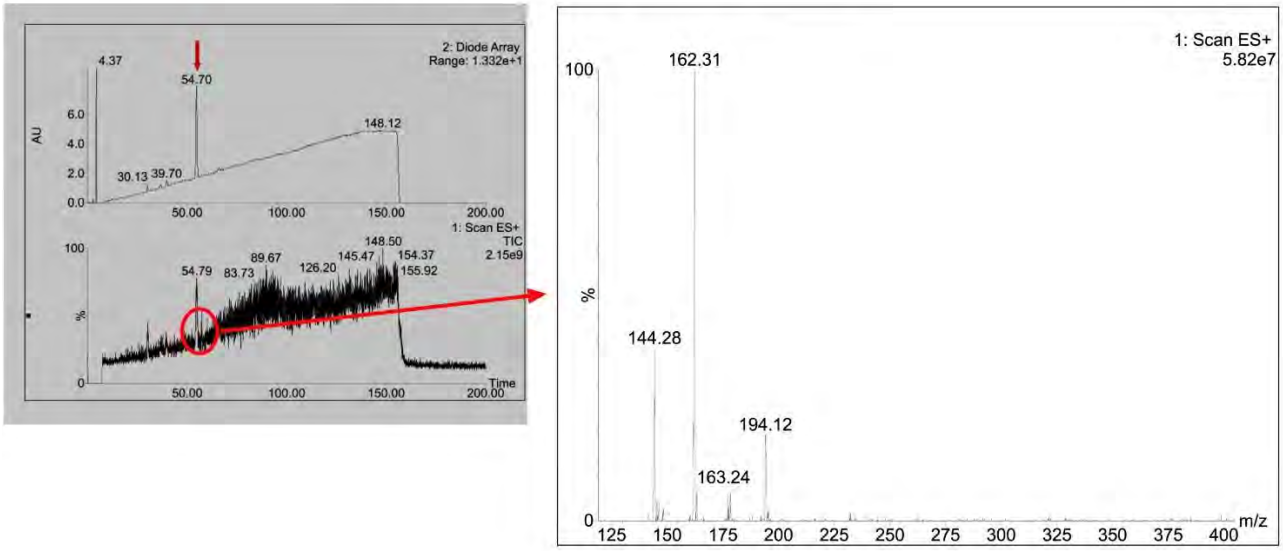


Figure 46: Mass spectrometry profile of HPLC peak 3.

Individual HPLC peak 3 was profile by high resolution mass spectrometry (HRMS). The highlighted region contains the peak of interest and subtraction of the background information reveals the masses that make peak 3. Presented data is from one of at least three biological repeats.

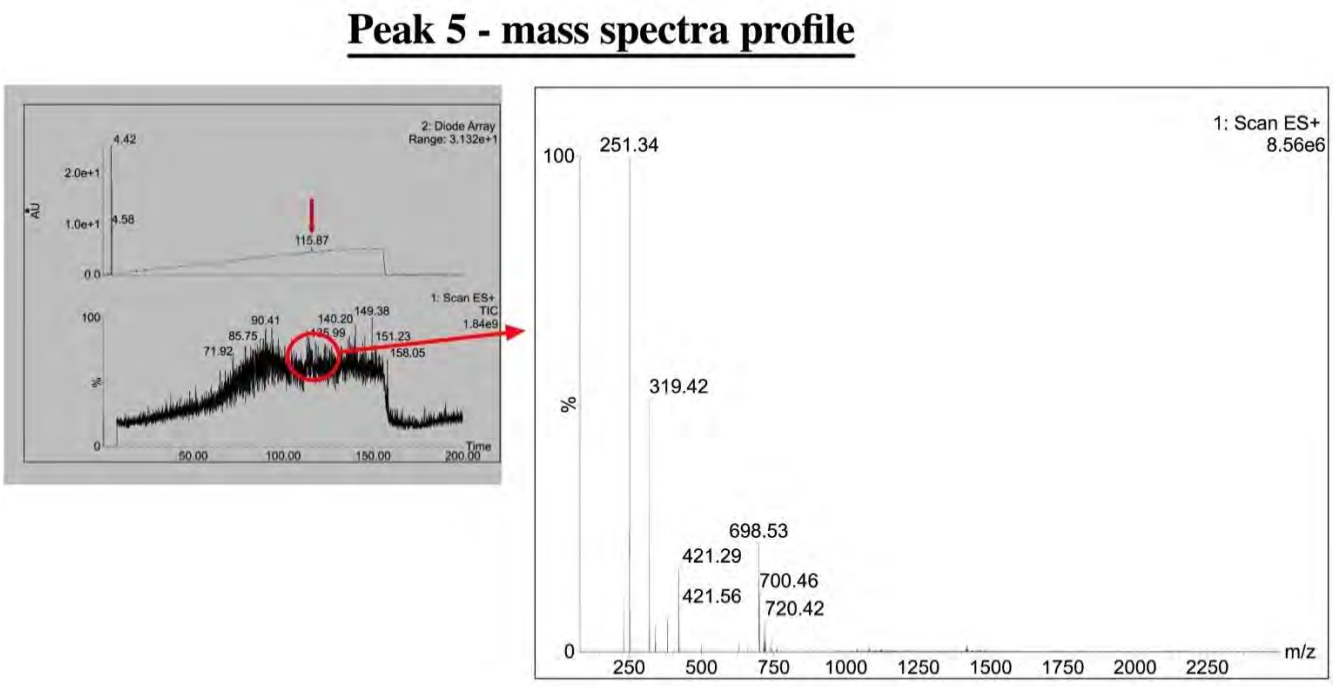


Figure 47: Mass spectrometry profile of HPLC peak 5.

Individual HPLC peak 5 was profile by high resolution mass spectrometry (HRMS). The highlighted region contains the peak of interest and subtraction of the background information reveals the masses that make peak 5. Presented data is from one of at least three biological repeats.

Peak 8 - mass spectra profile

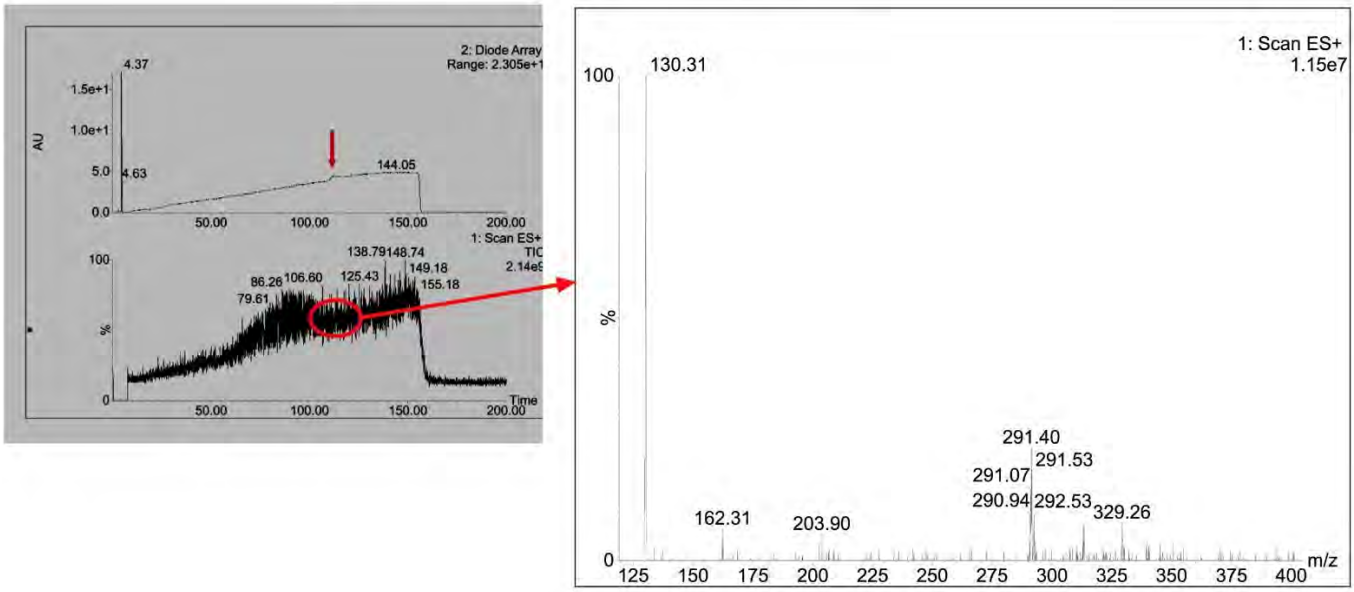


Figure 48: Mass spectrometry profiling of peak 8.

Individual HPLC peak 8 was profile by high resolution mass spectrometry (HRMS). The highlighted region contains the peak of interest and subtraction of the background information reveals the masses that make peak 8. Presented data is from one of at least three biological repeats.

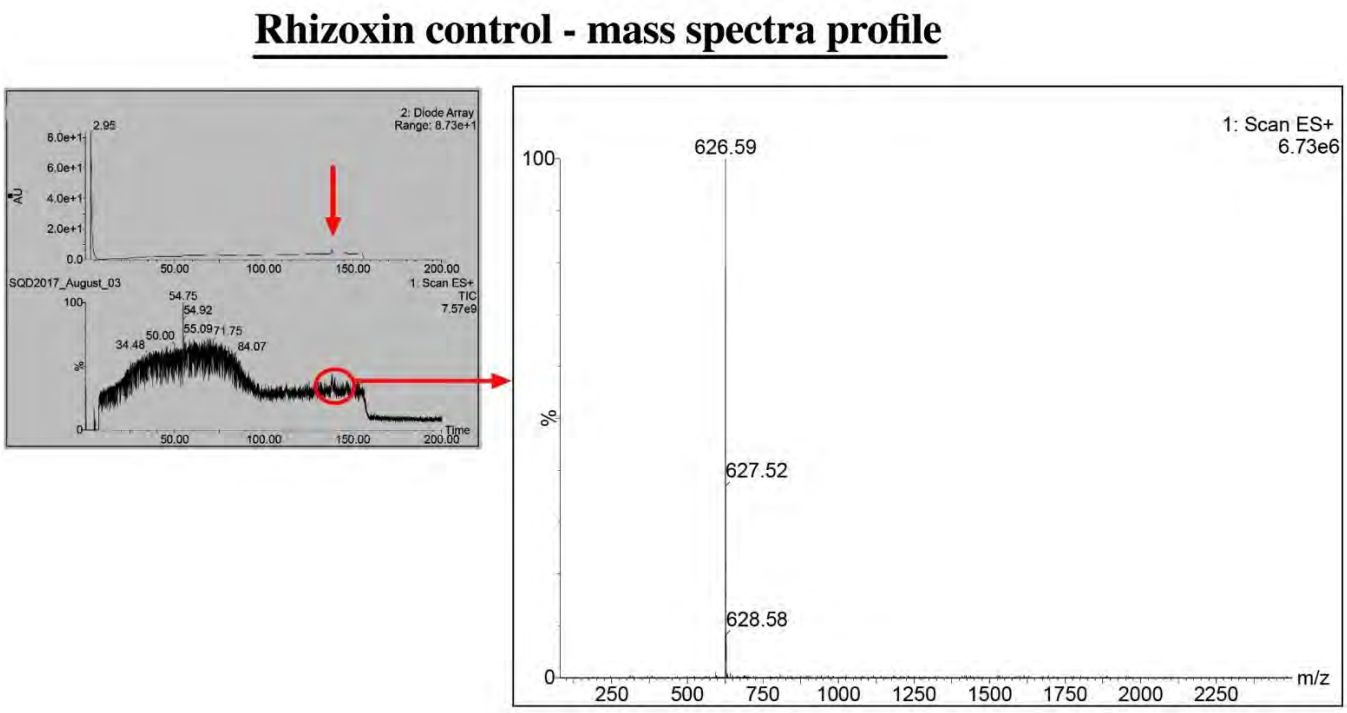


Figure 49: Mass spectrometry profile of Rhizoxin D.

High Resolution Mass Spectrometry profile of the rhizoxin D standard. The highlighted region contains the peak of interest and subtraction of the background information reveals the masses that make Rhizoxin D.

4.11 Discussion

Fungal metabolism is fundamental for successful evasion of host immunity. It is not uncommon for filamentous fungi to produce compounds such as mycotoxins and other metabolites in a diverse range of matrices. Depending on their biochemistry, these compounds can demonstrate a diverse range of effects on both plant and animal health. In fact, some of them are already implicated in food poisoning, water contamination and human disease pathogenesis (Oliveira et al., 2018).

Here, we report that mucormycete spore metabolism also induces expression of key secretory factors, with significant effects on host pathogen interactions particularly negatively impacting macrophage effector functions, although our understanding of the mechanisms involved is still very limited. In this regard, *R. microsporus* has recently been reported to be associated with both a macrocyclic polyketide phytotoxin metabolite, rhizoxin and a hepatotoxic cyclopeptide rhizonin (Partida-Martinez et al., 2007a, Partida-Martinez and Hertweck, 2005).

However, both are yet to be implicated in fungal pathogenicity and are produced by bacterial symbionts rather than the fungus (Lackner et al., 2011a, Araldi-Brondolo et al., 2017, Frey-Klett et al., 2011, Gee et al., 2011, Husnik and McCutcheon, 2018, Ibrahim et al., 2008b, Lackner et al., 2011b, Partida-Martinez et al., 2007a).

Interestingly, rhizoxin is associated with plant pathology and rhizonin hepatotoxic and agriculture produce contamination (Partida-Martinez et al., 2007a, Partida-Martinez and Hertweck, 2005). This raises the possibility that perhaps association with human pathogenesis is also possible, but has so far not been demonstrated.

The data presented here show that phenotypic (particularly cytotoxic) effects of spore metabolism on both murine and human cells are similar to those by rhizoxin and cytochalasin *in vitro*. Instigating the question of whether the supernatant factors in question here are similar in structure or function to either toxin types as already characterised by other studies (Qiao et al., 2011, Newman and Watt, 1988).

Yet, implicating the rhizo-toxins means implicating bacterial endosymbionts in host pathogenesis. However, this would not be the first-time; Chamilos et al (2007) had earlier alluded to possibly why the prevalence of mucormycosis has increased in recent years, suggesting that changes from use of antibiotics when managing opportunistic fungal infections then to now when they are particularly not being used as often (Chamilos et al., 2007). Suggesting that anti-bacterial usage then, perhaps even unknowingly was protective against targeted bacterial endosymbionts which in turn distabilised fungal pathogenicity. And as a result, mucormycosis was less prevalent as it is today.

Indeed, the role of bacterial endosymbionts in pathogenesis of mucormycosis is poorly characterized and somewhat ignored. So far, studies by Ibrahim *et al* (2008) and Partida-Martinez *et al* (2008) suggest that these bacteria may play no role in pathogenesis of mucormycosis (Ibrahim et al., 2008b, Partida-Martinez et al., 2008). However, both of these studies were not exhaustive enough and mainly focused on the late stages of the infection. Yet the significant damage may be caused early during colonization and infection, and our demonstration of the impact of metabolic process here could point to that.

From this stand point, we can conclude that spore metabolism plays a crucial role in evading innate anti-fungal immunity. We also suggest here that the evasion mechanism begins quite

early during infection. This is depicted through a demonstration of reduced phagocytic uptake, lack of macrophage killing and inhibition of phagosome maturation illustrated here.

On characterization of these metabolites, we note that the compound is not proteinus or a carbohydrate in nature but most likely a hydrophobic secondary metabolite or lipid. A chloroform extraction process of the supernatant gives a somewhat solid account to this. Further analysis reveals the active compounds' profiles to be consistent with the rhizo-toxins and cytochalasins at HPLC level; and with rhizoxin derivatives at mass spectrometry anlytical level. However, if we are to consider the similarities with rhizoxin we ought to contemplate the role of bacterial symbionts in the production of the active compound, excluding cytochalasin which is produced by *Aspergillus* species (Partida-Martinez and Hertweck, 2005, Scherlach et al., 2006, Partida-Martinez and Hertweck, 2007, Partida-Martinez et al., 2007b, Partida-Martinez et al., 2007a). Given the data here, it is seeming that the question of bacterial symbionts contributing towards pathogenicity of mucoralean fungi is becoming inevitable.

The data presented in this chapter demonstrates that pre-germinated mucormycete spores secrete a factor capable of inhibiting macrophage effector functions. We employed several assay tools and techniques including culture, fluorescence microscopy, phagocytosis, metabolomics and chemical extraction of the supernatant to describe the nature of the factor. However, most of the tools including HPLC, MS, metabolomics and fluorescence microscopy were qualitative rather than quantitative. Additionally, the effect of the supernatant factor was mainly assessed against macrophages and little attention was paid to other host parameters. The role of other cells such as neutrophils, red blood cells, other innate immune components such as complement can be a focus for future directions. More specialised and specific tools

such as NMR, MS/MS-LCS can also be considered for future directions to characterise this fungal factor.

CHAPTER FIVE: The Role of Bacterial Symbiosis in Pathogenesis of Mucormycosis

5.1 Chapter Overview

Some of the data presented in this chapter, was performed in collaboration with University of Aberdeen and University of California Los Angeles (USA). Specifically, Transmission Electron microscopy (TEM) work was performed with the help of Dr Elizabeth Ballou and Xin Zhou at the Aberdeen TEM facility. The mouse work was done in Prof Ashraf Ibrahim's Lab at UCLA by Teklegiorgis Gebremariam, Yiyou Gu and Lin Lin. Fish work was performed at the University Birmingham BSMU facility by myself with the help of Dr Kerstin Voelz, Dr Elizabeth Ballou, Mark Probert and Dr Joao Correia. Finally, bioinformatics work herewith was performed by myself but with technical support from Poppy Sephton Clark and Dr Elizabeth Ballou.

5.2 Background

Over time environmental and biological features of fungi have mainly been characterized through the study of associated abiotic factors such as pH or temperature. These, to some extent, have been extrapolated to explain fungal growth, gene expression and host-fungal pathogen interactions (Araldi-Brondolo et al., 2017, Husnik and McCutcheon, 2018). However, ecological modes of fungi are not only mediated by the environments where they thrive and their intrinsic features but also interactions with other microbial communities (Araldi-Brondolo et al., 2017).

In this regard, studies employing recent advancements such as whole genome sequencing highlight significant links between microbiome and microbiome networks which contribute towards disease pathogenesis. This is changing the way fungal phenotypes and hence associated diseases are perceived and studied (Witherden et al., 2017). For instance, study of the gut microbiome and mycobiome interactions seem to reveal an interplay between the two microbial communities and is now implicated in disease development such as chron's disease (CD) (Hoarau et al., 2016, Li et al., 2014, Sam et al., 2017, Hernandez-Santos and Klein, 2017,

Huseyin et al., 2017, Paterson et al., 2017, Botschuijver et al., 2017, Nash et al., 2017, Ackerman and Underhill, 2017, Leung et al., 2016, Husnik and McCutcheon, 2018).

Indeed, a definitive exploration of poly-microbial interactions including bacterial-fungal interfaces shows that bacteria can network with fungi at various levels, mediating shifts in substrate consumption, enzyme production, thermotolerance, sexuality, antifungal resistance, metabolism and virulence (Araldi-Brondolo et al., 2017, Salvioli et al., 2016, Shaffer et al., 2017, Moebius et al., 2014a, Mondo et al., 2017, Spraker et al., 2018, Hoffman et al., 2013, Lastovetsky et al., 2016, Nazir et al., 2014, Dixon and Hall, 2015, Harriott and Noverr, 2011, Hoffman and Arnold, 2010).

One way that fungi would interact with bacteria is by a complex mutualistic and heritable symbiotic relationship between fungal hosts and endo-hyphal bacterium. Recently, endo-hyphal bacteria have also been identified among mucoralean fungi, as already mentioned (Section 1.4.5.4). Although these are not yet linked with pathogenesis of human mucormycosis, their influence on sexuality, horizontal gene transfer, metabolism, antifungal resistance and plant pathology is highlighted (Moebius et al., 2014a, Mondo et al., 2017, Spraker et al., 2018, Spraker et al., 2016, Scherlach et al., 2006, Partida-Martinez et al., 2007c, Partida-Martinez and Hertweck, 2005, Partida-Martinez et al., 2007a, Mobius and Hertweck, 2009, Lackner et al., 2009b, Husnik and McCutcheon, 2018).

With this background, it is likely that some of these phenotypic shifts will also influence host-pathogen interactions. Studies by Lackner *et al* (2011), Mondo *et al* (2017), Murray *et al* (2017) and Spraker *et al* (2016) all attest to this possibility. For instance, Lackner *et al* (2011) show that endo-fungal bacteria including *B. rhizoxinica* are able to control fungal hosts such as *R. microsporus* through a hypersensitive response protein (*hrp*) type III secretion system, influencing sexuality and reproduction via toxinogenic derived alliances between bacteria and the fungus.

In context, taking *B. rhizoxinica* and *B. endofungorum* involvement in plant pathology and hepatotoxicity respectively, we realize that the impact of endosymbiont-driven modulations on manifestation of mucormycosis is not completely understood. Some studies, for instance Ibrahim *et al* (2008), showed no difference in the infection of endothelial cells by both parent and endosymbiont-free strains of *R. oryzae* in a DKA mouse model; while Partida-Martinez *et al* (2008) was unable to isolate endosymbiont bacterium from some clinical isolates of mucoralean fungi (Ibrahim et al., 2008b, Partida-Martinez et al., 2008).

However, one can make the argument that both of these studies were not exhaustive enough: Ibrahim *et al* employed an immunosuppressed mouse model whilst Partida-Martinez *et al* does not provide crucial information regarding anti-bacterial treatment history. Additionally, both studies focus on the latter events of pathogenesis, when possibly significant damage would have been done during early stages of infection, colonization or latency.

All taken together, it is important to recall that our findings here so far show a correlation between spore metabolism and immune evasion. Most importantly the data highlights that a bacterial endosymbiont could be the source of the active metabolites involved. However, if we are to implicate bacterial endosymbionts here, we ought to exhaustively establish what they are via specific characterisation and identification of the bacterium, associated metabolites and their modes of action. Thus, in this chapter we attempt to characterize the role of bacterial endosymbionts in mucormycete fungal pathogenicity, focusing mainly on the events that transpire during the early stages of infection.

5.3 Evidence of bacterial symbiosis among mucoralean fungi

In chapter 4, we showed the supernatant factors in question are similar to the rhizo-toxins which are secreted by bacterial endosymbionts. To rule out bacterial endosymbionts as producers here, screening for presence of the bacteria was investigated.

Previously studies by Ibrahim *et al* (2008), Nazir *et al* (20014), Moebius *et al* (2014), and Partida-Martinez and Hertweck (2005) have all demonstrated the presence of endosymbionts by using ciprofloxacin to cure the fungus of the bacteria and successfully confirming elimination of the bacteria by either localized screening with fluorescence microscopy, PCR for 16S rDNA or isolation of bacteria in pure culture (Moebius *et al*., 2014a, Mondo *et al*., 2017, Nazir *et al*., 2014, Partida-Martinez *et al*., 2007c, Partida-Martinez and Hertweck, 2005). Similarly, here bacterial elimination was performed on SDA plates containing ciprofloxacin. Spores were harvested, fermented in VK media for 2 to 4 days; and mycelia processed for microscopy, DNA isolated for PCR or bacteria isolated as described before (Sections 2.2, 2.4, 2.5 and 2.10).

5.3.1 Fluorescence microscopic screening reveals presence of endo-hyphal bacterial

For a fluorescence microscopic approach, LIVE/DEAD *Bac*klight staining kit, was used which is popular for demonstrating endo-hyphal bacteria (Partida-Martinez and Hertweck, 2005, Partida-Martinez *et al*., 2007c). The kit is made of two portions; A (a green fluorescing SYTO 9) and B (a red fluorescing propidium iodine (PI)). Both of these dyes are able to get through the fungal cell wall and specifically bind bacterial DNA (Partida-Martinez *et al*., 2007a, Partida-Martinez *et al*., 2007c). SYTO 9 will enter all cells while PI stains is limited to dead or damaged cells.

Notably, SYTO 9 staining revealed clusters of green stained bacterium along fungal filaments as demonstrated in **Figure 50a** for both the control and clinical strains of *R. microsporus*. Interestingly, treatment with ciprofloxacin abolished the staining signal **(Figure 50b)**, indicating elimination or absence of the bacteria. More importantly parent strains of other mucoralean fungi also demonstrated a staining signal for the bacterium as with *R. microsporus*

previously (**Figures 51-53**). These findings showed that indeed different mucoralean fungi may also harbour endo-hyphal bacterial.

