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PhD Thesis

Study of *Lomentospora prolificans* pathogenicity: Host immune response and fungal virulence factors

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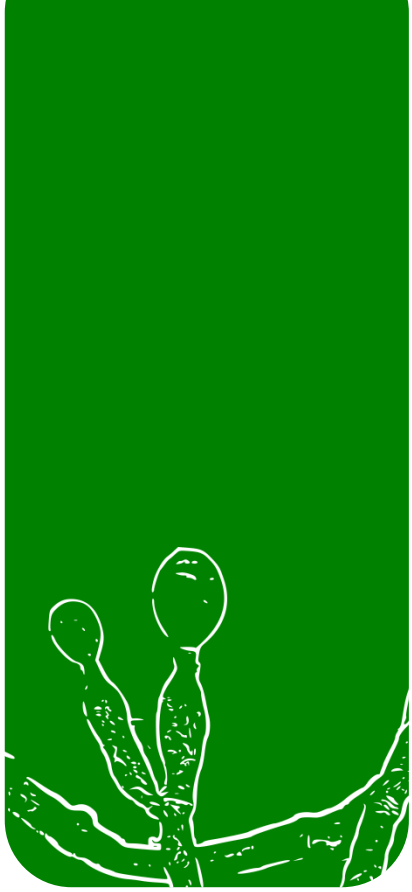
List of abbreviations

1-DE	One dimensional electrophoresis
2-DE	Two dimensional electrophoresis
AIDS	Acquired immune deficiency syndrome
bp	Base pair
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
CECT	Spanish Collection of Type Cultures (Colección Española de Cultivos Tipo)
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DMEM	Dulbecco's modified Eagle medium
DMSO	Dymethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotides tri-phosphate
DTT	Dithiothreitol
ECMM	European Confederation of Medical Mycology
EDTA	Ethylenediaminetetraacetic acid
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HBSS	Hank's balanced saline solution
HRP	Horseradish peroxidase
IAA	Iodoacetamide
IEF	Isoelectric focusing
IMDM	Iscove's modified Dulbecco's medium
ITS	Internal transcribed spacer
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MALDI	Matrix assisted laser desorption ionization
MIC	Minimum inhibitory concentration
MIC_{50}	Minimum inhibitory concentration for 50% inhibition of fungal growth
MOPS	3-(N-morpholino)propanesulphonic acid
Mr	Relative molecular mass
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCBI	National Center for Biotechnology Information
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
p*I*	Isoelectric point
PMF	Peptide mass fingerprinting
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SDS-PAGE	Polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate
SEM	Standard error of the mean
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TOF	Time of flight

Introduction



1. General considerations of *Lomentospora prolificans*

Lomentospora prolificans is a saprophytic filamentous fungus that can become pathogenic to both humans and animals especially when their immune system is compromised. This species is classified into the family *Microascaceae*, order *Microascales*, phylum *Ascomycota*, being its particular taxonomic status further presented later on.

The fungus is able to grow on a wide range of liquid and solid culture media, in which it forms colonies moderately fast that display a downy and moist surface with white colour at the beginning, later turning from brownish olive-grey to black when conidiation becomes evident (Figure 1 A). Microscopically, *L. prolificans* produces conidiophores with swollen bases, which is an important taxonomic feature of the species (Figure 1 D). On the contrary to its relatives, this fungus is not known to either present other asexual forms, such as *Graphium*-like conidiophores, or sexual reproduction (Cortez *et al.*, 2008). In addition, *L. prolificans* displays septate hyphae from which flask-shaped conidiophores may rise, being some isolates able to develop conidia directly from hyphae. Single-cell conidia are thick-walled, ovoid or pyriform, hyaline to pale brown, and measure 2 to 5 by 3 to 13 µm.

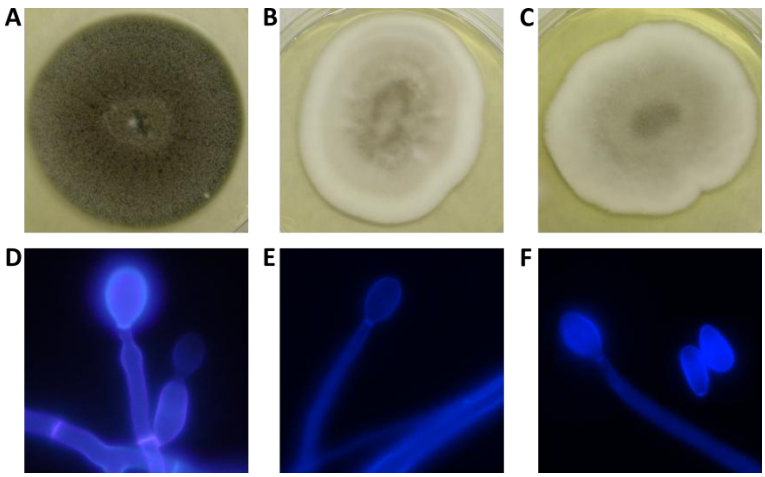


Figure 1. Macroscopic and microscopic views of *Lomentospora prolificans* and *Scedosporium* spp. Photographs of colonies growing for 7 days in potato dextrose agar and micrographs using calcofluor white of *L. prolificans* (A, D), *S. apiospermum* (B, E), and *S. aurantiacum* (C, F).

1.1. Taxonomic rearrangements: *Lomentospora prolificans* and the related genus *Scedosporium*

Lomentospora prolificans was firstly described by Hennebert & Desai in the soil of a greenhouse as a new hyphomycete (Hennebert and Desai, 1974). A decade later, Salkin & Malloch presented a novel species of the genus *Scedosporium*, named *S. inflatum* (due to the "inflated" conidiogenous cells), being detected as a pathogen causing osteomyelitis (Malloch and Salkin, 1984). Interestingly, several years later molecular and morphological similarities between the two abovementioned fungi were found. Hence, other authors suggested that nomenclatural synonymy existed and, in consequence, coined the new name *S. prolificans* (Gueho and de Hoog, 1991; Lennon *et al.*, 1994).

Until 2011, mycologists followed the classical botanical nomenclature based mainly on morphological and cultural techniques. Therefore, some species were classified by several distinct names, depending on e.g. the asexual or sexual morph. However, in 2011 the mycological scientific community achieved a general agreement in order to gradually eliminate this dual nomenclature, as established in the "Amsterdam Declaration on Fungal Nomenclature" (Hawksworth, 2011). In this way, Lackner and co-workers recently proposed a reorganization of the taxonomy concerning the family *Microascaceae* and, specifically the *Pseudallescheria*/*Scedosporium* species complex (Lackner *et al.*, 2014). In this study, the authors found inconvenient the use of two names, *Pseudallescheria* and *Scedosporium*, to refer to the different