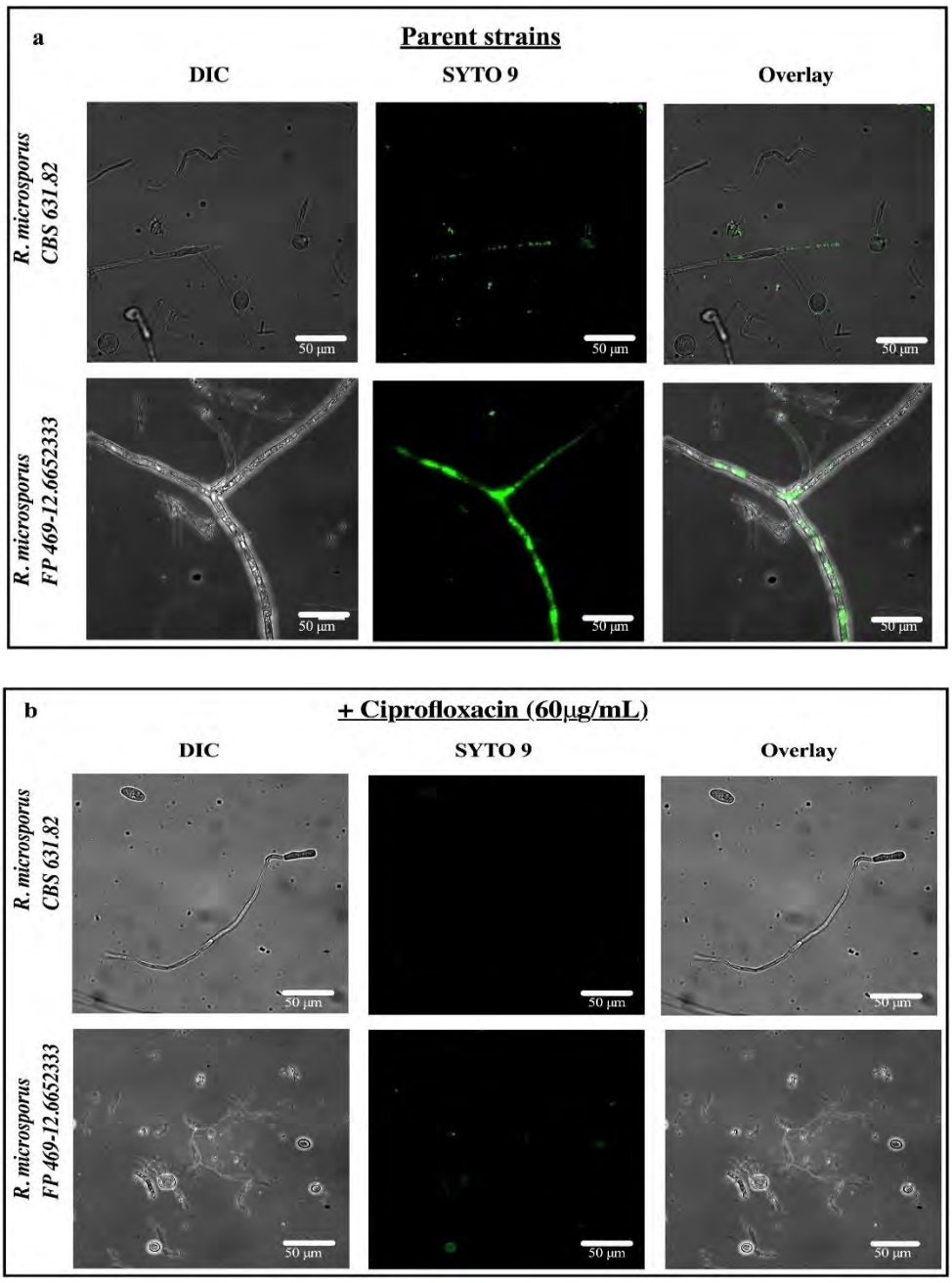


Figure 50: Endosymbiont bacteria can be located inside fungal hyphal mass with fluorescence microscopy.

Spores of (a) parent strains of a positive control (CBS-63182) and clinical isolate (FP469-12) of *R. microsporus* were fermented in VK, mycelia pellet submerged in NaCl, stained with SYTO9 and bacterial staining determined by fluorescence microscopy (**b**) The parent strains were then treated with ciprofloxacin (60 µg/mL) for a period of 8 weeks to eliminate the bacterial endosymbiont, spores collected and processed for staining with SYTO 9. Micrographs are representation from one of at least three biological repeats

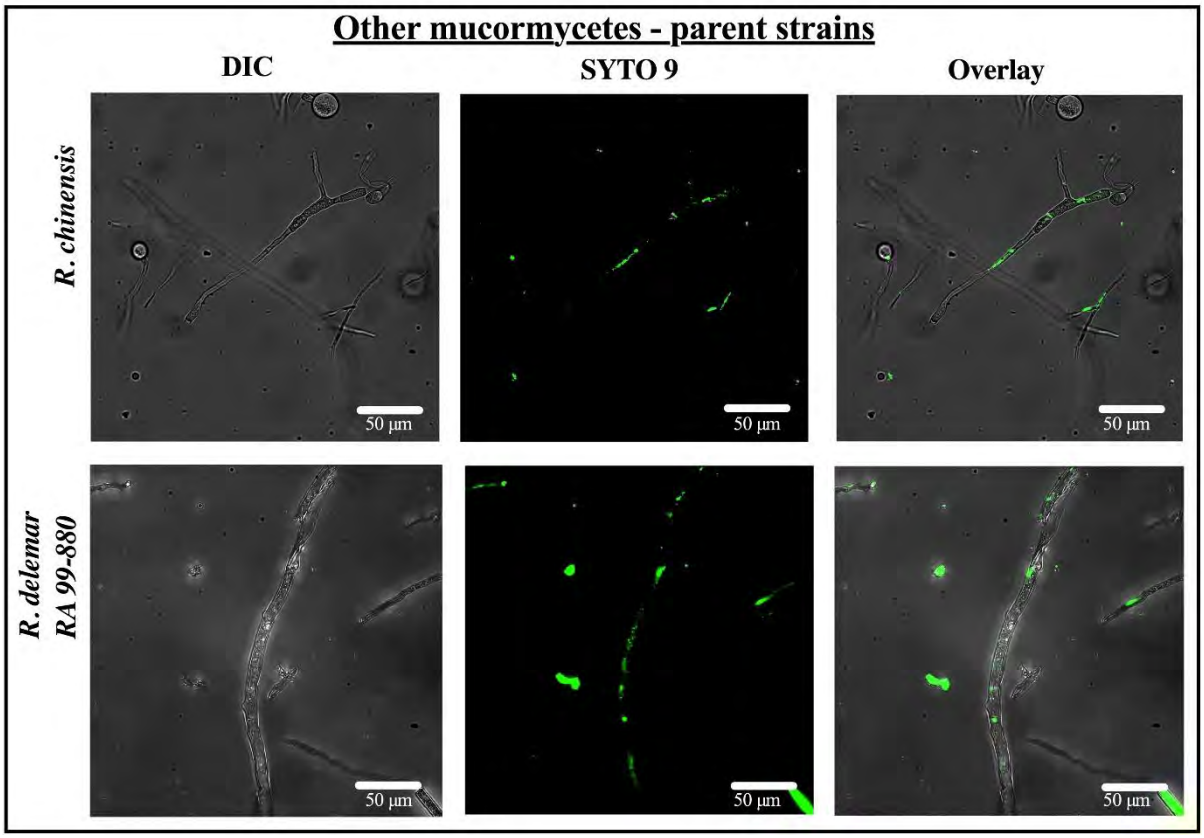


Figure 51: Endobactrerium located among other *Rhizopus* species

Collected spores of parent strains of other mucoralean fungi (*R. chinensis* and *R. delemar* RA-99-80) were fermented in VK, mycelia pellet submerged in NaCl and stained with SYTO9 and bacterial staining demonstrated by fluorescence microscopy. Representative micrographs from one of at least three staining biological repeats are shown.

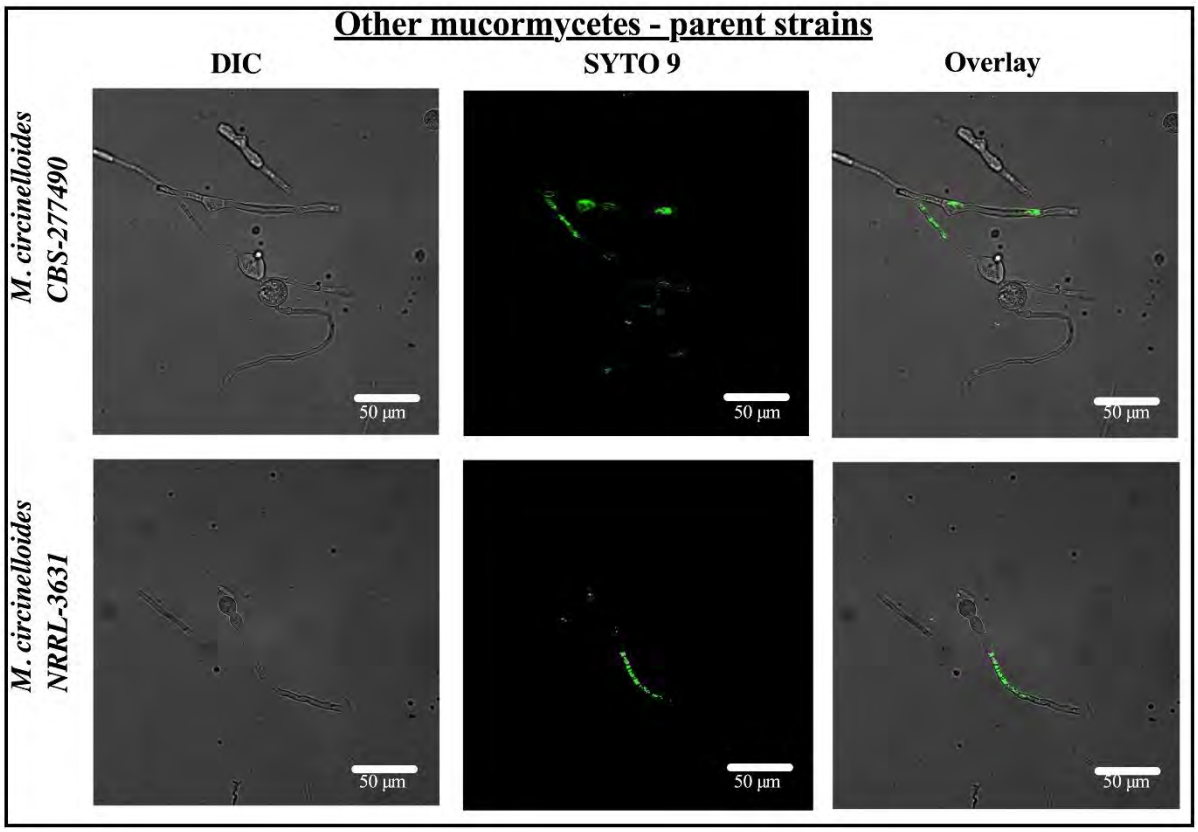


Figure 52: Endobacterium located among other mucoralean fungi specifically *Mucor* species.

Spores of parent strains of two other *M. circinelloides* isolates (NRRL-3631 and CBS-277490) were fermented in VK, mycelia pellet submerged in NaCl and stained with SYTO9 and bacterial staining demonstrated by fluorescence microscopy. Representative micrographs from one of at least three staining biological repeats are shown.

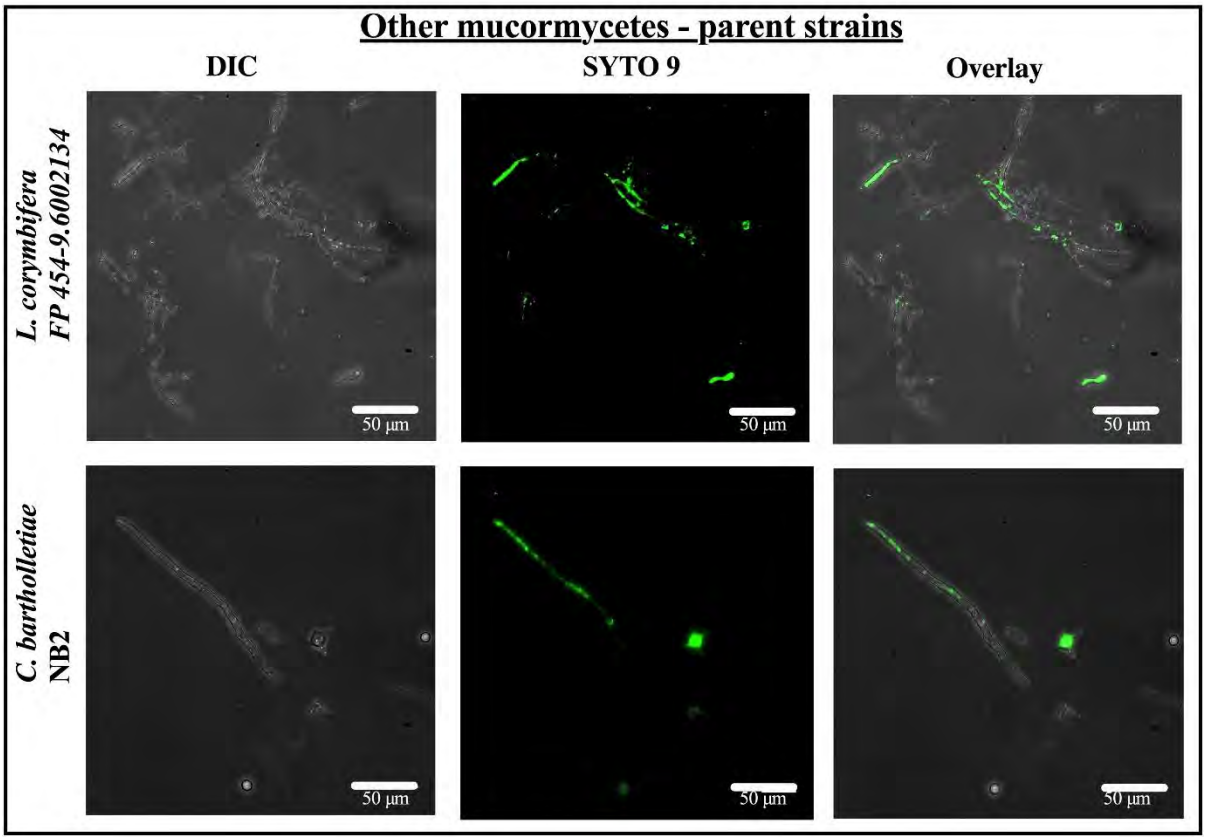


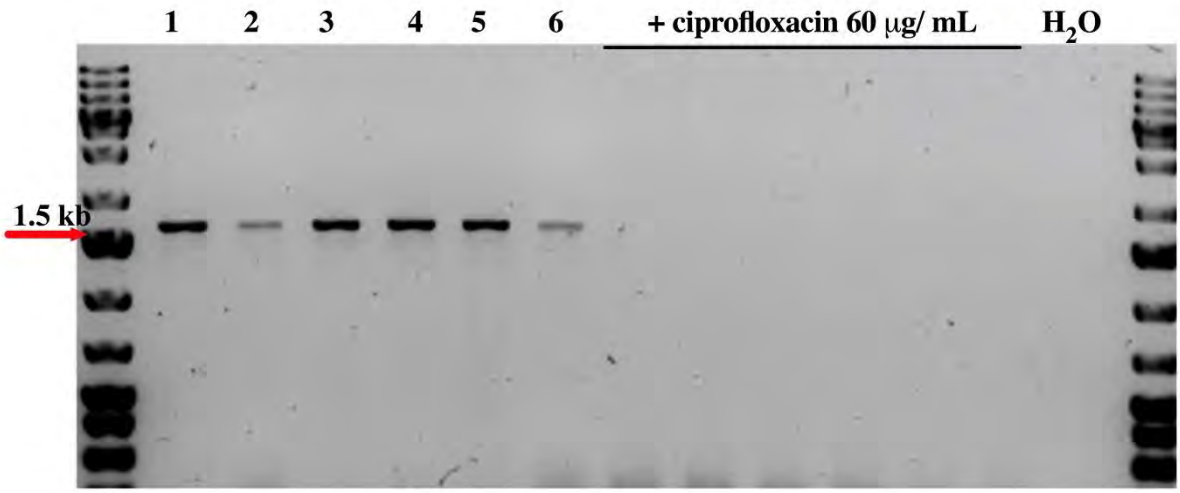
Figure 53: Endobacterium located among other mucoralean fungi specifically *Cunninghamella* and *Lichtheimia*

Spores of parent strains of other mucoralean fungi, *L. corymbifera* (FP454-9.6) and *C. bertholletiae* (NB2), were fermented in VK, mycelia pellet submerged in NaCl and stained with SYTO9 and bacterial staining demonstrated by fluorescence microscopy. Representative micrographs from one of at least three staining biological repeats are shown.

5.3.2 PCR screening reveals a band specific for bacterial 16S rDNA

The 16S rDNA sequence is a well conserved DNA region in bacterial species that can be used for identification of bacteria and is absent in fungal nuclear genomes. The equivalent in fungi is 18S rDNA. Therefore, using universal PCR primers, a band was amplified among DNA isolates from fermented mycelia for both parent and cured strains as described in previous studies (Partida-Martinez et al., 2007a, Partida-Martinez et al., 2008, Ibrahim et al., 2008b).

As shown in **Figure 54**, a 1.5 kb band specific for the bacterial 16S rDNA was amplified from parent but not ciprofloxacin treated (cured) strains. Showing that bacterial 16s rDNA is present among mucoralean fungi and can be eliminated with ciprofloxacin treatment further agreeing with the above shown microscopic observation. The application of ciprofloxacin here was based on reports by Sugar et al, who reported that this antibiotic had no intrinsic effects on fungus (Sugar and Liu, 2000, Sugar et al., 1997, Chamilos et al., 2007). Other antibiotics such as penicillin, streptomycin and gentamycin were also tested but proved ineffective against the endosymbiont.



1. *R. microsporus* (CBS 631.82)
2. *R. microsporus* (FP 469-12.6652333)
3. *M. circinelloides* (CBS 277940)
4. *M. circinelloides* (NRRL 3631)
5. *L. corymbifera* (FP 454-9.6002134)
6. *C. bertholletiae* (NB2)

Figure 54: PCR screening reveals a band consistent with bacterial 16S rDNA among mucoralean fungi.

Genomic DNA was isolated from both parent and cured strains of the indicated mucoralean fungi and screened by PCR for the amplification of a 1.5 kb band indicative of bacterial 16S rDNA. The image is representative of gel electrophoresis of PCR products from one of at least three different biological extraction repeats.

5.3.3 Transmission electron microscopy reveals the influence of the endobacteria on fungal cell wall organization

Partida-Martinez et al (2007) has unequivocally proven that *Burkholderia* bacterial species are true endosymbionts residing in the fungal cytosol, where they can be distinguished from the fungal organelles. Using a similar approach, we attempted to demonstrate the presence or absence of the bacteria in swollen spores of both parent and cured strains of *R. microsporus.*

Accordingly, the spores were harvested and metabolically activated for 4 has described before (section 2.3). Cells were then fixed by High Pressure Freezing and sectioned for Transmission Electron Microscopy (section 2.9)

As shown in **Figure 55**, TEM micrographs showed patches of low electron density, similar to those described by Partida-Martinez et al (2007) that are distinct from the fungal organelles. However, the more pronounced finding we noted with this experiment was the changes in the cell wall organisation between the parent and cured strains of *R. microsporus*. These findings point to influence of the endo-bacteria on cell wall organization but calls for more work in order to improve our understanding of these effects.

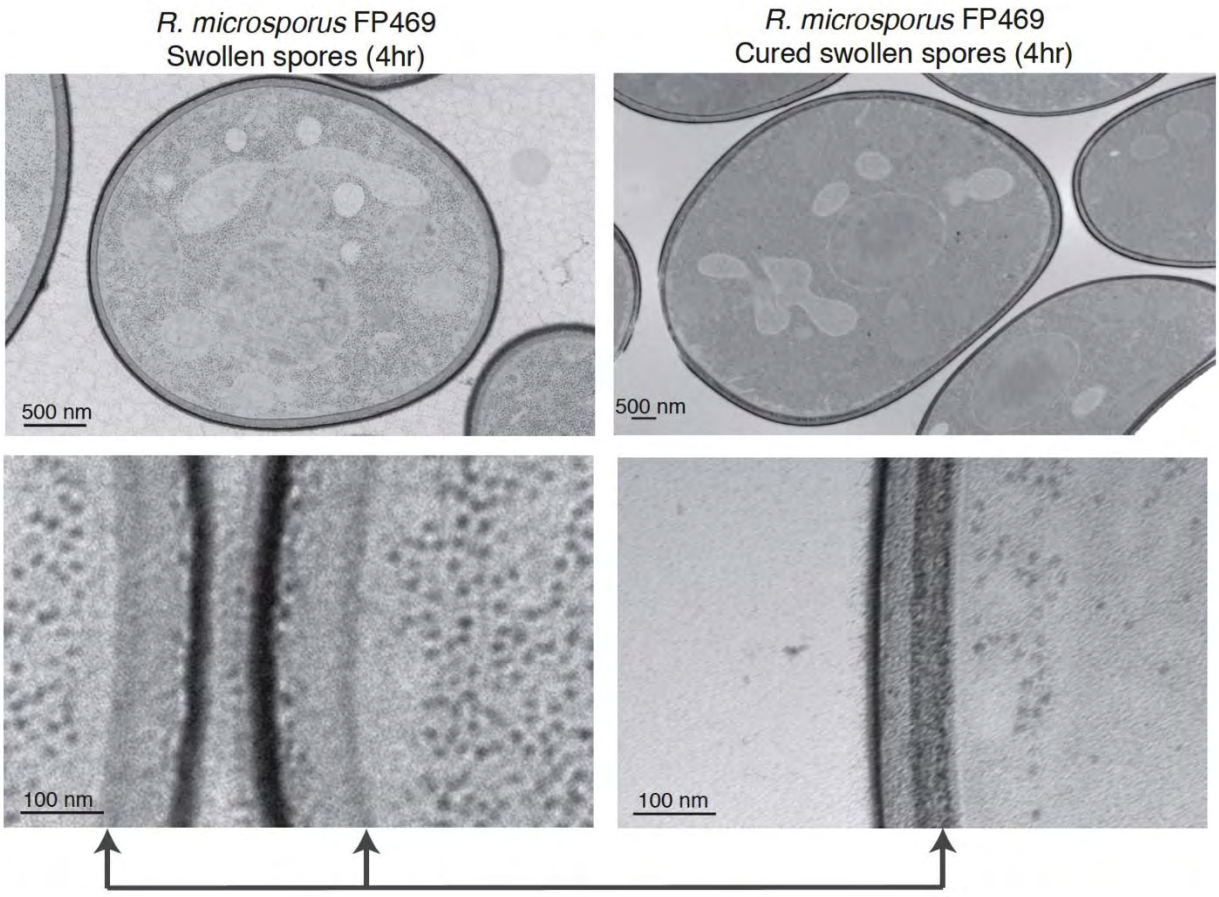


Figure 55: The Endobacterium influences fungal cell wall organisation.

Effect of endosymbiont status on *R. microsporus* F469-12 fungal structure. The TEM micrographs represent cross-sectional structures of both the parent and cured swollen spores, plus close up of the fungal cell wall in both cases. (Images acquired by Xin Zhou, unpublished data).

5.3.4 Endo-hyphal bacterial can be isolated in pure culture

The final approach to this was isolating the bacterium in pure culture, as has been done by others to definitively prove the presence of the endosymbiont and better characterise its effects ((Scherlach et al., 2006, Partida-Martinez and Hertweck, 2005)). Here, a modified version of the protocol described by Partida-Martinez *et al* (2005) and Scherlach *et al* (2006) was used as is described (Sections 2.2, 2.3 and 2.5) Spores were fermented (**Figure 53**), mycelia digested with Lyticase enzyme, and centrifuged, and the supernatant aliquots inoculated and incubated for fungal or bacterial growth as described before.

As shown in **Figure 56** and **Table 12,** bacterial isolation was successful in the following strains: *R. microsporus* (CBS 631.82), *R. microsporus* (FP 469-12), *M. circinelloides* (CBS 277940*), M. circinelloides (NRRL 3631), and C. bertholletiae* (NB2). Bacterial isolation failed in *L. corymbifera, R. chenensis* and *R. delemar*. However, supernatant aliquots from fungi where isolation was not possible also demonstrated presence of bacteria that failed to thrive in culture. This may be an example of an endosymbiotic bacteria demonstrating a reduced genome size and function due to loss of certain genes that would otherwise facilitate their survival when not living in a symbiotic association, as demonstrated by Uehling *et al* (Uehling et al., 2017).

Table 12: Characterisation of isolated endosymbiotic bacteria.

No.	Fungal strain ID	Isolated bacterial symbiont	Family	Colony and other description feature
1	*R. microsporus* (CBS 631.82)	*Ralstonia pickettii (Burkholderia pickettii)*	Bukholderiaceace	Colorless, shiny colonies producing a strong foul smell on Nutrient Agar.
2	*R. microsporus* (FP 469-12.6652333) clinical isolate	*Ralstonia pickettii (Burkholderia pickettii)*	Bukholderiaceace	Colorless, shiny colonies producing a strong foul smell on Nutrient Agar
3	*Mucor circinelloides* (CBS 277940)	*Micrococcus luteus*	Micrococcaceae	Yellowish odorless colonies on Nutrient Agar
4	*Mucor circinelloides* (NRRL 3631)	*Micrococcus luteus*	Micrococcaceae	Cream-ish odorless colonies on Nutrient Agar
5	*Cunninghamella bertholletiae* (NB2)	*Sphingomonas wittichii*	Sphingomonadaceae	Orange pinkish odorless colonies on nutrient Agar

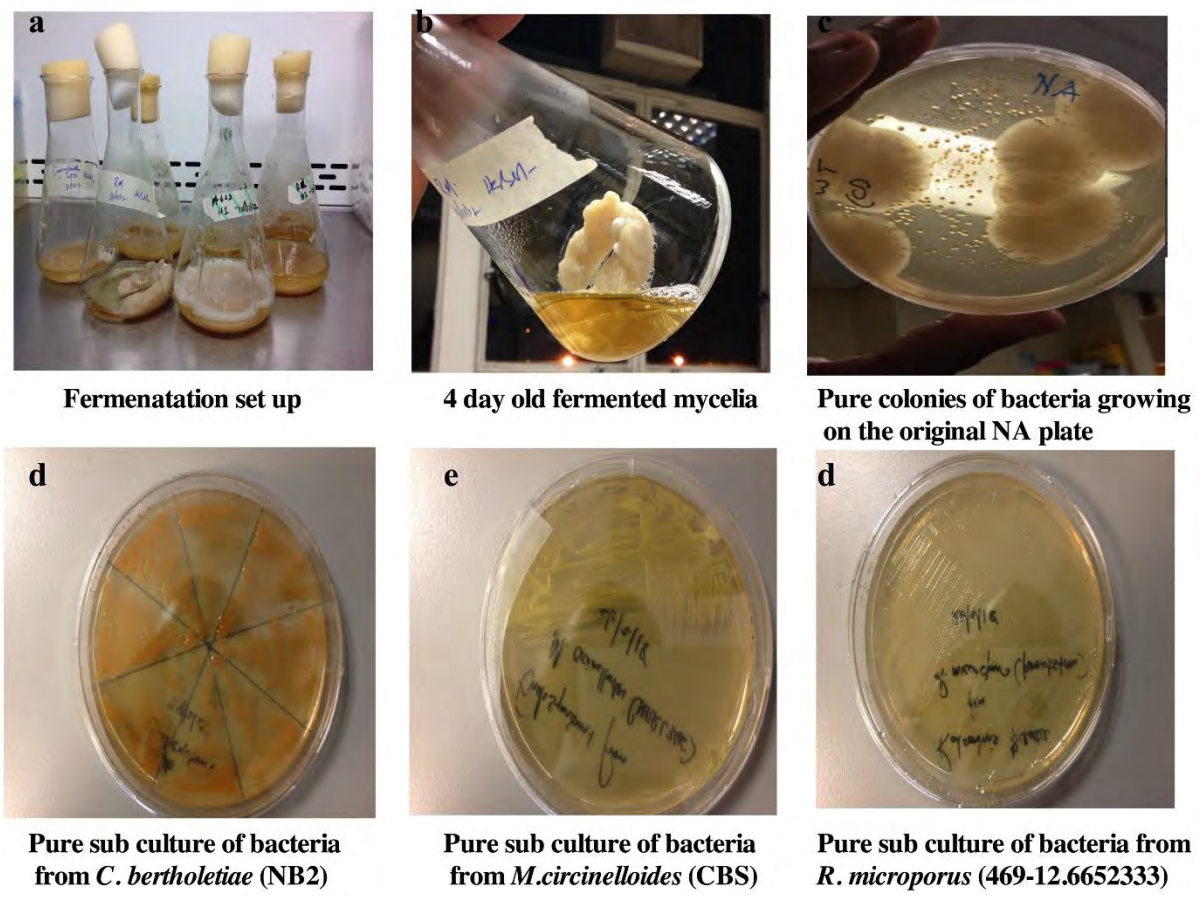
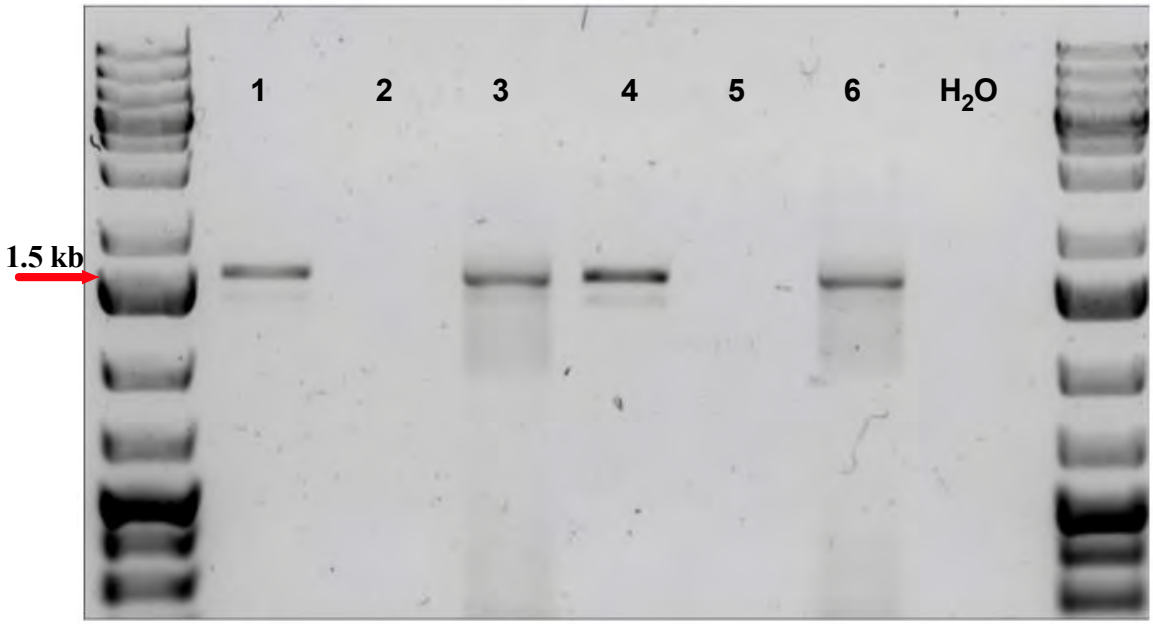


Figure 56: The endo-hyphal bacterium can be isolated in pure culture.

Typical fermentation set up (a) with 250 mL of VK media and incubated at 30 ºC with (80 rpm) for 2-4 days. (b) Typical 4 day fermented mycelial fungal ball. (**c**) Typical initial isolation of the bacteria on a nutrient agar plate after 3-5 days of incubation at 30 ºC and sub-cultured colonies of respective bacterial endosymbiont isolated from **(d**) *C. bertholletiae* (NB2), (**e**) *M. circinelloides* (CBS-277490) and (**d**) *R. microsporus* (FP469-12).

Examination of the isolated bacteria demonstrated various features: colonies ranged from colourless to pigmented colonial appearances (**Figure 56**) and cells were mainly of a coccobacilli-rod shaped morphology. The bacteria were identified through genotyping and were demonstrated to belong to different families, as shown in **Table 9**. Genotyping identified them as *Ralstonia pickettii* from both *R. microsporus* strains, *Micrococcus luteus* from both *Mucor* strains and *Sphingomonas wittichii* from *C. bertholletiae*. Interestingly, *R. pickettii* belongs to the same family as the *B. rhizoxinica* and *B. endofungorum* endosymbionts and16S amplicons of isolated bacteria here in comparison to the fungal Isolated DNA reveals similar results as before, demonstrated in **Figure 57.**

Finally, an attempt was made to re-introduce the endosymbiont to the fungal host. Bacteria and fungi were co-incubated following the protocol of Lackner *et al* (2011) (Lackner et al., 2011a). However, although uptake of the endosymbiont was observed at a low-level, we should note that the complemented (re-infected) fungal phenotype was not taken forward in this project because a stable mutualism could not be re-established in the time available. It is possible that the bacterium requires enough time or specific environmental conditions to adapt and re-establish its mutualistic relationship with the fungus.



1. *R. microsporus* (CBS 631.82)
2. *R. microsporus* (CBS631.82) + ciprofloxacin
3. *Ralstonia pickettii* from (CBS 631.82)
4. *R. microsporus* (469-12.6652333)
5. *R. microsporus* (469-12.665233) + ciprofloxacin
6. *Ralstonia pickettii* from 469 strain

Figure 57: PCR amplicons from isolated endobacterium reveal 16S rDNA.

Genomic DNA was isolated from fungal and isolated bacteria strains as indicated and screened by PCR for the amplification of a 1.5 kb band indicative of presence of a bacterial 16S rDNA. The image is representative of gel electrophoresis of PCR products from one of at least three different biological extraction repeats.

5.4 Effect of Bacterial elimination on Fungal Phenotype

Bacterial-fungal interactions (BFI) have a significant impact on phenotypes such as gene transfer, substrate consumption, virulence, sexuality and metabolism (Araldi-Brondolo et al., 2017, Moebius et al., 2014b, Husnik and McCutcheon, 2018, Mondo et al., 2017). Our data have now demonstrated the presence of bacteria symbionts among mucoralean fungi, including in the strain of interest here. Most importantly, we are able to eliminate the bacteria from the fungus and also isolate the endosymbiont in pure culture. This way interface mechanisms of BFI can now be studied together and separately.

Thus, using elimination and isolation as tools, the influence of the bacteria on modulation of fungal phenotype, particularly metabolism, was considered and investigated. We hypothesised that if the bacterium is the force behind the anti-phagocytic attributes described so far, then its elimination would abolish the effects demonstrated by the spores during infection. In addition, a similar loss of functional peaks would be reflected in the chemistry profiling described before (Sections 2.5 and 2.11).

5.4.1 Elimination of the bacteria reduces fungal sporulation

We have preliminarily investigated the impact of the bacteria on fungal sporulation. Accordingly, both parent and cured strains of *R. microsporus* were passaged on respective plates of SDA with or without ciprofloxacin as described before (Sections 2.4 and 2.5)

As shown in **Figure 58**, when continuously passaged on ciprofloxacin plates, sporulation significantly decreased with time. These findings are similar to what has been reported elsewhere, that bacterial symbionts influence sexuality in *R. microsporus* by *B. rhizoxinica* where the endosymbiont has been characterized to control fungal transcription through *RAS*2 gene which encode GTPases key for fungal reproduction (Mondo et al., 2017).

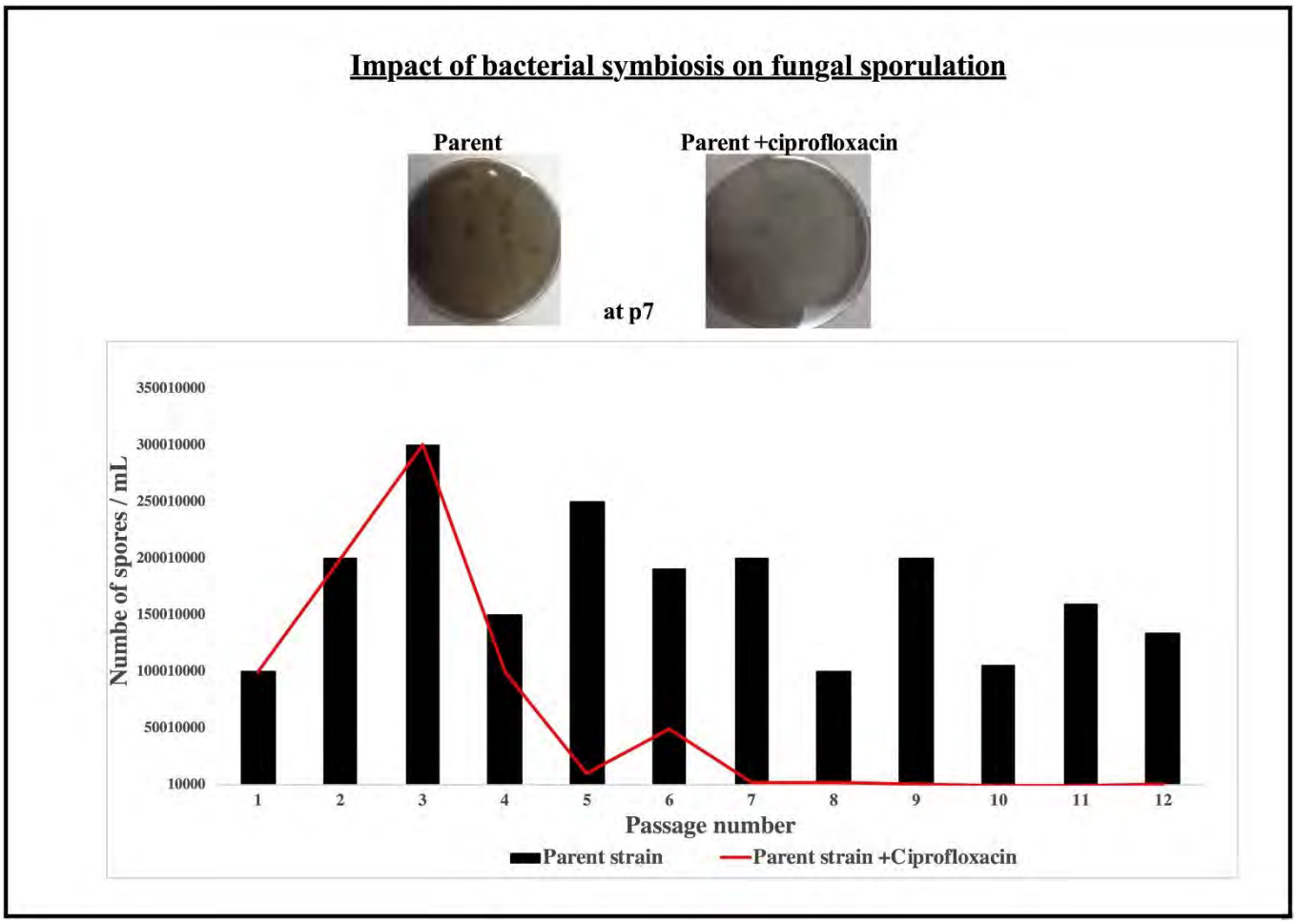


Figure 58: Elimination of bacterial endosymbiont reduces fungal sporulation.

The parent strain of *R. microsporus* was passaged on SDA plates with or without ciprofloxacin at room temperature every 2 weeks, spores harvested and counted. The data are presented as standard error of the means from at least three biological repeats at each passage.

5.4.2 Elimination of the bacteria restores phagocytosis of pre-germinated spores

We have already shown in Chapter 3 that metabolically active spores are not as readily phagocytosed by macrophages. To determine the impact of the endosymbiont bacteria on this outcome, endosymbiont free spores were pre-germinated and phagocytic uptake determined as described previously (Sections 2.3, 2.7 and 2.11).

As demonstrated in **Figure 60**, there are significant differences in the uptake rate of parent spores as opposed to endosymbiont free strain. For instance, dormant spores of the cured strain demonstrated an increased uptake form 76% by parent strain to 87% ($p<0.01$), while swollen spores also demonstrated increased uptake rate from 25% by parent to 79% by cured ($p<0.0001$) at 2 h; and from 15% by parent to 78% by cured ($p<0.0001$) at 4 h (Figure **60**). This reversal in phagocytic uptake of cured swollen spores' points to the influence of bacterial endosymbiont in the evasion of phagocytic uptake.

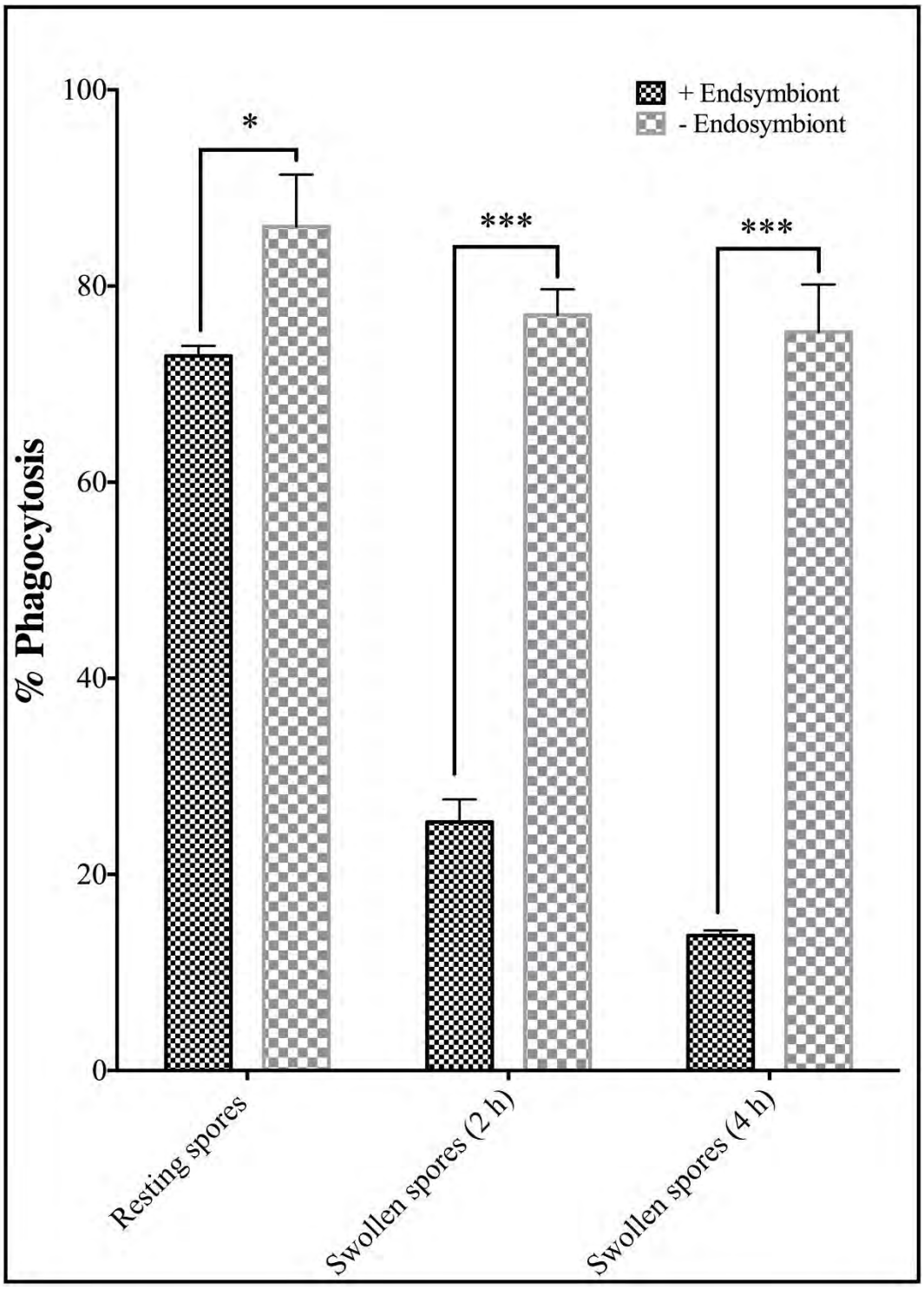


Figure 59: The bacterial endosymbiont influences fungal secretome and interaction with mammalian cells in vitro.

Phagocytosis of parent or cured resting or swollen (2 and 4 h) spores of *R. microsporus* FP469-12 by J774A.1 macrophages (n=3 biological replicates, One-way ANOVA with Tukey's correction for multiple comparisons) and for the examined repeats errors bars represent s.e.m. (*= $p< 0.01$, ***=$p< 0.0001$).

5.4.3 Supernatant from cured spores demonstrates loss of activity

To determine the effect of bacterial elimination on expression of the supernatant (SN) factors, cured strain (endosymbiont free) supernatants were collected and their activity against phagocytosis, cytoskeleton, and phagosome maturation determined as described before (Sections 2.3, 2.5, 2.7 and 2.9) As shown in **Figures 61-63**, treatment of macrophages with the two (+/- endosymbiont) supernatant types variably impacts macrophage phenotypic functions. For instance, phagocytosis is inhibited from an average percentage uptake of 85% (± 6 SEM) by the control to on average 35% (± 5 SEM) upon treatment with the parent supernatant ($p < 0.00001$) but no significant difference was noted between the control and cured strain supernatant registering a reduction from 85% to 63 (± 7 SEM) ($p=0.725$) (**Figure 61**).

Regarding phagosome maturation: Both spore categories (+/- endosymbiont) significantly inhibit phagosome maturation when compared with control ($p < 0.05$) (**Figure 62a**). Inhibition by parent spores is significantly lower than by endosymbiont free spores ($p < 0.001$) (**Figure 62a**); and there is no difference in phagosome inhibition between resting and swollen spores of both strains ($p > 0.05$) (**Figure 62a**).

Regarding supernatants, treatment with parent SN ($p<0.0001$) significantly inhibits phagosome maturation compared to endosymbiont free SN ($p> 0.05$) (**Figure 62b**)

Previously, we demonstrated that treatment of phagocytes with *R. microsporus* swollen spore supernatant reduces overall staining with Phalloidin dye and anti-beta tubulin immunostaining, indicative of reduced cytoskeletal integrity. This was also reflected in the rounding of cells (chapter 4 **Figures 33** (actin) and **34** (beta-tubulin)) (Section 4.6.2) Here we also investigated the impact of bacterial symbiosis on cytotoxic effects. As shown in **Figure 63**, it was interesting to see that whilst parent SN significantly inhibited both actin and beta tubulin ($p < 0.0001$) respectively. This activity was lost following treatment with endosymbiont free SN ($p > 0.05$). Indeed, there were significant differences in the activity parent vs cured supernatants

against actin or beta tubulin polymerisation. These findings further show that the bacterial endosymbiont may be involved in the metabolic attributes of fungal spores.

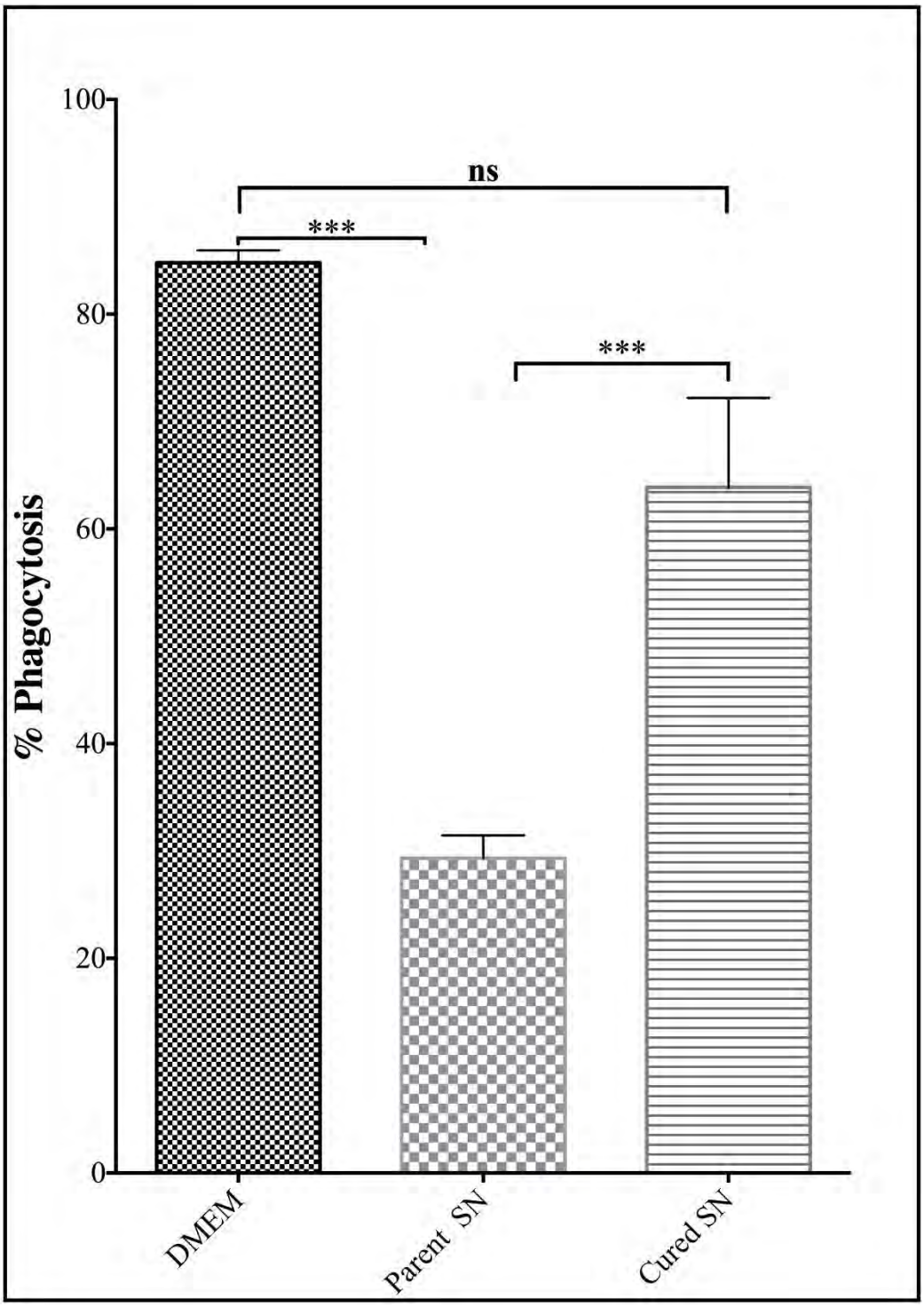


Figure 60: The bacterial endosymbiont influences fungal secretome and interaction with mammalian cells.

Effect of bacterial endosymbiont supernatants isolated from indicated fungal hosts on phagocytosis of *R. microsporus* resting spores. For both conditions, the number of phagocytes containing at least one spore were counted after 1 hour (n=9000, One way ANOVA with Tukey's correction for multiple comparisons). In each graph, three biological replicates were examined and error bars represent s.e.m. (**ns**=$p>0.05$, ***=$p<0.0001$).

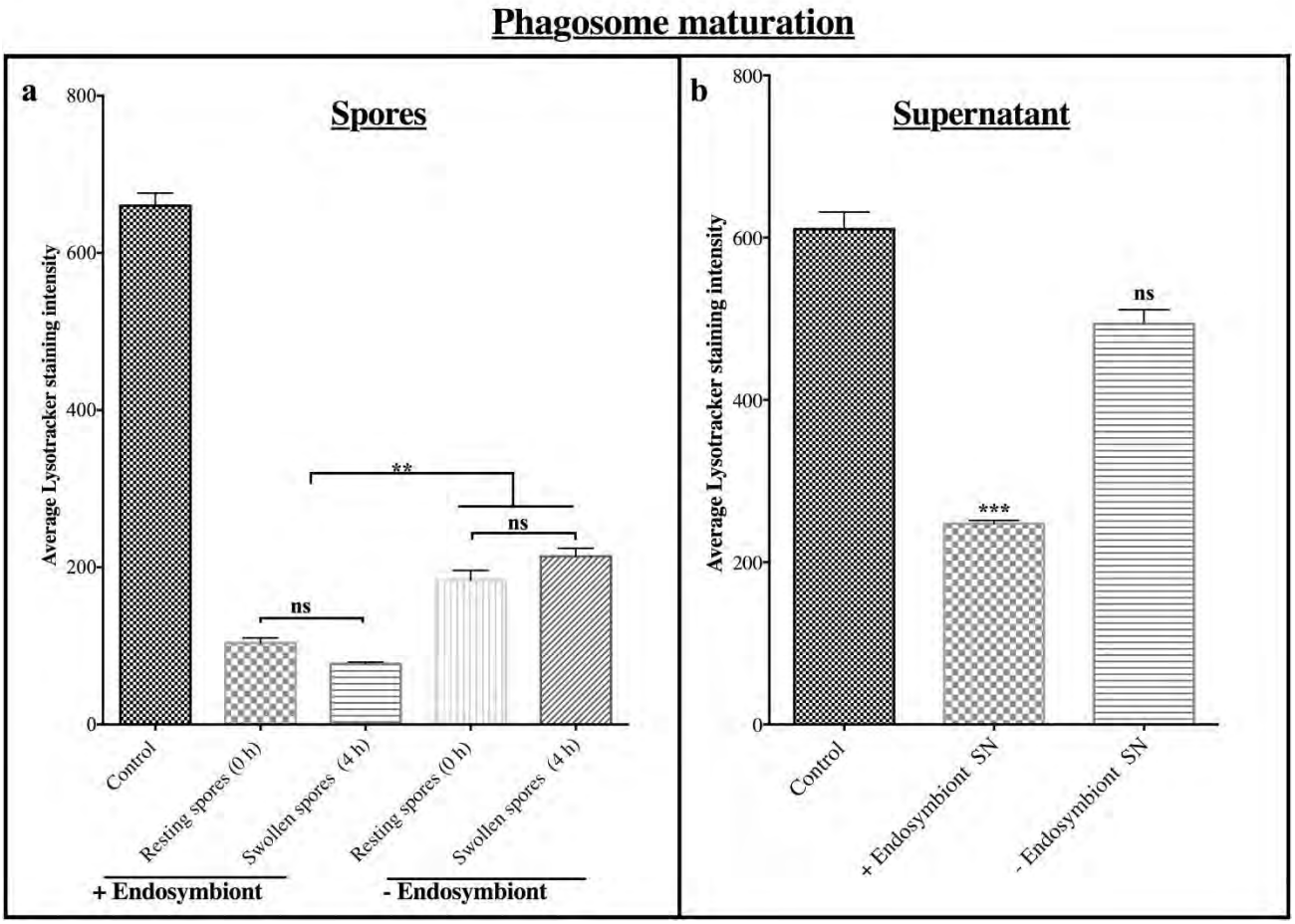


Figure 61*:* The bacterial endosymbiont influences phagosome maturation *in vitro.*

Harvested (**a**) parent and cured spores resting or pre-germinated spores (in SDA at 30º C for 4 h) were co-incubated with J774 phagocytes for 1 h and phagosome maturation assessed using LysoTracker staining. (**b**) Supernatants were collected from both parent and cured strains of *R. microsporus.* These were then used to treat J774 phagocytes which were co-incubated with *R. microsporus* swollen fixed killed spores for 2 h, phagosome maturation determined with LysoTracker staining and quantified with image J. (n=300 (three biological repeats), one way ANOVA with Turkeys correction for multiple comparisons and error bars represent s.d (ns=$p>0.05$, **=$p<0.001$ and ***$p<0.0001$).

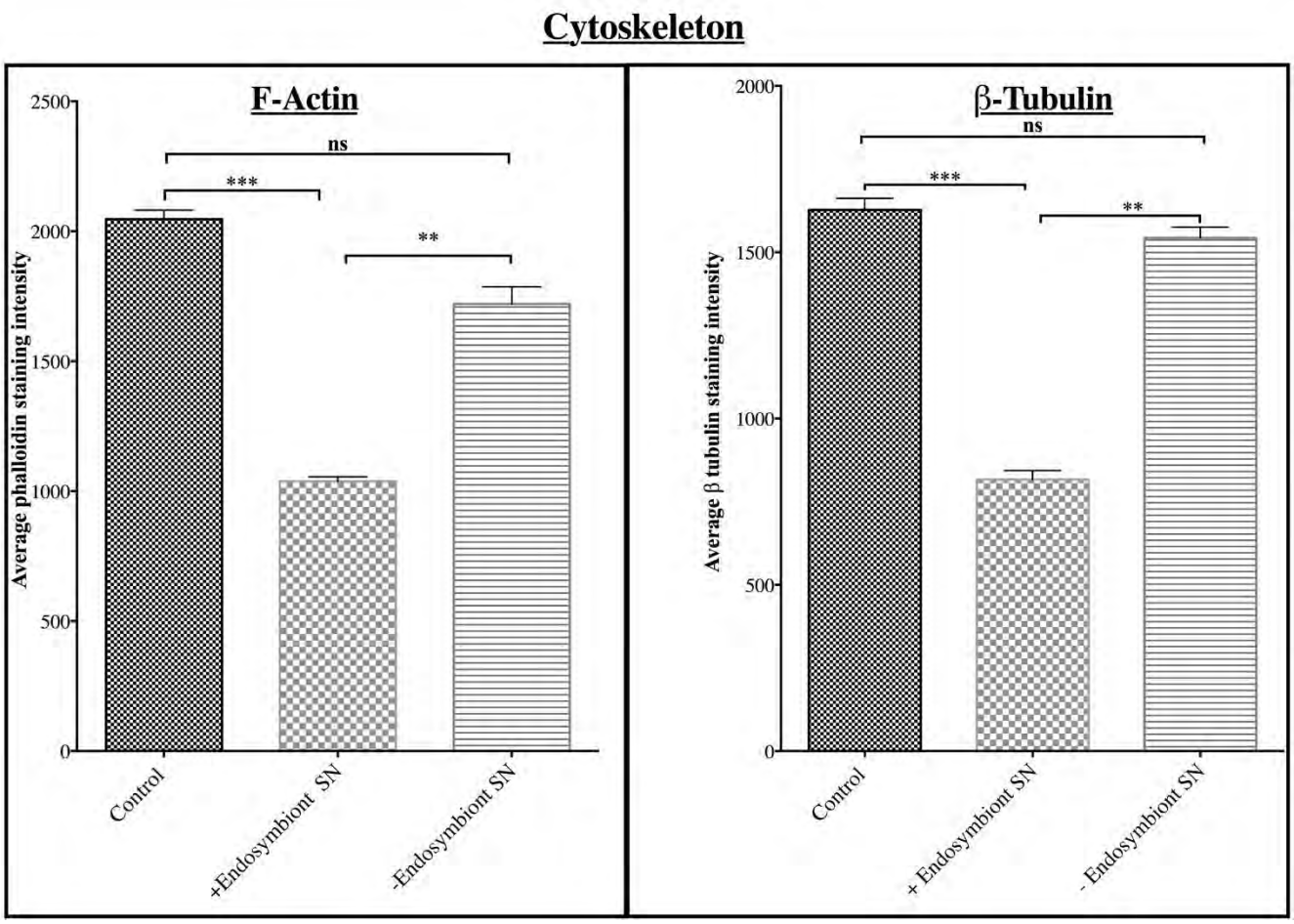


Figure 62: The bacterial endosymbiont influences cytoskeletal organisation *in vitro*.

Supernatants were collected from parent and cured strains of *R. microsporus*. These were then used to treat J774 phagocytes for 1 h. (**a)** phagocytes were then stained for F-actin with phalloidin for 15 min. (**b)** Phagocyte were stained for Beta- tubulin with anti-beta tubulin antibodies for 2 h. Stain quantification was scored with image J. Both assays, n=450, Statistical significance was assessed via one-way ANOVA with Turkey's correction for multiple comparisons. In each graph, three biological repeats were examined and error bars represents s.e.m. (ns=$p>0.05$, **=$p<0.001$), ***$p<0.0001$).

5.4.3 Endosymbiont free spores are more susceptible to macrophage killing and stress

We previously shown that *R. microsporus* parent spores are resistant to both macrophages killing and environmental stress factors (Section 3.3.2). Here, the impact of the bacterial symbiosis on the spore's ability to resist macrophage killing and other stresses was also determined as before (Sections 2.5, 2.6 and 2.7).

As demonstrated in **Figures 63-64**; when compared with survival of parent spores, we saw that, after treatment with ciprofloxacin to cure the endosymbiont, both resting and swollen spores' ability to resist macrophage killing was compromised. There was no difference in the survival of parent and endosymbiont free spores at 0 h ($p > 0.05$). But then spore survival significantly declined from 100 % to 80 % after 6 h ($p < 0.01$), to 50 % after 24 h ($p < 0.0001$) for resting spores; and from 98 % to 60 % after 6 h ($p < 0.001$), to 45% after 24 h ($p < 0.0001$) for swollen spores (**Figure 63**). These data demonstrate that bacterial symbiosis may play a key role in fungal survival and evasion of innate immunity during infection.

In regard to response to other stress factors, endosymbiont free spores were generally susceptible to individual stresses including cell wall damage due to osmotic and SDS ($p<0.05$), nitrosative stress by sodium nitroxide ($p<0.05$), oxidative stress by hydrogen peroxide $p<0.00001$) and antifungal stress by AmB treatments $p<0.05$) (**Figure 64**). The findings here demonstrate that in spite of the state of the spores, losing the endosymbiont compromises the ability of the fungus to survive against stress factors. These findings are similar to those obtained from the macrophage killing assay. These findings point to how crucial the endosymbiont might be for fungal survival and flexibility, both in the wider environments and during infection.

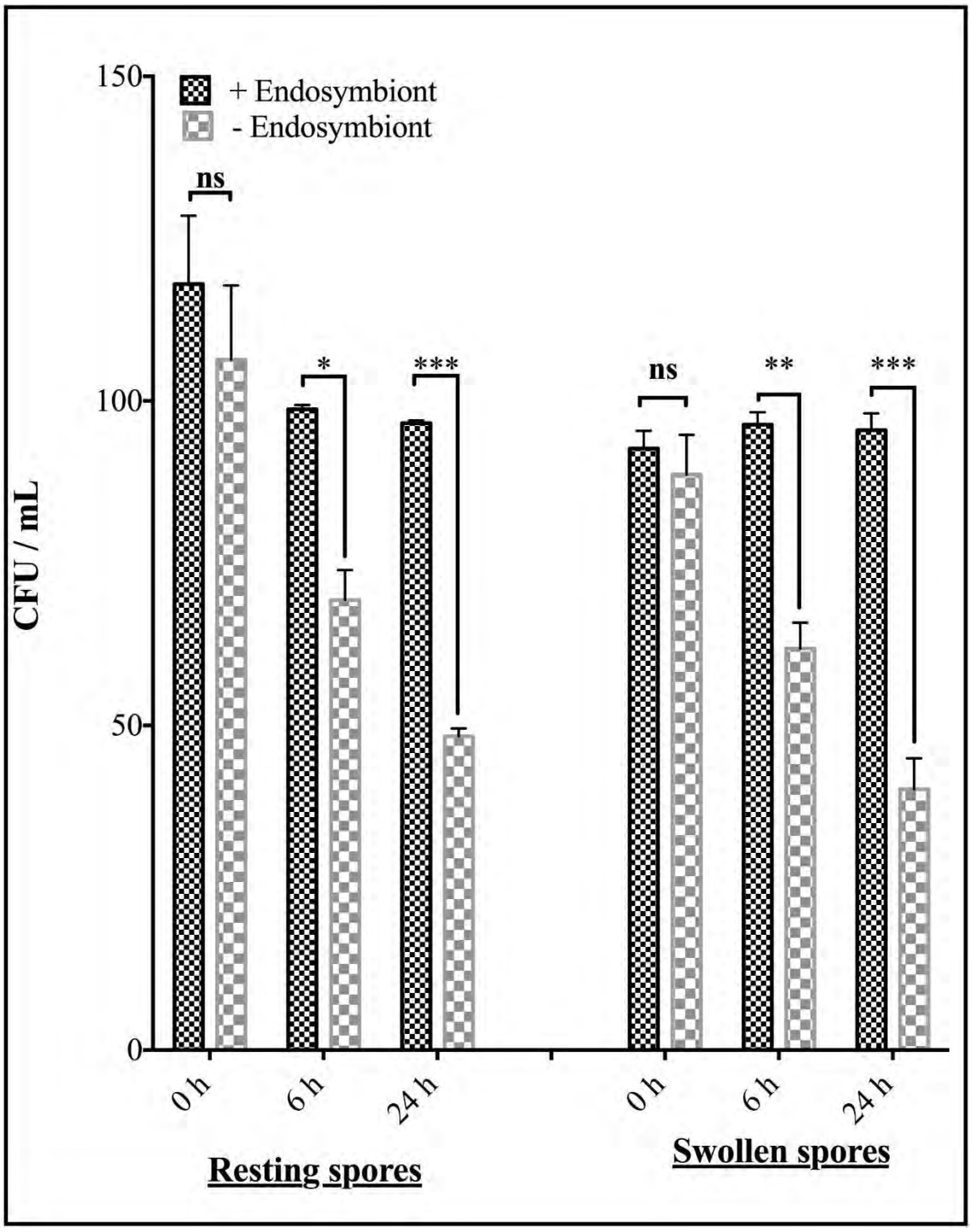


Figure 63: Loss of a bacterial endosymbiont compromises fungal survival following phagocytic uptake.

Impact of bacterial endosymbionts on fungal (*R. microsporus* F469-12) parent or cured spore's survival following co-culture with J774 macrophage for the indicated time points. Three biological repeats were examined and error bars represent s.e.m. Statistical significance was assessed via One way ANOVA with Tukey's correction for multiple comparisons (ns=$p>0.05$, *=$p<0.001$, **=$p=0.001$, ***=$p<0.0001$).

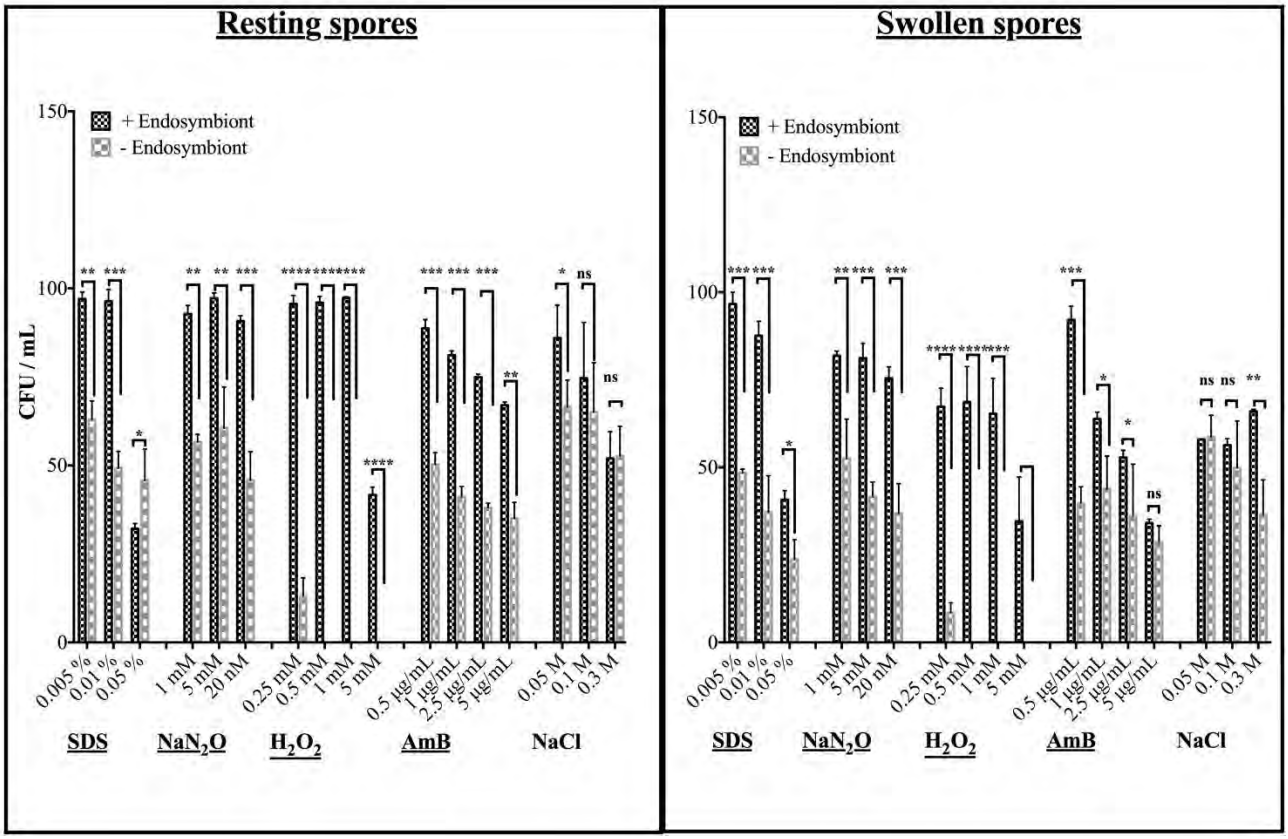


Figure 64: Endosymbiont free spores show increased susceptibility to environmental stress factors.

Impact of bacterial endosymbionts on fungal (*R. microsporus* F469-12) parent or cured resting spores' susceptibility to stress factors include cell wall, nitrosative, oxidative and antifungal stresses at the indicated concentrations. Three biological repeats were examined and error bars represent s.e.m. Statistical significance was assessed via One way ANOVA with Tukey's correction for multiple comparisons (***ns***=p>0.05, **=p<0.001, ***=p=0.0001, ****=p<0.00001).

5.5 Chemistry based evidence of bacterial symbiosis

5.5.1 Elimination of the bacteria abolishes expression of active HPLC peaks

HPLC profiling of *R. microsporus* parent SN revealed several peaks, particularly peaks 3, 5 and 8, as being the most active against phagocytosis. Furthermore, MS analysis of these peaks also revealed masses consistent with rhizo-toxins, particularly from peaks 5 and 8 (refer to chapter 4 (Section 4.9; **Figures 46-48**).

Given the fact that rhizo-toxins are secreted by the endosymbionts, as mentioned earlier (Section 1.4.5.4), the effect of eliminating the bacteria on expression of these peaks was also investigated here. We hypothesised that the supernatant factors are either secreted by a bacterial endosymbiont or by the fungus but only when in association with the bacteria.

Accordingly, both supernatant types (+/- endosymbiont) were extracted, and profiled by HPLC as described before (Sections 2.11). As shown in **Figure 65**, we saw that all active peaks 3, 5 and 8 are abolished following elimination of the bacteria. Further highlighting the loss of activity demonstrated in the endosymbiont free supernatant as shown earlier, most importantly confirming that bacterial symbiosis may significantly impact on fungal metabolism and perhaps infection.

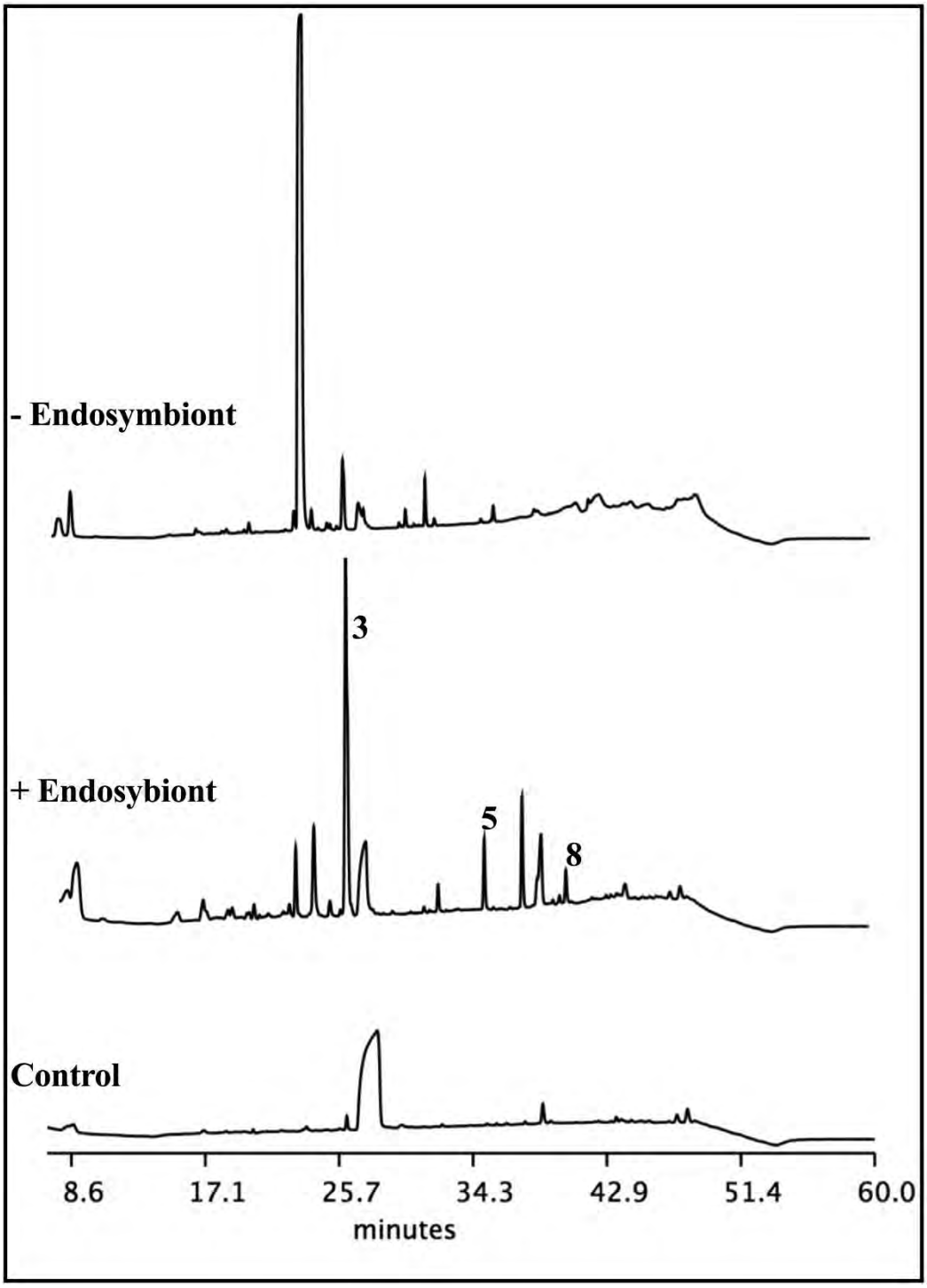


Figure 65: Elimination of the endobacterium abolishes all active HPLC peaks.

R. microsporus (parent and cured) strains were fermented in sfDMEM at 30ºC, 80 rpm for 2-4 days. Supernatant was collected, extracted with chloroform and HPLC profiled using C18 column. A medium control is included. The Chromatograms shown are from one of at least three biological repeats.

5.6 Bacterial derived supernatants

5.6.1 Bacterial supernatants show reduced activity

We have so far explored the effect of spore metabolism through examining supernatants from parent and endosymbiont knockout spores. This way, we established a correlation between spore metabolism and bacterial symbiosis. Therefore, following isolation of the bacteria in pure culture, we sought to explore the biological activity in three different strains: wildtype (parent), cured (endosymbiont free) fungal strains, and the isolated bacteria in pure culture both separately and co-cultured together with the endosymbiont-free fungal host (discussed later in Chapter 6).

Given this background, we sought to examine activity of bacterial-derived supernatants against macrophage effector functions to establish if the bacteria are the producers of the supernatant factors in question. Accordingly, the bacteria were grown in sfDMEM, the supernatant was collected and activity against phagocytosis, phagosome maturation and cytoskeleton determined as described before (Sections 2.4, 2.5, 2.7 and 2.9)).

As shown in **Figure 66**, supernatants of bacteria isolated from *R. microsporus* and other mucoralean fungi were all evaluated for activity against phagocytosis. In addition, supernatants from both parent and cured fungal categories (resting and swollen) and a wild type strain of *Burkholderia cepacia*, a close relative of *R. pickettii,* were evaluated as controls. While parent SN significantly reduces phagocytosis from 78% to 30% ($p<0.00001$), bacterial supernatants demonstrated reduced activity against phagocytosis to an average of 55% ($p<0.001$). There was however a significant difference in the activity of bacterial SN to that of the wildtype *B. cepacia* strain ($p<0.001$).

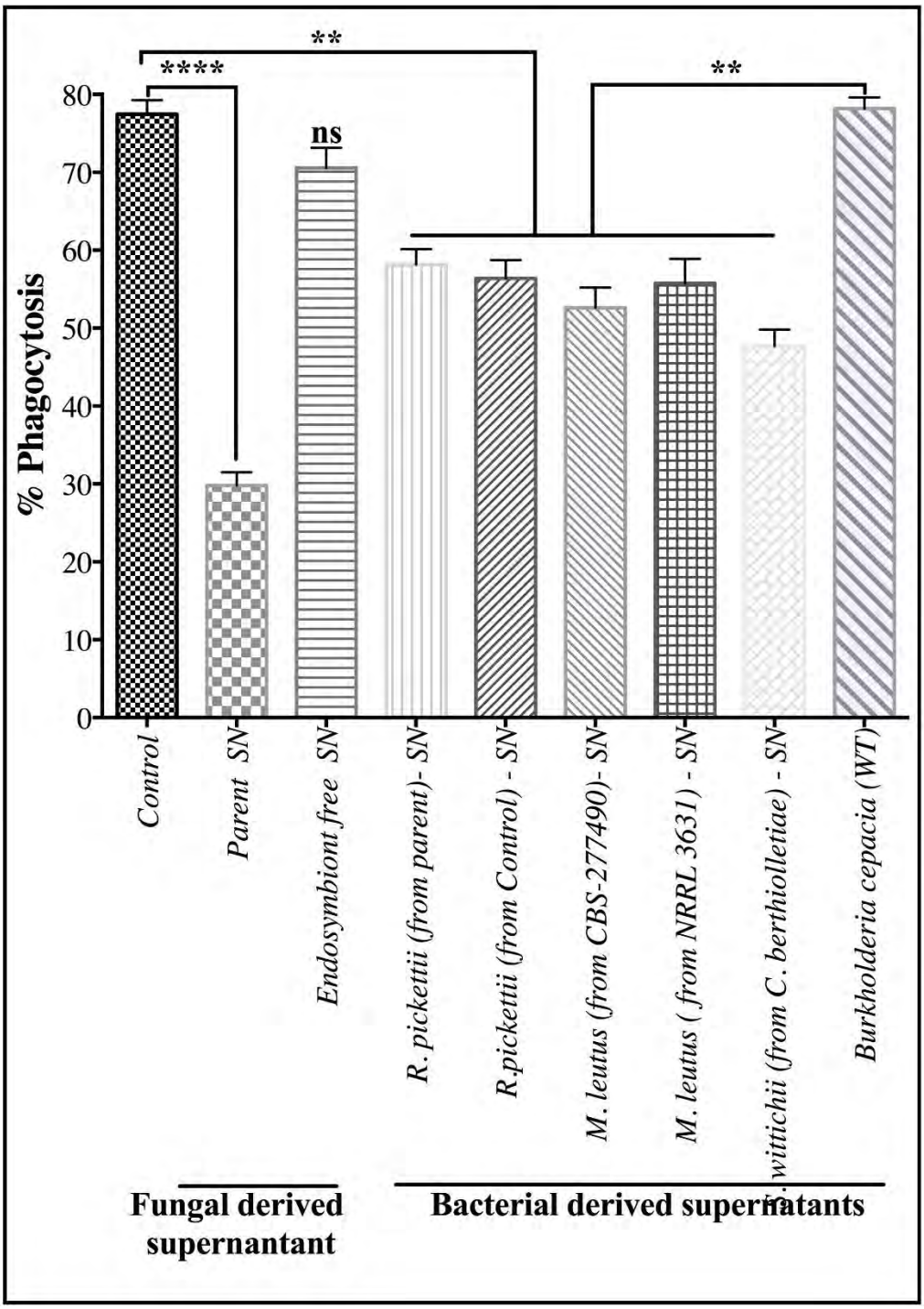


Figure 66: Bacterial endosymbiont culture supernatants show reduced activity against phagocytic uptake by J774 macrophages.

Supernatants were collected from indicated fungal hosts and their respective isolated bacterial strains. These were then used to treat J774 phagocytes which were co-incubated with *R. microsporus* dormant spores for 1 h and uptake rate assessed. Three biological repeats were examined and error bars represent s.e.m. Statistical significance was assessed via One way ANOVA with Tukey's correction for multiple comparisons (**ns**=$p>0.05$, **=$p<0.001$, ****=$p<0.00001$).

Also stemming from cytotoxic effect demonstration already, the effect of bacterial endosymbiont supernatants on cytotoxic effects was tested. As shown in (**Figure 68**), significant and comparable levels of activity were seen by both *R. pickettii* and parent supernatants against phagosome maturation ($p < 0.01$) (**Figure 67a**); actin polymerisation ($p < 0.01$) (**Figure 67b)** and no significant differences were noted for beta tubulin ($p>0.05$) (**Figure 67c**). For all assays, there were significant differences between activity of the bacterial endosymbiont supernatants and endosymbiont free fungal supernatants ($p<0.05$) (**Figure 67**). Although the bacterial supernatant demonstrated reduced activity against phagocytosis (see **figure 67**), its activity against other macrophage effector functions was comparable to supernatant from parent swollen fungal supernatants. However, these findings can suggest possibility of metabolic confounding factors that could contribute to this difference in activity.

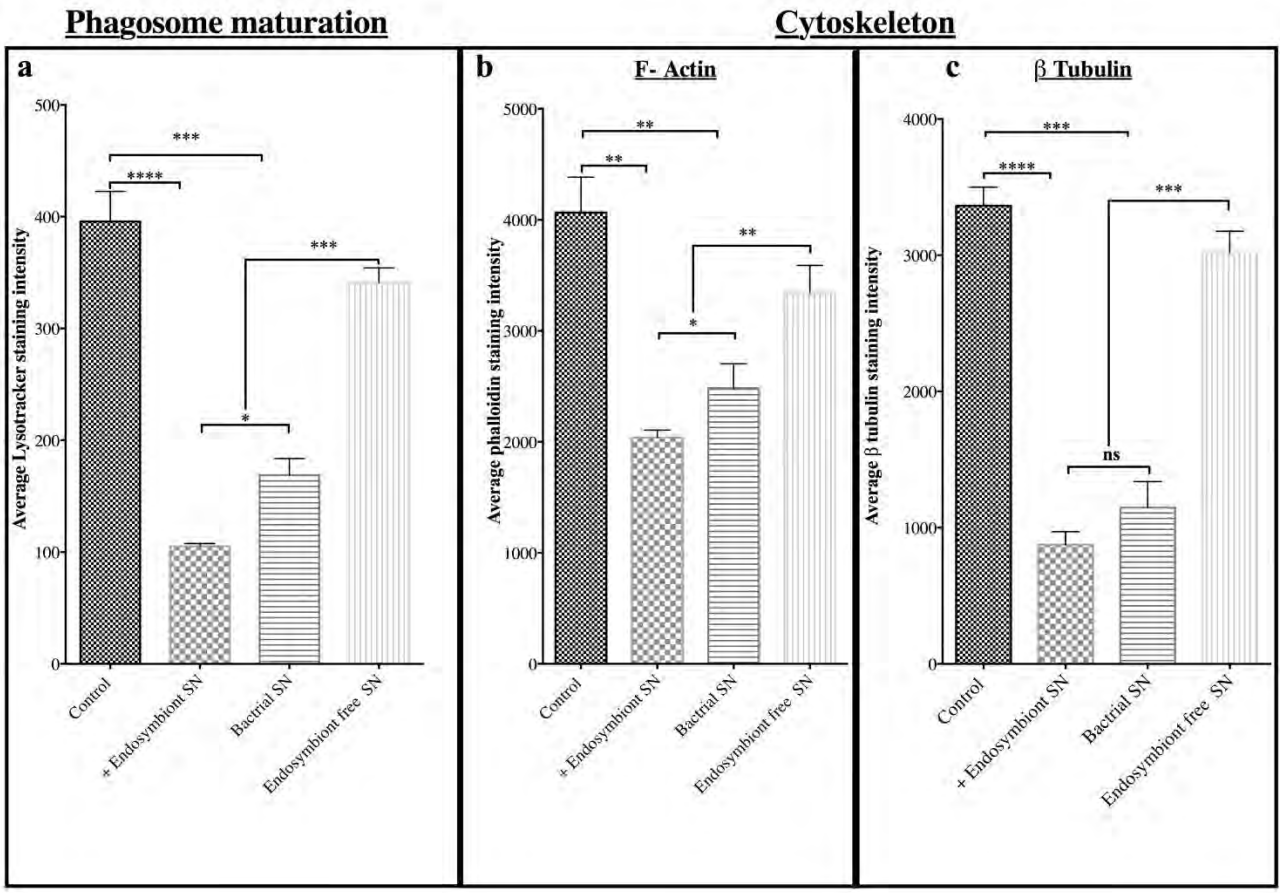


Figure 67: Bacterial endosymbiont culture supernatants show similar levels of activity against phagosome maturation and cellular cytoskeleton.

Supernatants were collected from *Ralstonia pickettii* (isolated from *R. microsporus*). This was then used to treat J774 phagocytes for 1 h. (**a**) The phagocytes were then co-incubated with swollen fixed killed spores of *R. microsporus* and phagosome maturation determined with LysoTracker staining for 2 h. (**b)** Phagocytes were stained for F-actin with rhodamine-conjugated phalloidin for 15 min. (**c)** Phagocyte were stained for Beta- tubulin with anti-beta tubulin antibodies for 2 h. For all tests staining intensity was scored with image J. For all assays, **(a)** n=100, (**b** and **c**) n=450, Statistical significance was assessed via one way ANOVA with Turkey's correction for multiple comparisons. In each graph, three biological repeats were examined and error bars represents s.e.m.(ns=$p>0.05$, *=$p<0.01$, **=$p<0.001$), ***$p<0.0001$ and ****=$p<0.00001$).

5.6.2 Bacterial endosymbiont supernatant shows loss of the most active peak (5)

Reduced activity against phagocytosis by bacterial supernatant was rather intriguing, prompting further analysis and thus, was also profiled using HPLC, as previously described (Sections 2.5 and 2.11). As shown in **Figure 68**, it was quite interesting to see that of the active peaks present in the wildtype fungal supernatant, only peaks 3 and 8 are present in bacterial supernatant. The most active one, peak 5, is absent. Taken together with the observation that endosymbiont supernatant has reduced anti-phagocytic activity compared to wildtype fungal supernatant, this could mean that peaks 8 and 3 have some limited activity. These data also further confirm that peak 5 is the most active against phagocytosis, as demonstrated before (Section 4.9). Whether peaks 3 and 8 are metabolites of the same product remains to be determined. This finding also highlights the potential role of different growth conditions on expression of the metabolic factors, and this is explored below. Specifically, we investigate whether the expression of peak 5 is dependent on presence or absence of particular substrates.

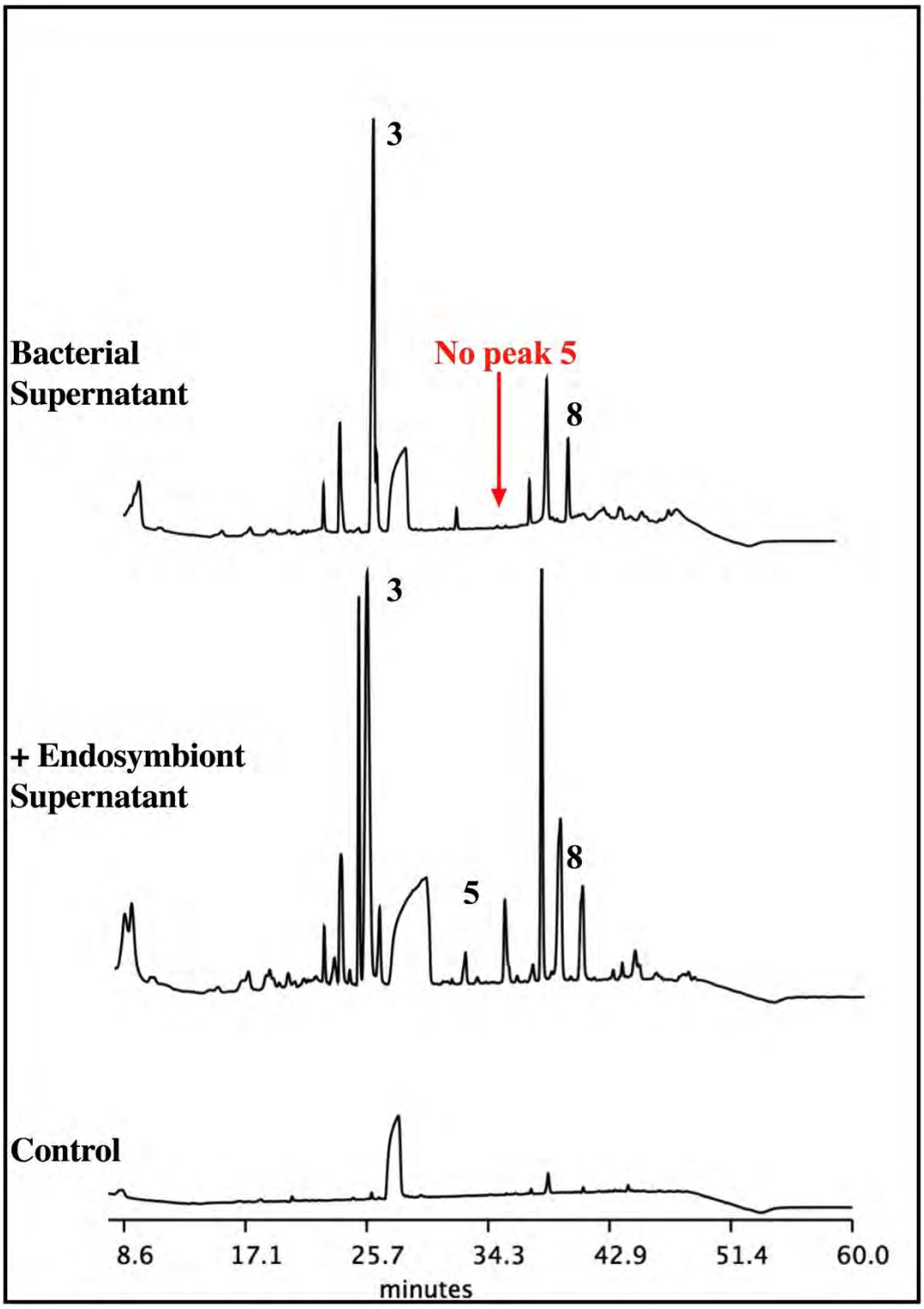


Figure 68: The bacterial endosymbiont supernatant shows loss of HPLC peak 5.

R. microsporus (parent and cured) and *R. pickettii* strains were fermented in sfDMEM at 30ºC, 80 rpm for 2-4 days. The Supernatant was collected, extracted with chloroform and HPLC profiled using C18 column. A medium control is included. Chromatograms shown are from one of at least three biological repeats.

5.6.3 Growth conditions significantly impact metabolism of both the bacterium and fungus

The above findings pointed to differences in metabolic process by both the fungus and bacterium while living in symbiotic association or separate. Thus, to examine this aspect, we used a chemistry approach to profile the impact of substrate availability on metabolism of the parent, cured fungal and isolated bacterial strains. Accordingly, the strains were grown in sfDMEM, VK or HL5 medium, supernatants collected, extracted and HPLC profiled as described before (Sections 2.2 and 2.11). The host-relevant medium DMEM is a nutrient-poor medium for bacterial and fungal culture and supports limited growth. We therefore asked whether other nutrient-rich media would support expression of peak 5, the most active peak. HL-5 is a common medium for the cultivation of the mini-host model organism *Dictyostelium discoideum* a natural predator of the mucorales

As shown in **Figure 69**, it was interesting to see that there were significant changes in the HPLC profiles by both fungi and bacteria under the different medium growth conditions. Both the fungus and bacteria produced a larger range of products in HL5 and VK than in DMEM medium, indicating that production of the active compounds could be significantly affected by the growth conditions. These conditions may reflect those that the bacteria experiences during symbiosis but perhaps not while cultured in DMEM medium. However, more work is needed to fully understand the impact of different substrates on symbiotic metabolism attributes.

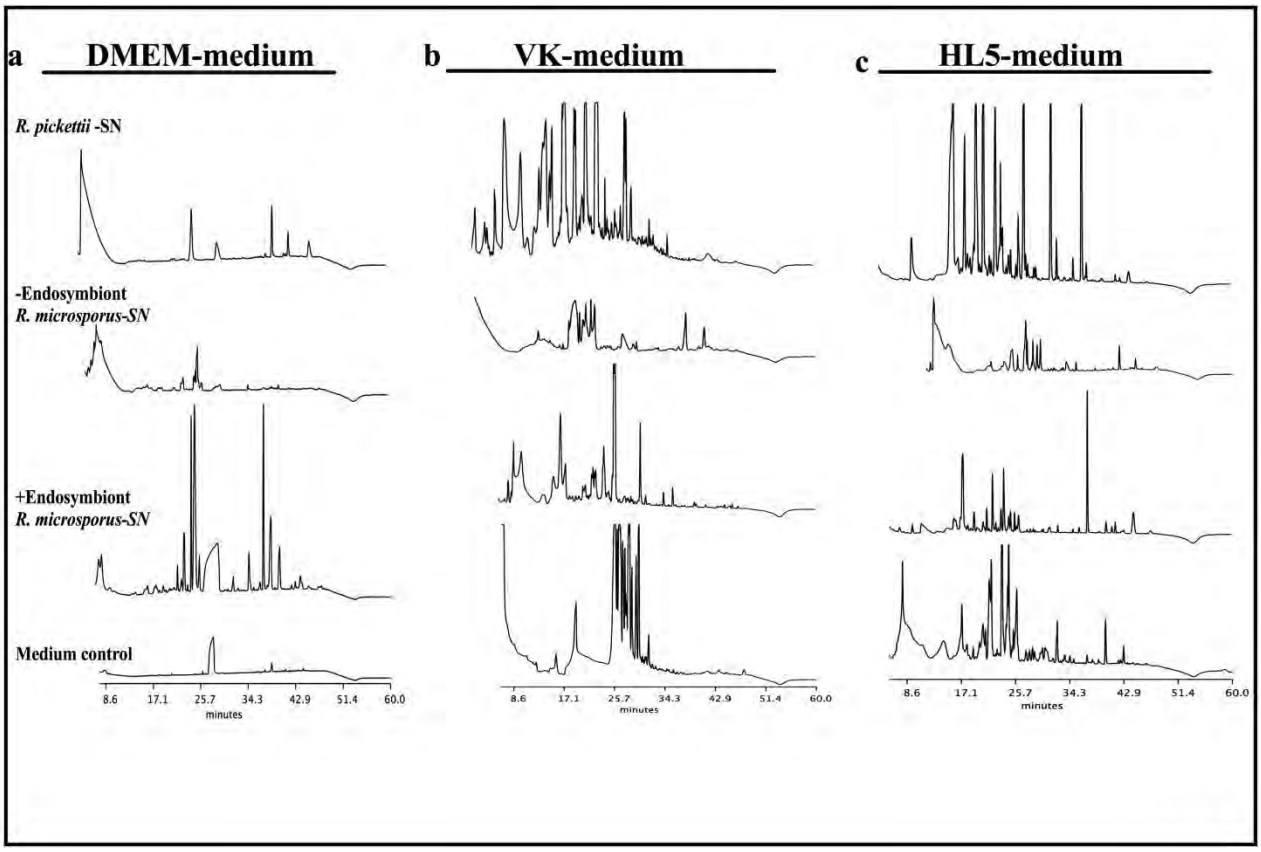


Figure 69: Different growth conditions influence both bacterial and fungal metabolism.

R. microsporus (parent and cured) and *R. pickettii* strains were fermented in different culture medium as indicated at 30ºC, 80 rpm for 2-4 days. Supernatants were collected, extracted with chloroform and HPLC profiled using C18 column. Medium controls were also included. Chromatograms shown are from one of at least three biological repeats.

5.7 Genetical evidence

Loss of peak 5 from bacterial supernatant was an unexpected finding if we are to refer to the rhizo-toxins which are produced by *Paraburkholderia rhizoxinica* or *Paraburkholderia endofungorum* (Partida-Martinez et al., 2007b, Partida-Martinez et al., 2007a). Thus, genetic analysis of the bacteria was also performed. The isolate in our case is *R. pickettii*, a phylogenetically close relative of *Paraburkholderia* species belonging to same Bukholderiaceace family (**Figure 70**). The question at this stage was whether *R. pickettii* also produced the likes of rhizo-toxins. This was done by examining the metabolic cluster in the bacterial genome.

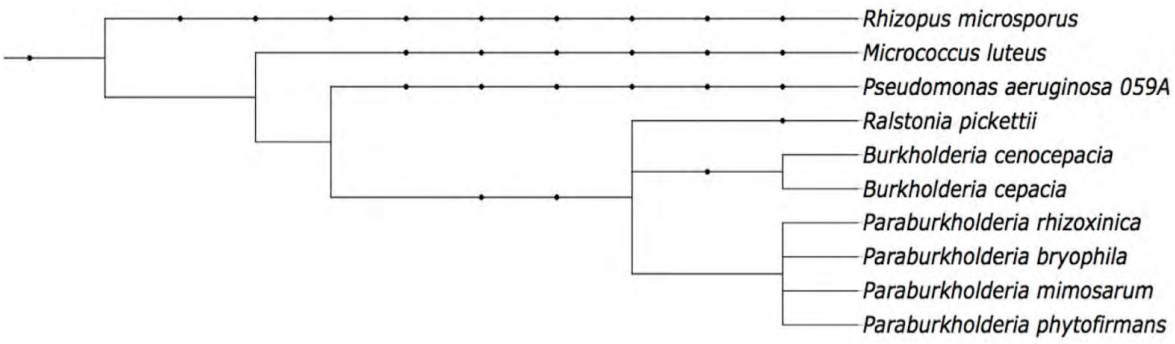


Figure 70: *R. pickettii* is phylogenetically related to Paraburkholderia.

Genomic DNA was isolated from bacterial endosymbionts and whole genome sequence performed to determine identity and phylogenetic relations. The tree shows the phylogenetic relationship between *R. pickettii*, other mucoralean bacterial endosymbionts and other bacterial strains.

5.7.1 Genome sequence of *R. pickettii* shows no evidence of *rhi* gene cluster for rhizoxin

We ought to elucidate both the molecular and genetic properties of *R. pickettii* to specifically characterize and identify the supernatant factors in question. Stemming from the similarities in function to the rhizo-toxins, comparisons between the two were drawn. Rhizoxin biosynthesis is driven by both a polyketide synthase (PKS) and a single non-ribosomal peptide synthetase (NRPS). Several reports have shown the existence of type I PKS gene clusters among metagenomes of rhizoxin producing fungi and isolated endosymbionts (Partida-Martinez and Hertweck, 2007). The *rhi* gene cluster has also been identified and encodes rhizoxin (*rhi*) mega synthase and some accessory enzymes. It is an 81 kb in size with 10 open reading frames (ORFs) A-J flanked by two transposase genes. Five of these ORFs (*rhi* A-F) encode proteins that represent a giant PKS-NRPS thio-template system with domains organized into modules (Partida-Martinez and Hertweck, 2007, Scherlach et al., 2006).

Given this background prediction, a search for similar domains was performed on the *Ralstonia* genome using BLAST, Anti-smash and Pfam tools.

However, all searches failed to return matches for *rhi* homologs in *R. pickettii*. Interestingly though, Pfam prediction yielded a series of domains with similar function to some of the individual genes in the *rhi* cluster (**Figure 71**). The anti-smash search for PKS and NRPS on the other hand returned mainly unidentified gene clusters, with one possible type 1 PKS cluster (**Figure 72a**). The prediction for this was based on an assumed PKS/NRPS collinearity rather than the actual structure (**Figure 72b**) giving similar findings as identified by Pfam. It was also interesting to note that *R. pickettii* shared homologs with other species of *Ralstonia* and *Burkholderia* (**Figure 72c**)

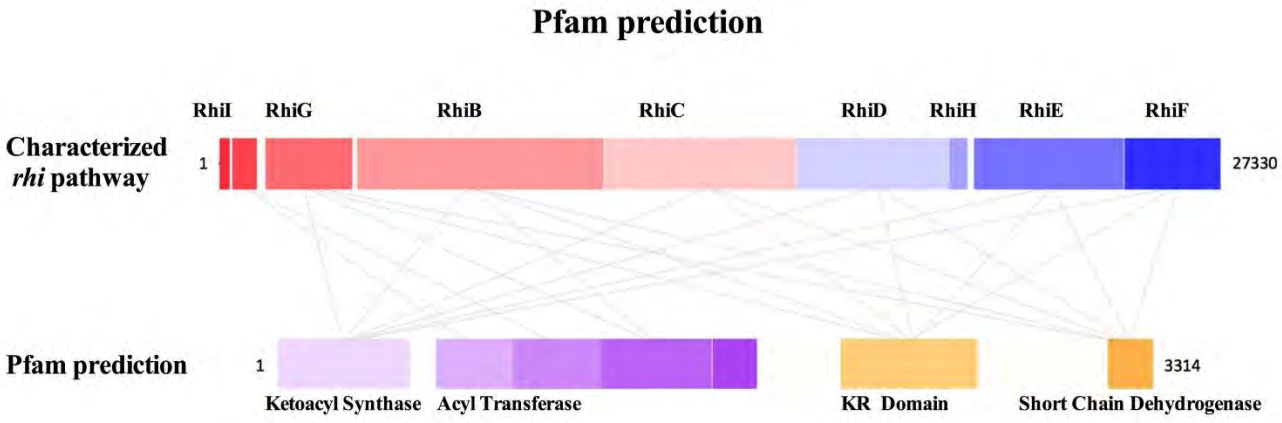


Figure 71: Pfam based prediction for rhi homologs.

Genomic sequence data was used to search for homologs of the rhi gene cluster (PKS) or (NRPS) in *R. pickettii* using the pfam domain data base. The cartoon shows domains in *R. pickettii* with similar predicted functions (*prepared by Poppy Sephton-Clark, unpublished*).

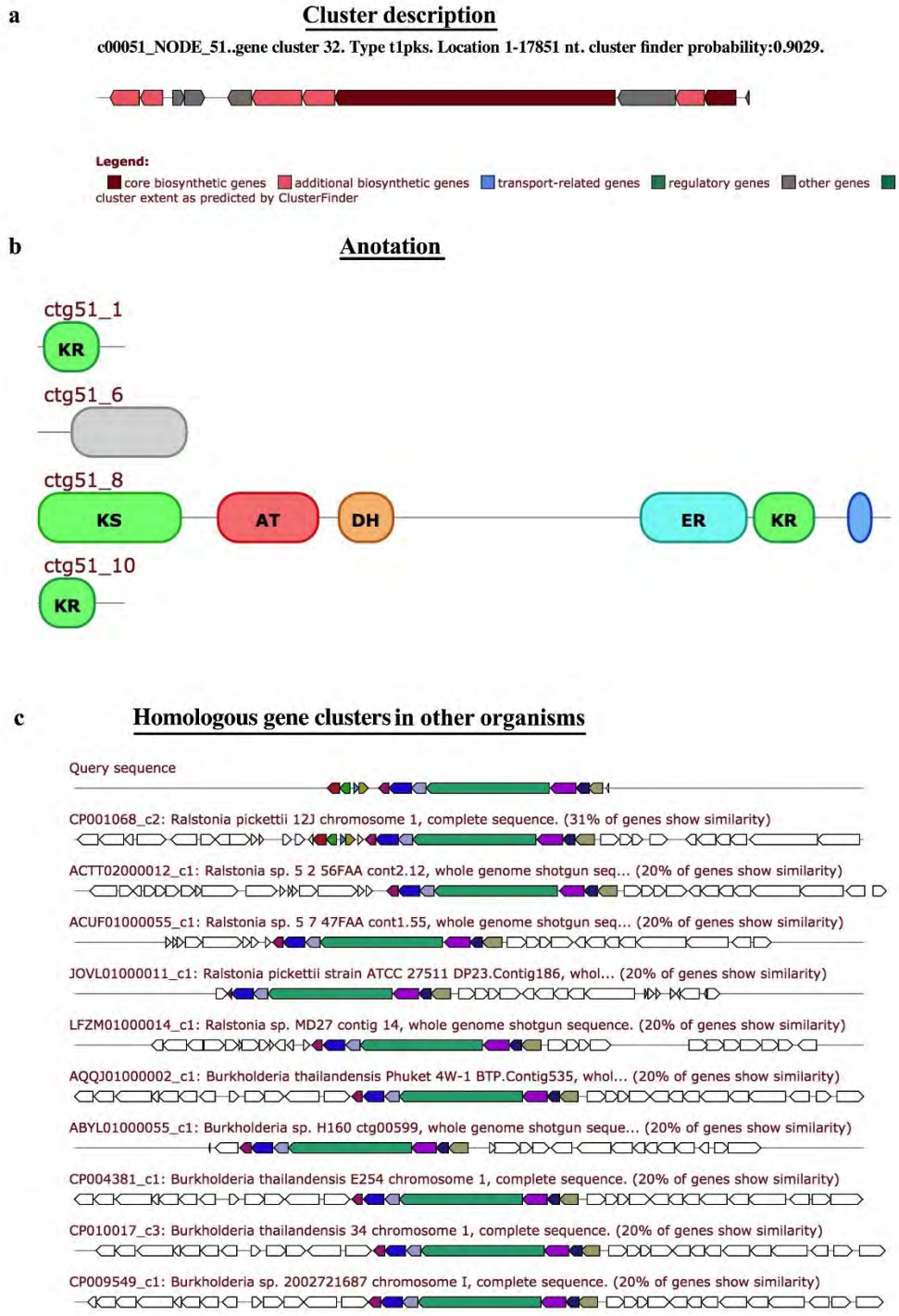


Figure 72: Anti-smash search for polyketide synthetase (PKS) or non-ribosomal polyketide synthase (NRPS) gene clusters.

Genomic sequence data was used to search for homologs of the rhi gene cluster (PKS) or (NRPS) in *R. pickettii* using the Anti-Smash data base for gene clusters. The cartoons represent location of gene, annotation and homologs in other organisms. Anti-smash search reveals (i) a possible type 1 PKS with (ii) co-linearity and similar functional domains to those predicted by pfam. (iii) Homologs of this cluster are also found in other species of *Ralstonia* and *Burkholderia*.

Whilst the findings here showed that *R. pickettii* lacks the explicit *rhi* gene cluster previously reported, it did indicate that domains with similar predicted functions to some of those by *rhi* individual genes may exist. Whether this alludes to the existence of an alternative pathway for rhizo-toxin biosynthesis remains to be explored. Therefore, we can at this stage state that the supernatant factor in question might be similar to the rhizo-toxins in function but perhaps different in the structural signature. This thus remains to be fully identified and characterized.

5.8 Impact of bacterial symbiosis *in vivo*

In vivo dynamics of mucormycete infections and disease progression are poorly understood. Several *in vivo* lab systems are being created to address this concern, and studies by Chamilos *et al* (2008), Ibrahim et al (2008), Voelz *et al* (2015) and Inglesfield *et al* (2018) have all pioneered this in different systems including the fly, mouse and zebrafish models (Chamilos et al., 2008a, Voelz et al., 2015, Inglesfield et al., 2018, Ibrahim et al., 2008b).

In order to place our findings into a more physiologically relevant context, we used *in vivo* models of infection. We employed three systems: Zebrafish, *Galleria mellonella* wax moth, and Mouse models. Whilst fish and wax moth larvae work were done primarily at University of Birmingham, mouse studies were performed in collaborations with Ibrahim Ashraf at the University of California Los Angeles (UCLA) (Section 2.13).

5.8.1 Why these particular models?

The zebrafish (*Danio rerio*) model is extensively used in biomedical research mainly to study embryonic development and haematopoiesis. However, its application has expanded to other fields including immunology. Our application of the model here is based on the fact that following fertilization, the larva survives with only the innate immune system in the early

stages of development. Adaptive immunity in the fish develops morphological and functionally in the later stages post fertilization.

This somewhat temporary separation enables an independent *in vivo* study of innate immunity without interruption of the adaptive system. However, in addition to this there are several other advantages of the zebrafish system that make it suitable for a multitude of vertebrate based studies. These include but are not limited to transparency, which enables real-time visual imaging, its small size, its rapid life cycle, and the fact that it can be easily manipulated genetically (Benard et al., 2012, Brothers and Wheeler, 2012, Ellett and Lieschke, 2010, Harvie and Huttenlocher, 2015, Meeker and Trede, 2008, Novoa and Figueras, 2012, Voelz et al., 2015).

The mouse model was used here because it is more closely related to the human system and thus a more suited model to study human disease (Ibrahim et al., 2008b, Ibrahim et al., 2011). Other advantages of this model include but not limited to; genome has been sequenced, breed fast, relatively cost effective and smaller compared to humans.

The application of the Galleria model here was mainly as an alternative system probing whether mucormycetes can be studied using this set up in future studies (Huang et al., 2015, Maurer et al., 2015, Won et al., 2017, Andrejko et al., 2014, Tsai et al., 2016).

5.8.2 Bacterial symbiosis promotes fungal pathogenicity in *Galleria mellonella* wax moth larvae model

The *Galleria mellonella* wax moth is extensively used for the study of bacterial pathogenesis, and is used for fungal studies with growing frequency, most commonly as an alternative *in vivo* model. It offers an alternative to reduce the number of animals used, as recommended by the National Centre for the Reduction, Replacement and Refinement of animals in Research (NC3R^{s}) (Tsai et al., 2016). Previously the model has been tried with several filamentous and dimorphic fungi including *Candida albicans*, *Penicillium marneffei* and *Aspergillus* in addition

to bacteria studies (Huang et al., 2015, Maurer et al., 2015, Won et al., 2017, Andrejko et al., 2014, Tsai et al., 2016). Thus, application of this model system here was also considered as a possible future alternative system for studying mucoralean fungi *in vivo*. Accordingly, the larvae were injected with two separate doses (low (10^4) and high (10^6)) of resting or pre-germinated spores (+/- endosymbiont) and survival of the larvae determined as described (Section 2.11)

As shown in **Figure 73**, for low dose (LD) infections there were significant differences in mortality of the larvae between parent and cured spores ($p<0.05$). Similar patterns were seen with high dose (HD) infections for swollen spores ($p = 0.0012$). For HD resting spores, there was no statistically significant difference ($p > 0.05$). These findings also show how important bacterial symbiosis could be for fungal pathogenicity. Although the findings here are similar to those obtained using the fish model, more studies are still needed to certify this system for mucoralean fungi.

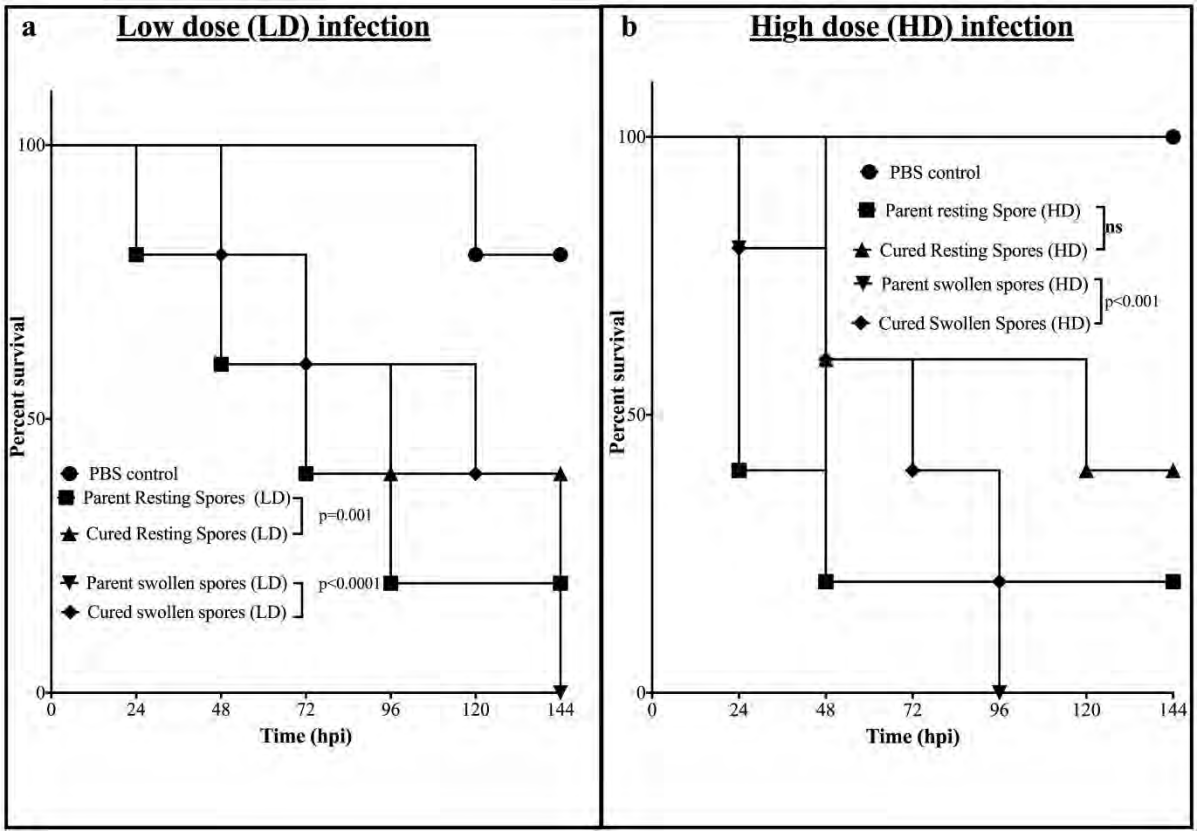


Figure 73: Bacterial symbiosis promotes virulence of *R. microsporus* FP-469-12in Galleria mellonella.

Spores were harvested, pre-germinated or maintained in a resting state and stained before infections were made. Wild type *Galleria* larva were infected with parent or cured strains of *R. microsporus* by injecting with low (10^4/10μL) or high (10^6/μL) doses of the spores as indicated into pro-limbs of the larvae. Three biological replicates of populations of 5 larva each were examined (n=15). Statistical differences were determined using Mantel-Cox with Bonferroni's correction for multiple comparisons (5% family-wise significance threshold = 0.025).

5.8.3 Bacterial symbiosis enables fungal pathogenicity in zebrafish

Based on previous reports by Voelz *et al* (2015) and recently Inglesfield *et al* (2018) that *M. circinelloides* induces early tissue-specific innate immune responses by rapid recruitment of phagocytes, the impact of bacterial symbiosis on both fish and fungal survival was examined. Accordingly, ~24 h old embryos of wildtype (AB) zebrafish were inoculated in the hindbrain ventricle as shown in **Figure 74a** with resting or swollen *Rhizopus* spores (+/- endosymbiont), and survival rates of both fish and the fungus determined. Also, the impact of ciprofloxacin treatment of the fish on both host and pathogen survival was evaluated as described (Section 2.13).

Notably, there was no statistical significance in the rate of mortality due to PVP control and by either resting or swollen endosymbiont free spores ($p > 0.05$). However, significant differences in mortality were seen between parent vs cured resting spores ($p = 0.0006$); and between parent vs cured swollen spores ($p = 0.00323$) (**Figure 74b)**. Fungal survival reveals a similar pattern, with significantly more fish clearing cured as opposed to parent spores of both categories. However, as already shown *in vitro*, swollen spores survived better than resting spores (**Figure 74c**). Interestingly, following treatment with ciprofloxacin, we also saw significant differences in mortality of the fish for both + Cipro and – Cipro for resting spores ($p < 0.0001$); and for swollen spores ($p = 0.0393$). A similar pattern is reflected in fungal survival, although the difference was larger with resting spore, where fungal CFUs are reduced in the treated as opposed to the untreated fish cohort infected with resting spores. Survival of the swollen spores was not significantly affected (**Figure 75**).

It possible that although survival is not as affected, perhaps treatment halted other metabolic processes of the spores, thus slowing down their growth rate and thus the progression of infection. These findings further highlight the importance of the endosymbiont *in vivo,*

particularly in facilitating an infection. They also point to the importance of ciprofloxacin in improving survival of the host. Whether this can be extrapolated clinically for prophylactic treatment of individuals at risk remains to be determined.

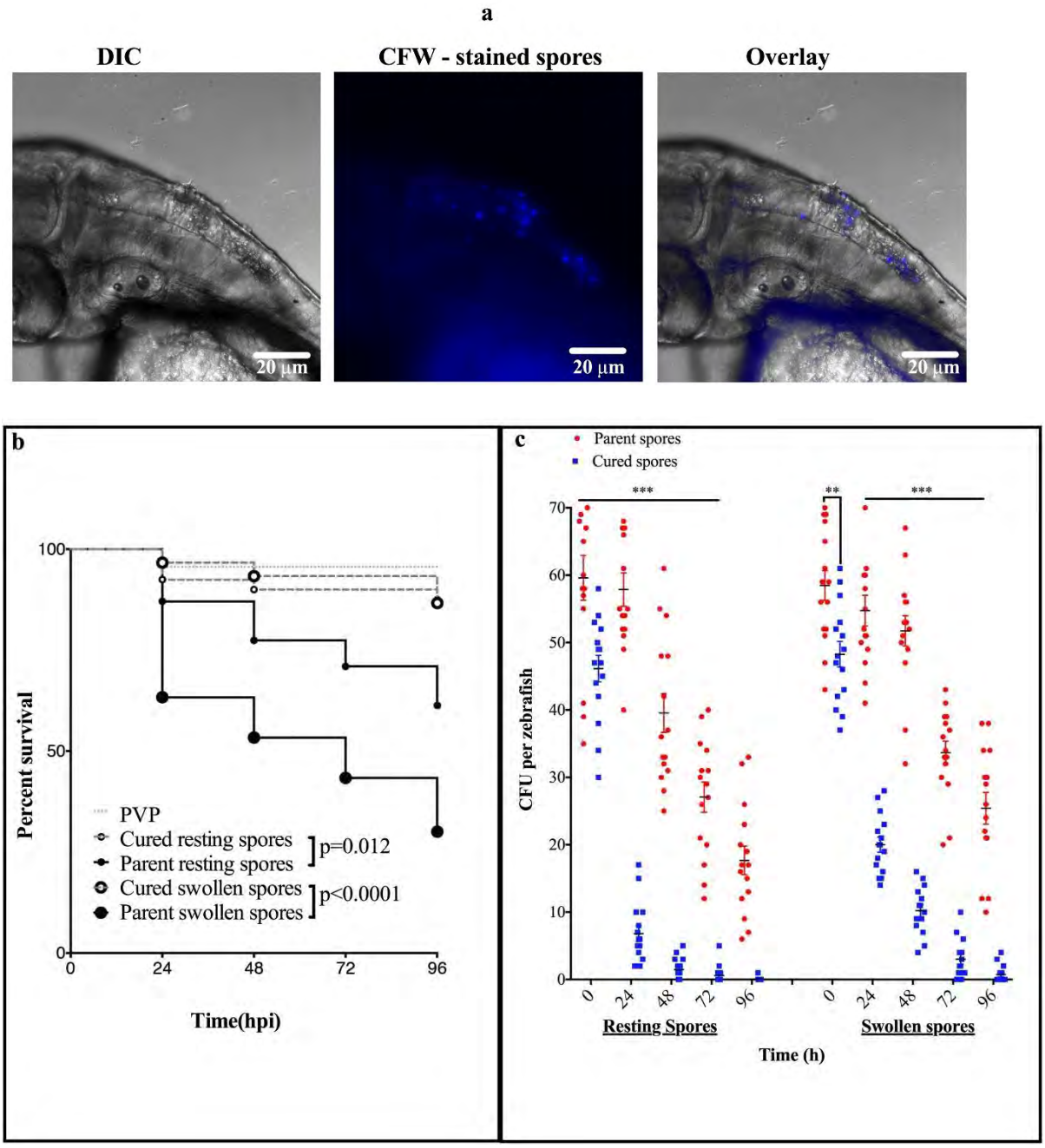


Figure 74: Bacterial symbiosis enables fungal survival *in vivo*.

Spores were harvested, pre-germinated or maintained in a resting state and stained with calcofluor white. (**a)** AB wild type zebrafish were infected with parent or cured strains of *R. microsporus* by injecting into the hindbrain. **(b**) Injected fish were then cultivated in E3 medium and monitored every 24 h.p.i for total of 96 h.p.i with mortality rates assessed every 24 h.p.i. (**c**) Infected fish were sacrificed and homogenized in E3 containing antibiotics every 24 h.p.i for a total of 96 h.p.i, homogenate plated on SDA and incubated at room temperature for colony forming units (CFUs). For **(b)** Three biological replicates of populations of 10 fish each were examined (n=30). Statistical differences were determined using Mantel-Cox with Bonferroni's correction for multiple comparisons (5% family-wise significance threshold = 0.025). For **(c)** Statistical significance was assessed by Two-way ANOVA with Tukey's correction for multiple comparisons (**=p<0.001, ***=p<0.0001).

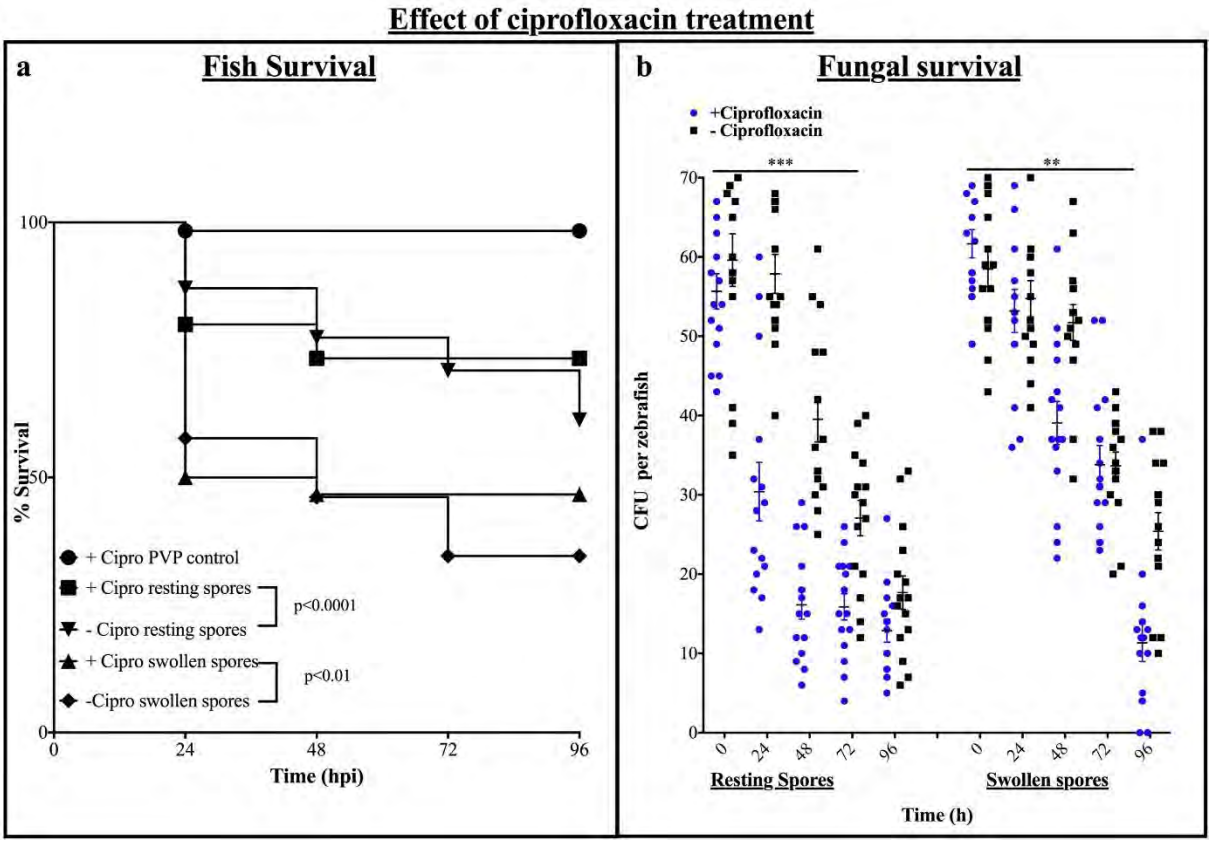


Figure 75: Treatment with ciprofloxacin is protective against infection by *R. microsporus* FP-469-12.

Spores were harvested, pre-germinated or maintained in a resting state and stained with calcofluor white. AB wild type zebrafish were infected with parent or cured strains of *R. microsporus* by injecting into the hindbrain. (a) Injected fish were then cultivated in E3 medium with or without 60 ug/ml of ciprofloxacin and monitored every 24 h.p.i for total of 96 h.p.i with mortality rates assessed every 24 h.p.i. (b) Infected fish were sacrificed and homogenized in E3 containing antibiotics (penicillin, streptomycin and gentamycin) every 24 h.p.i for a total of 96 h.p.i, homogenate plated on SDA and incubated at room temperature for colony forming units (CFUs). **(a)** Three biological replicates of populations of 10 fish each were examined (n=30). Statistical differences were determined using Mantel-Cox with Bonferroni's correction for multiple comparisons (5% family-wise significance threshold = 0.025). **(c)** Statistical significance was assessed by Two-way ANOVA with Tukey's correction for multiple comparisons (**=p<0.001, ***=p<0.0001).

5.8.4 Phagocytes are readily recruited and crucial for containing the infection

We have learned that defective phagocytic effector functions predispose one to mucormycosis (Riley et al., 2016, Ibrahim and Voelz, 2017). However, it also appears that even with a competent phagocyte system one is not entirely free from an infection by mucoralean fungi because of their ability to evade phagocyte killing and possibly establish latency. Inglesfield et al (2018) attests to this by showing formation of granulomas where spores are kept dormant, facilitating a latent infection that is activated when conducive immunosuppressive conditions manifest (Inglesfield et al., 2018). Further, these data suggested that whilst phagocytes are able to recognize and control fungal spores, they can also serve as a reservoir for dormant infection in at risk individuals.

Thus, the impact of bacterial symbiosis on the dynamics of phagocyte recruitment was examined, building on the hypothesis that bacterial symbiosis may prevent phagocyte recruitment. To answer this question, transgenic zebrafish lines with fluorescently tagged macrophages [Tg (mpeg1: G/U: Nfs B-mCherry)] or neutrophils [Tg (mpx: GFP)] were employed as described by Inglesfield *et al* (2018) to determine 1) survival of transgenic fish and 2) phagocyte recruitment following infection. Recruitment was determined and registered both in fish that survived throughout the entire experimental time line (survivors) and those that succumbed to the infection during the experiment (the non-survivors).

As shown in **Figures 76-78,** a similar and comparable pattern in mortality was observed in transgenic and wild type fish. In all models, parent spores killed significantly more fish than cured spores of either category ($p < 0.05$) (**Figures 76a and 76b**). Regarding phagocyte recruitment, it was interesting to see that macrophages (**Figure 77**) and neutrophils (**Figure 78**) are both rapidly recruited to the hindbrain following infection by either category of spores,

with an average cell count of about 20 macrophages and 25 neutrophils after 6 h.p.i post infection ($p < 0.05$) (**Figures 77a, 77b, 78a and 78b**). At this point, however, the data begin to stratify by survival outcome: survivors exhibit distinct patterns of phagocyte recruitment compared to non-survivors.

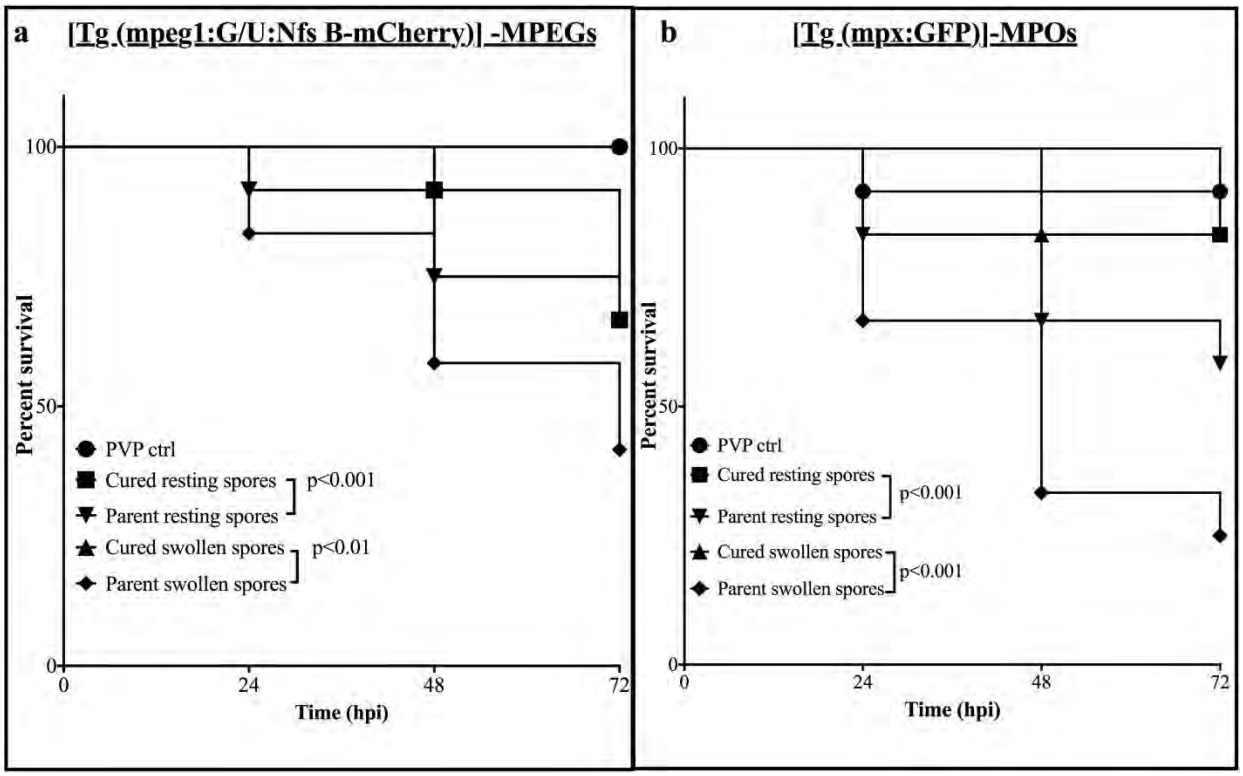


Figure 76: Bacterial symbiosis enables fungal survival following infection of transgenic fish.

Spores were harvested, pre-germinated or maintained in a resting state and stained with calcofluor white before infections were made. Transgenic zebrafish were infected with parent or cured strains of *R. microsporus* by injecting into the hindbrain. Injected fish were then cultivated in E3 medium monitored every 24 h.p.i for total of 96 h.p.i with mortality rates assessed every 24 h.p.i. (a) Mpeg fish expressed mCherry labelled macrophages while **(b)** MPOs expressed GFP labelled neutrophils. (**a)** Three biological replicates of populations of 10 fish each were examined (n=30). Statistical differences were determined using Mantel-Cox with Bonferroni's correction for multiple comparisons (5% family-wise significance threshold = 0.025).

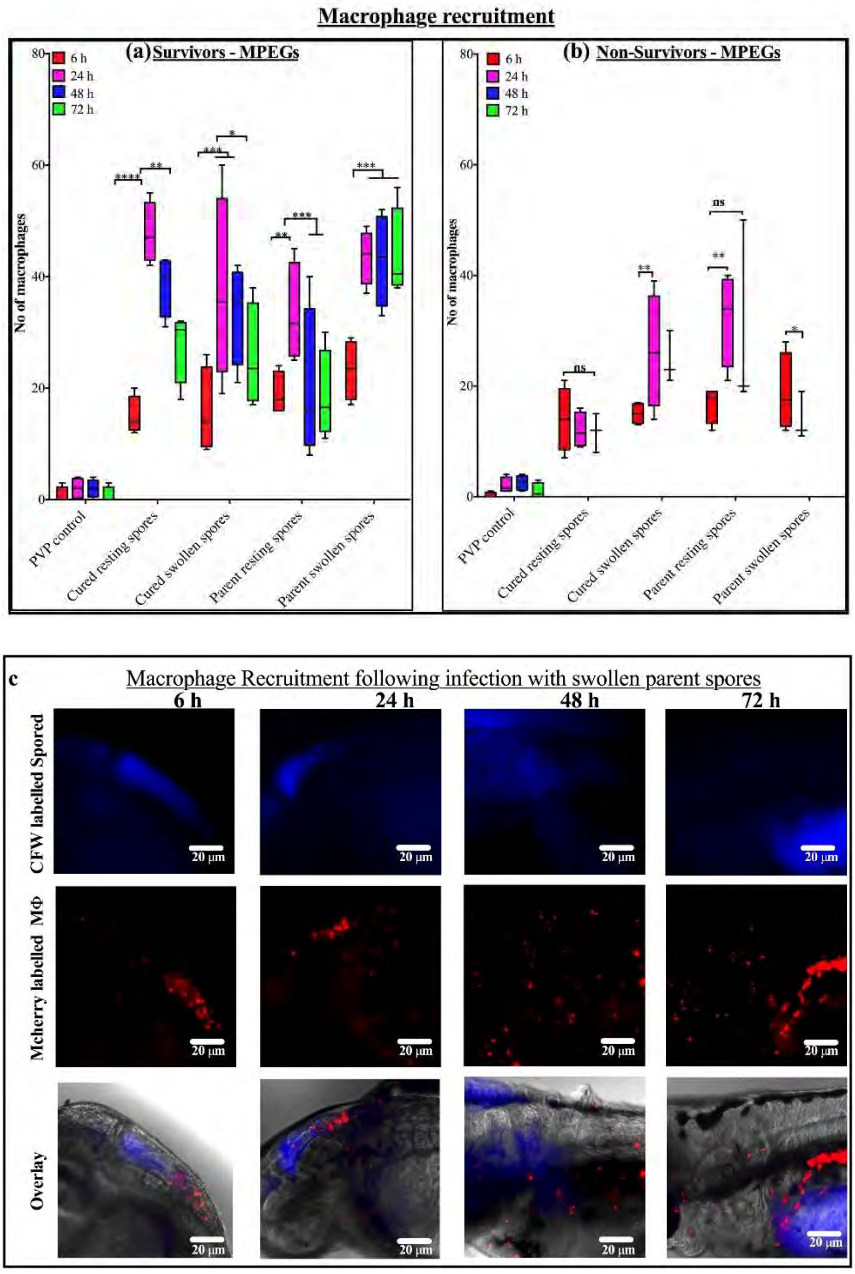


Figure 77: Effect of bacterial symbiosis on macrophage recruitment.

Spores were harvested, pre-germinated or maintained in a resting state and stained with calcofluor white before infections were made. MPEG transgenic zebrafish expressing mCherry macrophages were infected with parent or cured strains of *R. microsporus* by injecting into the hindbrain. The injected fish were then cultivated in E3 medium evaluated for macrophage recruitment. (**a**) fish surviving throughout the entire experiment and (**b**) those that succumbed to the infection during the course of the experiment was recorded. Three biological replicates of populations of 4 fish per condition were examined (n=12). Statistical significance was assessed by Two-way ANOVA with Tukey's correction for multiple comparisons or pairwise t-tests where sample number was unequal due to fish death (**ns**=$p>0.05$, *=$p<0.05$; **=$p<0.001$; ***=$p<0.0001$ and ****=$p<0.00001$). (**c**) Representative micrographs showing recruitment following infection with swollen parent spores are shown.

In the surviving fish, we note a significant and steady increase in recruitment of both cell types from 6 h.p.i to an average count of about 40 macrophages and 55 neutrophils after 24 h.p.i ($p < 0.05$); this was more pronounced for both cell types upon infection with cured spores rather than parent spores (**Figures 77a** and **78a**).

For infections with both cured and parent resting spore types, after 48 and 72 h.p.i there is a significant decline in macrophage recruitment, to about 30 and 20 macrophages respectively ($p < 0.05$) (**Figure 77a**). A similar decline was not observed for parent swollen spores.

Neutrophil recruitment significantly increases after 48 h.p.i ($p < 0.05$) and drops after 72 h.p.i for both cured spore types and more significantly with swollen cured spores ($p < 0.05$) (**Figure 78a**). However, neutrophil recruitment for parent spores significantly increased after 48 ($p < 0.05$) and 72 h.p.i. This effect was more pronounced with swollen spores ($p < 0.00001$) compared to resting spores ($p > 0.05$) (**Figures 77a, 77b, 78a and 78b**).

Additionally, among the survivors, macrophage recruitment variably and significantly ($p < 0.05$) increases in first 24 h.p.i, but then significantly drops after 48 and 72 h.p.i for both endosymbiont free and parent spore metabolic category (**Figure 77a**). Whereas, neutrophil recruitment variably and significantly ($p < 0.05$) increases in the first 24 h.p.i for all spore category, and significantly ($p < 0.05$) drops after 48 and 72 h.p.i for endosymbiont free spores; recruitment is maintained is respectively maintained for parent resting and significantly ($p< 0.05$) increased for parent swollen spores after 48 and 72 h.p.i (**Figure 78a**). A similar pattern is observed among the non-survivors, with significant ($p< 0.05$) increase in both macrophage and neutrophil recruitment but followed with only a significant ($p < 0.05$) decrease in recruitment post 24 h.p.i (**Figures 76b and 77b**).

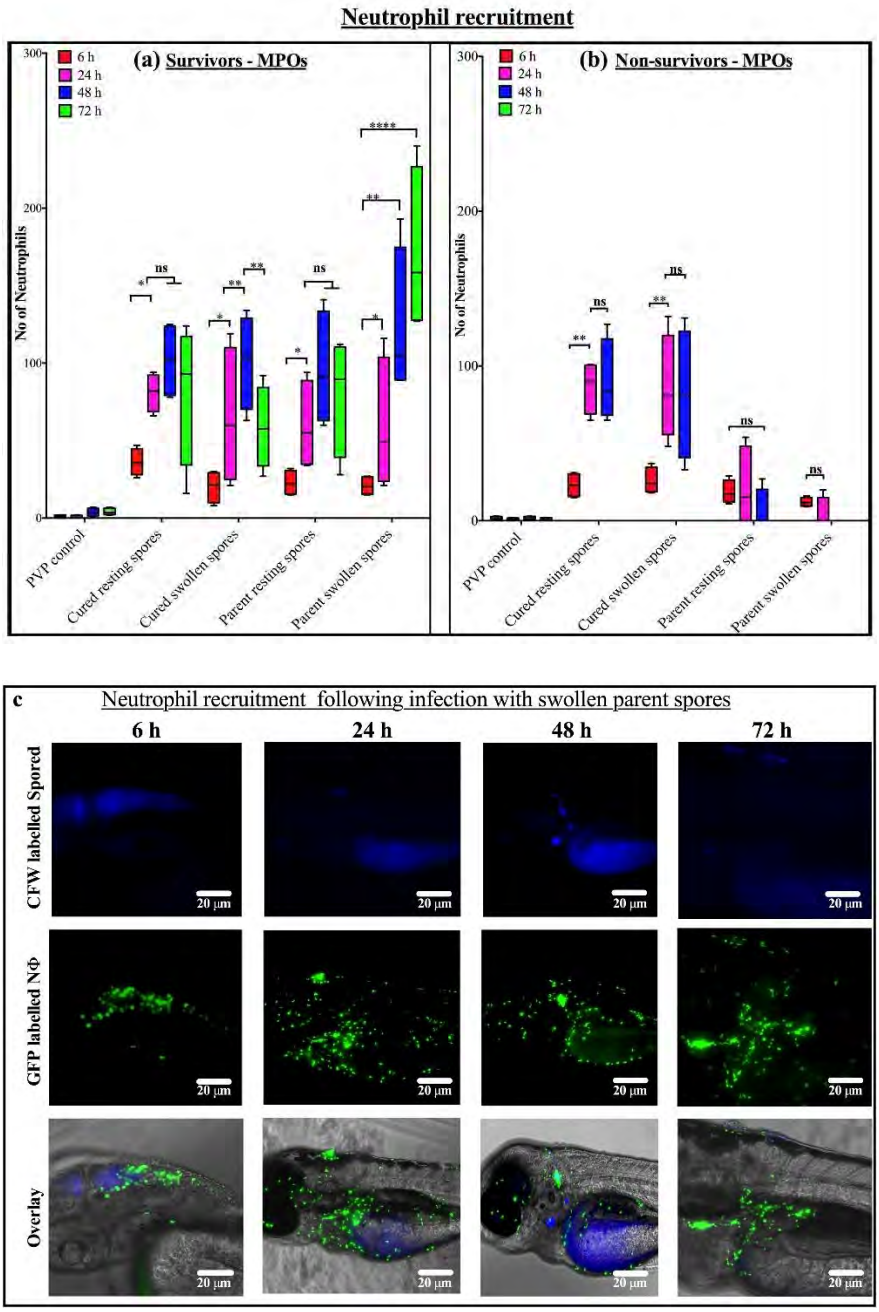


Figure 78: Effect of bacterial symbiosis on neutrophil recruitment.

Spores were harvested, pre-germinated or maintained in a resting state and stained with calcofluor white before infections were made. Transgenic zebrafish expressing GFP neutrophils were infected with parent or cured strains of *R. microsporus* by injecting into the hindbrain. The injected fish were then cultivated in E3 medium evaluated for macrophage recruitment for the indicated time points. (**a**) fish surviving throughout the entire experiment and (**b**) those that succumbed to the infection during the course of the experiment was recorded. Three biological replicates of populations of 4 fish per condition were examined (n=12). Statistical significance was assessed by Two-way ANOVA with Tukey's correction for multiple comparisons or pairwise t-tests where sample number was unequal due to fish death (**ns**=$p>0.05$, *=$p<0.05$; **=$p<0.001$; ****=$p<0.00001$). (c) Representative micrographs showing recruitment following infection with swollen parent spores are shown.

It would appear that recruitment of both macrophages and neutrophils is key but fish survival seems depend more on neutrophil recruitment following infection. This was better demonstrated during infection with parent swollen spores where neutrophils are recruited continually in the surviving fish, while an impairment in their recruitment leads to mortality of the fish as in the non-surviving fish. Indeed, phagocyte recruitment in the non-survivors was the opposite of that observed in the survivors: most of non-survivors demonstrating a defect in recruitment of phagocytes to combat the infection **(Figures 77b and 78b).**

Thus, the findings here clearly highlight the importance of innate immunity, particularly phagocytes, in containing the infections, especially in early stages of infection. Most importantly the impact of bacterial symbiosis on disease outcome is again significantly shown here. With a bacteria endosymbiont, the fungus may still evade host immunity, even with a competent phagocytic system. However, the evasion may not necessarily be solely through prevention of recruitment but also through inhibition of other phagocyte effector functions including phagocyte killing

5.8.5 Bacterial symbiosis promotes fungal pathogenicity in an immunocompetent mouse model

Both the zebrafish larvae and wax moth larvae models have limitations compared to the mouse model. The mouse is a close relative of humans and thus making it physiologically suitable for systemic studies. Thus, to establish the role of bacterial symbiosis in *R. microsporus* virulence during mammalian infection, a mouse mode was employed.

As already mention this part of the project was performed in collaborated with Ibrahim Ashraf, University of California Los Angeles. Accordingly, 10 immunocompetent mice per category were inoculated with resting or pre-germinated spores (+/- endosymbiont). The mice were sacrificed after 4 or 48 h.p.i., lungs harvested, and CFUs determined as described before.

As demonstrated in **Figure 80**, it was interesting to see that parent spores generally survived better than cured spores. For instance, at both 4 and 48 h.p.i we see a significant drop in the recovery of cured spores from 4.3 for parent to 2 .0 CFUs /g for cured resting spores ($p = 0.00102$); and even a further drop at 48 h.p.i from 2.9 for parent to 0.5 CFUs /g for cured resting spores (below the limit of detection) ($p < 0.0001$). A similar pattern is seen with swollen spores, with significant drops at 4 h.p.i from 3.5 for parent to 2.5 CFUs /g for cured swollen spores ($p = 0.01384$); and from 2.0 for parent 1.0 CFUs /g for cured swollen spores ($p = 0.012$).

Generally, there was a significant decline in survival of the fungus from 4 to 48 h.p.i ($p < 0.05$) (**Figure 79**). However, these findings revealed a lack of efficient clearance of spores with the endosymbiont as opposed to ones without. Whether latency is a possible outcome here remains to be explored. The results here further affirm that bacterial symbiosis might play a key role during fungal survival *in vivo*. Whether this impacts on both disease progression remains to be fully explored.

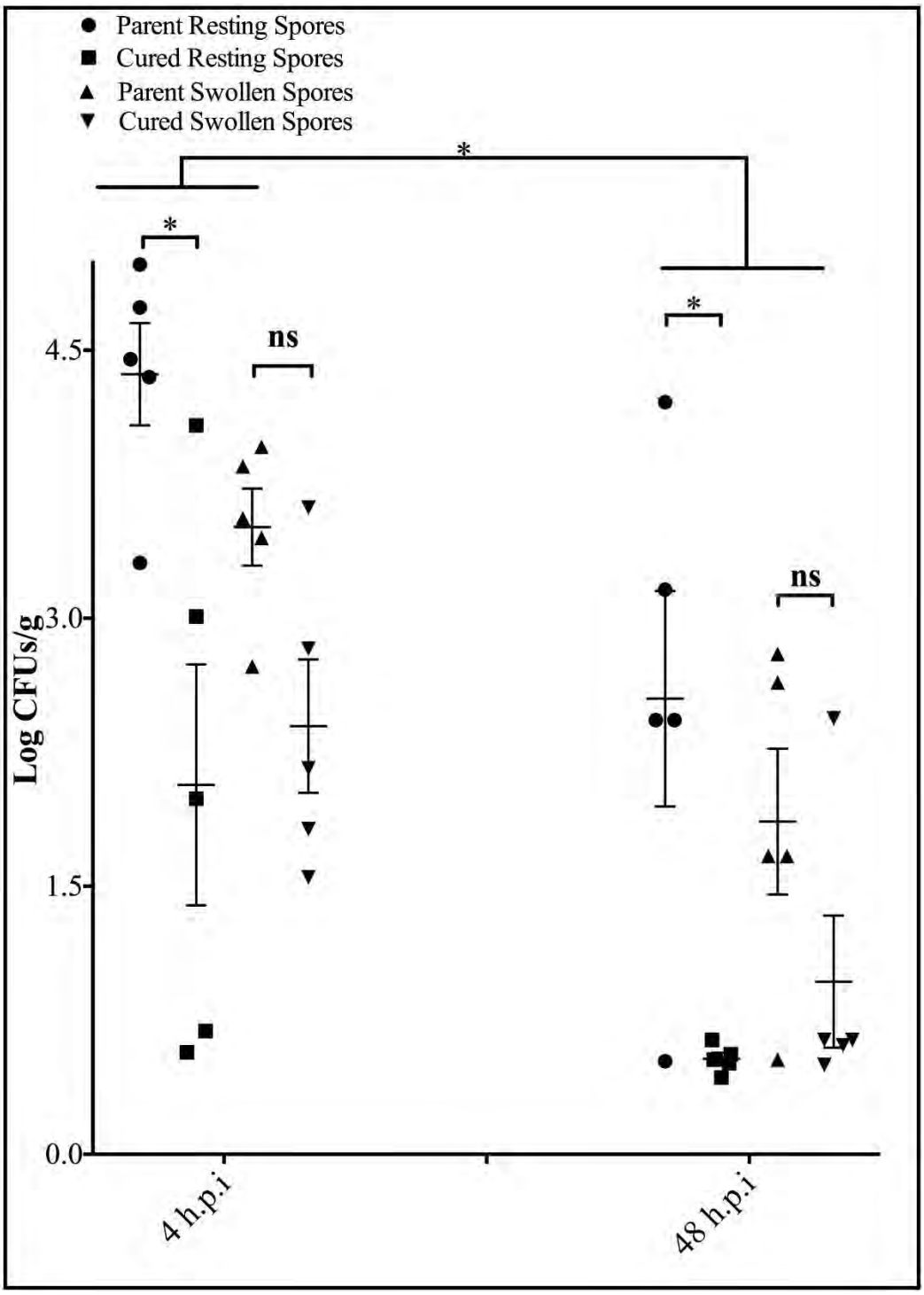


Figure 79: Bacterial symbiosis influences fungal pathogenicity in a mouse model.

Spores were harvested, pre-germinated or maintained in a resting state before infections were made. Immunocompetent CD-1 male mice were infected with 10^6/25 µL with parent or cured strains of *R. microsporus* intratracheally. The mice were sacrificed at 4 and 48 h.p.i, lungs collected, homogenized and homogenate plated on PDA +0.1% triton at 37ºC. Effect of endosymbiont status on fungal survival (CFUs) following intra-tracheal infection of mice at 4 and 48 hours with resting and swollen spores (n=5). Statistical significance was assessed by Two-way ANOVA with Tukey's correction for multiple comparisons (**ns**=$p>0.05$; ***=**$p < 0.01$).

5.9 Discussion

In this work, we demonstrate that the human fungal pathogen *R. microsporus* harbours an endosymbiont capable of influencing fungal interactions with mammalian phagocytes and modulating fungal virulence. Our findings highlight an emerging theme in fungal pathogenesis that urges a broader view of fungal growth and virulence to encompass cross-kingdom interactions reflecting the ecological origins of infecting species. Recent work has shown that several fungi like *Fusarium* and Mycorrhizal fungal species harbour bacterial endosymbionts that influence fungal phenotypes (Shaffer et al., 2017, Husnik and McCutcheon, 2018, Hoffman et al., 2013, Frey-Klett et al., 2011, Araldi-Brondolo et al., 2017).

The mucormycetes, particularly *R. microsporus*, one of the prevalent causative agents of fatal mucormycosis, has been reported to harbour bacterial endosymbionts including *Burkholderia rhizoxinica* and *Burkholderia endofungorum* species (Gee et al., 2011, Lackner et al., 2009b, Partida-Martinez et al., 2007a, Partida-Martinez et al., 2007b, Partida-Martinez and Hertweck, 2005, Scherlach et al., 2006, Partida-Martinez et al., 2007c). It is becoming evident that *Rhizopus* and these endosymbionts form a highly specific and complex association that significantly influences the fungal host's metabolic and sexual phenotypes (Moebius et al., 2014a, Mondo et al., 2017). Additionally, endobacteria are a source of potent antimitotic and hepatotoxic mycotoxins such as rhizoxin and rhizonin and can influence fungal pathogenesis in plants (Scherlach et al., 2006, Partida-Martinez and Hertweck, 2007, Partida-Martinez and Hertweck, 2005, Partida-Martinez et al., 2007b, Partida-Martinez et al., 2007a, Lackner et al., 2009b). However, the role of these endosymbionts in pathogenesis during mucormycosis has yet to be clinically defined. In this study, we report that a clinical isolate of *R. microsporus*, and other mucoralean fungi harbour bacterial endosymbionts that influence host–pathogen interactions including phagocytosis and macrophage killing.

We further show that *R. microsporus* spore survival following phagocytosis by macrophages is dependent on the presence of an endobacterium. Elimination of the bacterial endosymbiont by ciprofloxacin treatment facilitates macrophage killing and *in vivo* clearance of the fungal spore. In this study, we also observed that this is facilitated by the production of bacterial toxin consistent with rhizoxin. We further noted that this toxin exhibits several anti-phagocytic effects including impacting mammalian cell morphology and cytoskeleton organisation, particularly actin and beta tubulin polymerisation. These findings agree with reports that show that bacterial symbiosis facilitates plant pathogenesis by *R. microsporus* through production of the phytotoxin rhizoxin, an antimitotic agent that targets beta tubulin (Lackner et al., 2009b).

Two groups recently reported that although bacterial endosymbionts are widely found among mucormycete clinical isolates and produce the toxins rhizoxin or rhizonin, they did not impact pathogenesis during mucormycosis in both mice and fly models (Ibrahim et al., 2008b, Partida-Martinez et al., 2008). Our findings differ from these reports because elimination of the endobacterium restores phagocytosis, and promotes macrophage killing.

Several reports continue to report existance of endobacteria among fungi and their influence on fungal survival on several fronts, particularly on their metabolism, sexuality, antifungal reistance, and this is clearly being noticed across an array of fungal families (Shaffer et al., 2017, Nazir et al., 2014, Mondo et al., 2017, Moebius et al., 2014a, Frey-Klett et al., 2011, Benoit et al., 2015, Zheng et al., 2017, Salvioli et al., 2016, Hoffman et al., 2013, Araldi-Brondolo et al., 2017). From this stand point, it is early to write off the importance of bacterial symbiosis in the pathogenesis of mucormycosis. Furthermore, harnesing the role of bacterial symbiosis could prove key in revolutionalising mucormycotic management through combination therapy of anifungal and antibiotics.

In summary, we have shown that *R. microsporus* and other mucormycetes harbour a bacterial

endosymbiont. In *R. microsporus* this endosymbiont secretes a hydrophobic compound that affects several phagocytic effector functions including cell morphology and its elimination reverses all the effects. Whilst these anti-phagocytic phenotypes are consistent with those established with rhizoxin here, it is too early to implicate rhizoxin but rather late to explore the role of bacterial endosymbiosis in the pathogenesis of mucormycosis.

The data presented here describes ways of screening mucoralean fungi for bacterial endosymbionts using microscopy coupled with PCR and *in vitro* isolation; and the effects of endobacterium on fungal host phenotypes including phagocytosis, HPLC and *in vivo* studies. All these methods permit high through put screening for endobacterium among filamentous fungi. However, other more sensitive and specific techniques such as NMR, MS/LCS would be required to characterise this anti-phagocytic compound better. Furthermore, the effect of the factor on other cell lines such as PBMCs would be required to increase the relevance of the findings. On the other hand, *in vivo* model used in this study are adequate (Section 5.7.1) but our findings would require more repeats especially using the mouse to improve relevancy of the findings. In Future, the impact of the endo bacterium of cytokine profile can also be investigated and other infection models such the amoebae can also be employed. Ciprofloxacin was the antibiotic employed here in comparison with other authors, but these a gap to try other antibiotics against endobacterium. Finally, the genetics findings presented here are superficial and would require more detailed analysis especially on the co-evolutionary relationship front.

CHAPTER SIX: Additional work

We know now that *R. microsporus* spores secrete compounds with anti-phagocytic activity. Most importantly the compounds are secreted by a bacterial endosymbiont highlighting the role of the bacteria in fungal survival and metabolic attributes. Whether the secreted factor here is similar to what has been characterised such as rhizo-toxins remains unknown.

Stemming from the data presented in this thesis, we have proposed three hypotheses based on the chemistry data particularly peak 5.

1. That the fungus + endosymbiont make peak 5.
2. The fungus is a signal for the endosymbiont to make peak 5.
3. That the endosymbiont is a signal for the fungus to make peak 5.

Firstly; we tried to establish which of the two (bacteria or fungus) is the signal for the other to produce the metabolic compounds in question. This mainly stems from the data that show bacteria as producers of a large range of products in HL5 and VK compared to DMEM medium. Indicating that production of the active compounds could be significantly affected by the growth conditions suggesting that perhaps these conditions reflect those when living in symbiosis with the fungus (**Figure 82**)

So, to examine this, the bacteria were co-incubated with both live and heat killed mashed fungal substrate grown in under different conditions, supernatants collected and activity against phagocytosis determined as described before (Section 2.5)

As shown in **Figure 83**, co-incubation with mashed but live fungal substrate showed relatively more activity as opposed to that with heat killed substrate. This may suggest that the fungus is the signal production of the active compound by the bacteria but requires the fungus to be live for that to happen. More work is still needed to fully establish this.

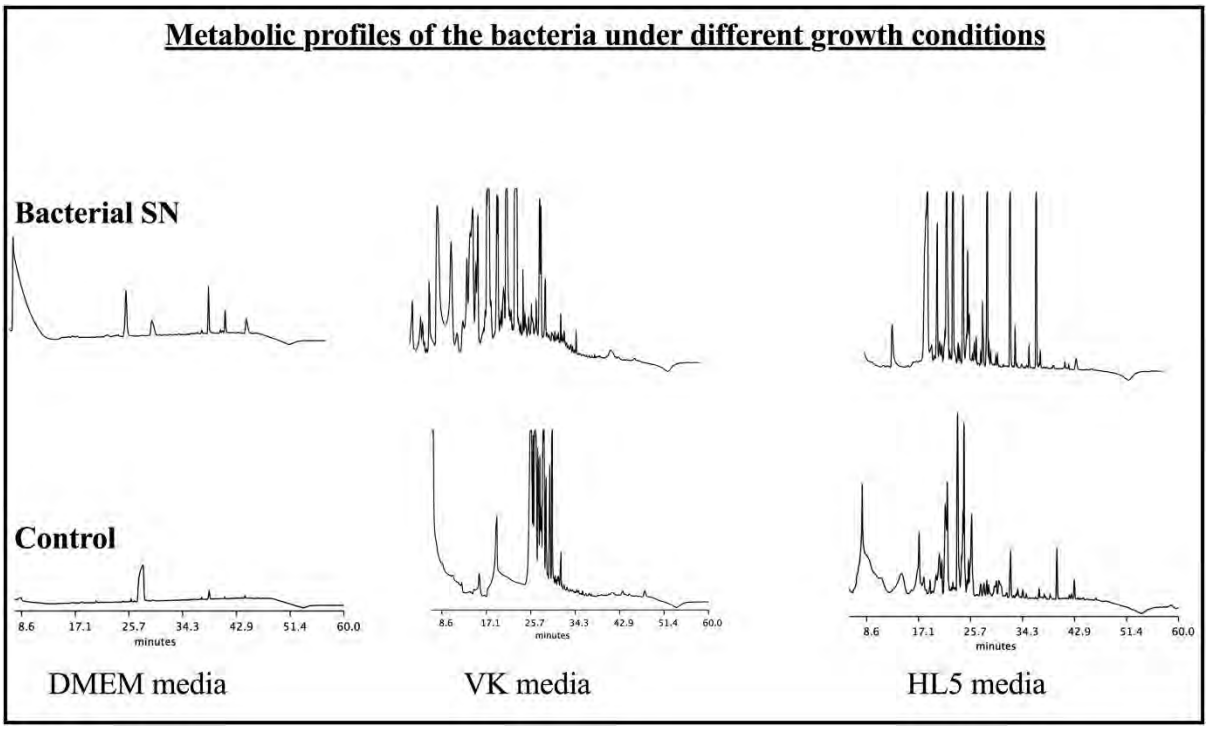


Figure 80: Growth conditions can influence bacterial metabolism.

R. pickettii bacteria was fermented in different culture medium as indicated at 30°C, 80 rpm for 2-4 days. Supernatants were collected, extracted with chloroform and HPLC profiled using C18 column. Medium controls were also included. Chromatograms presented are from one of at least three biological repeats.

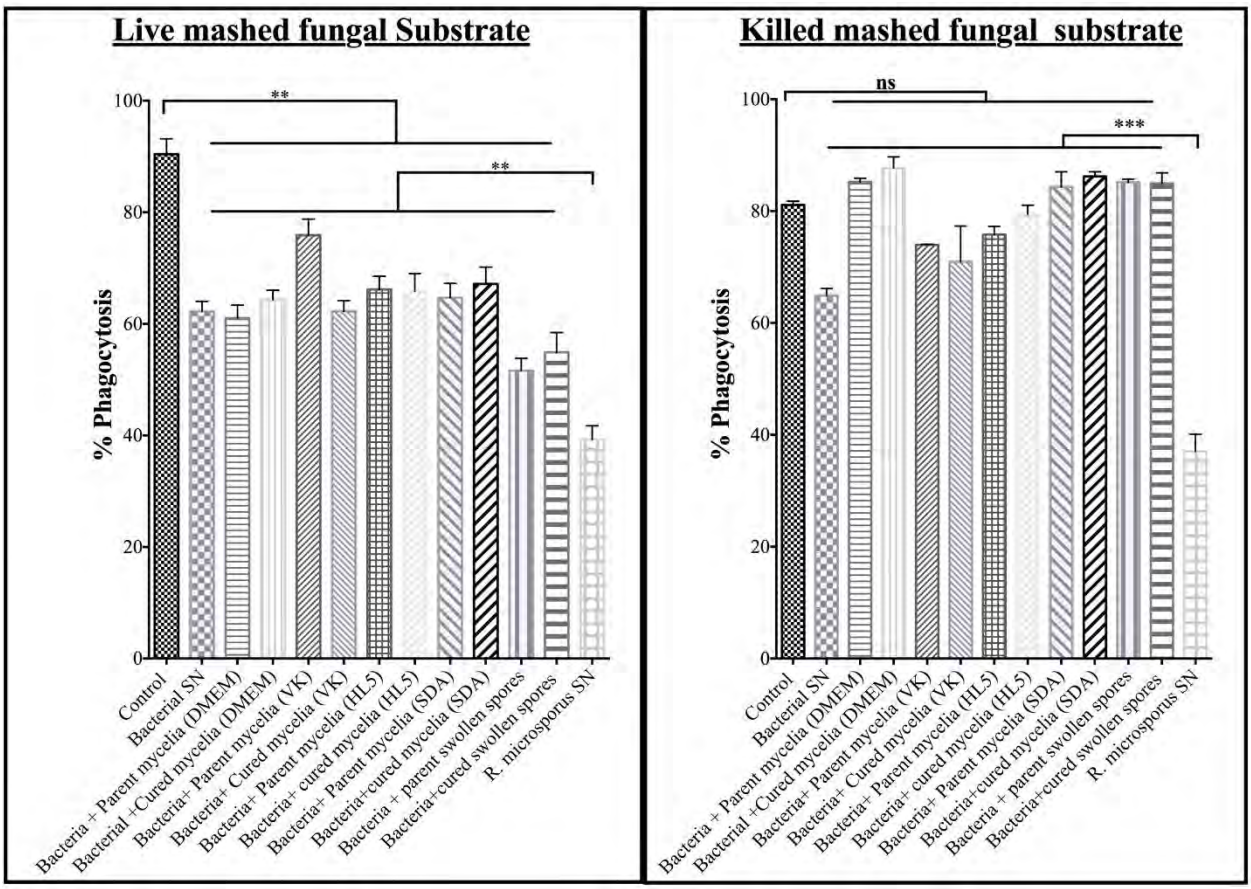


Figure 81: Effect of filtered supernatants from co-cultures of bacterial symbionts and different fungal substrates on phagocytosis by J774 macrophages.

Both the parent and cured strains were grown in different media conditions, mycelia collected, processed as indicated and co-incubated with bacteria in sfDMEM. The resultant supernatants were collected and used to treat J774 macrophages for 1 h then co-incubated with dormant spores and phagocytosis determined. For all conditions, the number of phagocytes containing at least one spore were counted after 1 hour (n=9000, One way ANOVA with Tukey's correction for multiple comparisons). In each graph, three biological replicates were examined and error bars represent s.e.m. (**ns**=$p>0.05$, **=$p<0.001$, ***=$p<0.0001$).

CHAPTER SEVEN: Final Discussion

7.1 Final discussion

This project has explored interaction mechanisms between the infecting particles of mucormycetes and the host cell lines in addition to utilising a series of animal models. Through employing a clinical isolate of *R. microsporus*, we have made vital observations, specifically that this pathogen and other mucoralean fungi harbour bacterial endosymbionts.

The data presented herein shows that these bacterial endosymbionts can modulate host-fungal interactions. Yet this happens to be an emerging theme that has already been explored to some extent in plant pathology (Shaffer et al., 2017, Husnik and McCutcheon, 2018, Hoffman et al., 2013, Frey-Klett et al., 2011, Araldi-Brondolo et al., 2017).

Fungi appear to harbour a wide diversity of bacterial endosymbionts that are specifically adapted to colonize respective fungal hosts and significantly influence host-relevant fungal phenotypes, including metabolism, cell wall organization, development, and plant host colonization. Indeed, these endosymbionts can be a source of potent mycotoxins that can influence fungal pathogenesis (Moebius et al., 2014a, Mondo et al., 2017, Shaffer et al., 2017, Hoffman et al., 2013, Araldi-Brondolo et al., 2017, Uehling et al., 2017, Ibrahim et al., 2008b, Takashima et al., 2018, Gee et al., 2011, Partida-Martinez et al., 2007b, Partida-Martinez and Hertweck, 2005, Scherlach et al., 2006, Nazir et al., 2014, Frey-Klett et al., 2011, Benoit et al., 2015, Zheng et al., 2017, Salvioli et al., 2016).

The role of these endosymbionts clinically is still un appreciated, representing a significant gap in our understanding of the pathogenicity of mucormycetes. Here we provide evidence that a bacterial endosymbiont may significantly influence its interaction with mammalian hosts. We show that during the early steps of spore-germination, the bacterium secretes a factor that inhibits phagocytosis. In addition, *R. microsporus* spore survival following phagocytosis by macrophages is dependent on the presence of the endobacterium. Elimination of the bacterial

endosymbiont by ciprofloxacin treatment facilitates macrophage killing and *in vivo* clearance of fungal spores.

While previous work has mainly focused on *Burkholderia-Rhizopus* endosymbiosis, we identify a previously unappreciated niche for the related gram-negative bacterium *Ralstonia pickettii* as an endosymbiont of *R. microsporus.* Consistent with long-term mutualism, we observed an overall reduction in RNS/ROS stress resistance and virulence in a cured *R. microsporus* isolate. We also observed reduced sporulation and overall fitness in this strain after repeated passage of fungal growth on SDA plates with cirpofloxacin. Mondo *et al.*, showed that bacterial endosymbionts can impact fungal sporulation and development through controlling *RAS2,* a key GTPase that regulates fungal sexual reproduction (Mondo et al., 2017). Additionally, consistent with a change in lipid biogenesis, we observe changes in the fungal plasma membrane in cured spores, as well as increased sensitivity to Amphotercin B, indicative of altered ergosterol content. Loss of the endosymbiont had profound impacts on *R. microsporus* response to phagocytosis. Together, our findings support a mutualistic lifestyle for *R. pickettii* and *R. microsporus.*

In addition to supporting fungal development and stress resistance, we show that *R. pickettii* inhibits phagocytosis by macrophages during the initial steps of germination. We demonstrate that this is facilitated by the production of a unique bacterial factor. We further note that this factor exhibits several anti-phagocytic effects including impacting mammalian cell morphology and cytoskeleton organisation, particularly actin and β-tubulin polymerization as well as phagosome maturation. These findings add to reports of bacterial endosymbionts that facilitate pathogenesis, first revealed by the finding that *B. Rhizoxina* increases *R. microsporus* pathogenesis through production of the phytotoxin Rhizoxin, an antimitotic agent that targets the cytoskeleton (Lackner et al., 2009b).

However, this factor appears to be distinct from the Rhizo-toxins in activity and biosynthesis, as evidenced by analysis of secondary metabolite clusters in the *R. pickettii* genome. Further, we show that the presence of the endosymbiont reduces fungal sensitivity to host phagocyte antimicrobial activity and that this can have implications for pathogenesis during the earliest stages of mammalian infection.

Two groups recently reported that although bacterial endosymbionts, including Rhizoxin and Rhizonin producers, are widely found among mucormycete clinical isolates, these endosymbionts did not impact pathogenesis during mucormycosis in both mouse and fly models (Ibrahim et al., 2008b, Partida-Martinez et al., 2008). The influence of endosymbiont-derived Rhizoxin on pathogenesis was assessed in a diabetic mouse model (Ibrahim et al., 2008b). Diabetic mice were infected with *Rhizopus* isolates harbouring Rhizoxin-producing *Burkholderia* endosymbionts and then treated with either Amphotericin B antifungal or Ciprofloxacin antibacterial or both 24 hours post-infection. No impact for Ciprofloxacin treatment was observed in that work (Ibrahim et al., 2008b).

In contrast, in our work, when immune-competent mice were infected with resting spores pre-treated with Ciprofloxacin, CFUs were reduced compared to untreated within 4 hours and spores could no longer be recovered after 48 hours. It should be noted that the work here was performed in a small number of mice (5 per group) and therefore is prone to the challenges associated with underpowered models of infection. However, we observed similar patterns in the zebrafish model of infection, which simultaneously allows larger study populations and detailed dissection of mechanisms of innate immune defence. We again observed a profound improvement in zebrafish larval survival upon infection with spores lacking the endosymbiont.

In both models, the endosymbiont appears to play a more significant role during the transition from resting to swollen spore, however direct infection with swollen cured spores was still associated with reduced host death. We hypothesize that in our model, the elimination of the endobacterium restores robust phagocyte recruitment and promotes macrophage killing of fungal spores, factors that remain defective in the diabetic or immune-suppressed host models.

During *in vivo* infection of immunocompetent mice, the closely related fungus *R. oryzae* was shown to be phagocytosed but not killed by alveolar macrophages, while un engulfed spores were associated with neutrophil recruitment (Andrianaki et al., 2018a). Swollen spore survival was linked to resistance to iron limitation and melanin-mediated arrest of phagosome maturation. These findings are consistent with observations in zebrafish showing the formation an early granuloma comprised of macrophages and neutrophils in response to *M. circinelloides* that likewise controlled but failed to kill spores and suggest a similar host response to a variety of infecting Mucorales species (Voelz et al., 2015, Inglesfield et al., 2018). Spore survival within macrophages *in vitro* and within early granuloma in immunocompetent mice indicate a potential reservoir for latent fungal infection. Indeed, an emerging trend in clinical reports of mucormycosis is the unappreciated burden of indolent or chronic mucormycosis (Rodriguez-Lobato et al., 2017, Desoubeaux et al., 2014, Gamarra et al., 2018). The observation that elimination of bacterial endosymbionts impacts cell wall organization, resistance to host relevant stress, and resistance to macrophage-mediated killing raises questions on the possibility that modulation of fungal endosymbiont status may impact fungal latency and allow clearance.

Together, our findings highlight the importance of bacterial symbiosis in the pathogenesis of mucormycosis. However, we urge caution in extending our findings to all Mucorales or fungal endosymbionts. While a number of studies have demonstrated general features common to

host-Mucorales interactions, there may be species-specific aspects that influence Mucorales pathogenesis. In addition, not all clinical isolates profiled do harbour endosymbionts, suggesting that endosymbionts may augment, but are not constitutively required for, pathogenesis (Ibrahim et al., 2008b). Overall, our findings point to a role for the bacterial endosymbiont in fungal immune evasion during infection via a secreted factor and raise the possibility of a role in reactivation of latent infections with resting spores in patients undergoing immunosuppressive therapy (Brunet et al., 2018). Harnesing the role of bacterial symbiosis could prove key in revolutionalising mucormycotic management through combination therapy of anifungal and antibiotics.

SUMMARY

Mucormycosis is a major health concern and its prevalence continues to grow among an array of at-risk individuals. Aetiology of the disease is mainly dominated by *Rhizopus* species and understanding their pathogenicity is key to our exploration of disease progression. In this regard, we also report here that metabolic spores of *R. microsporus* are able to evade phagocyte effector functions as recently shown in *R. oryzae.* Interestingly, our findings show that this metabolic process is attributed to bacterial symbiosis influencing pathogenicity of *R. microsporus* both *in vivo* and *in vitro.* Fascinatingly these findings come at the time when several studies exploring poly-microbial interactions including bacterial-fungal interactions have reported that bacteria are capable of networking with fungi at various levels, subsequently leading to modulations in substrate consumption, enzyme production, thermotolerance, sexuality, antifungal resistance, metabolism and virulence

Although bacterial endosymbionts such as *B. rhizoxinica* and *B. endofungorum* have been implicated in plant pathology via rhizo-toxin production and studies have so far shown that these bacteria may not contribute towards human pathogenesis of mucormycosis. Here, we have shown that *R. microsporus* may harbour other symbionts such as *R. pickettii*, a close relative of *B. rhizoxinica* in the family Bukholderiaceace. This is heavily involved fungal metabolism and promotes evasion of innate immunity by the fungus via production of a factor similar to the rhizo-toxins in function. To the best of our understanding this is the first report to show that bacterial symbiosis may contribute towards mucoralean pathogenicity and further examination of this concept can heavily contribute towards clinical management of mucormycosis. It has already been hypothesised before that endobacteria may contribute towards pathogenicity of mucoralean fungal hosts, and the evidence provided in this thesis serves as an initial step into providing answers for this question.

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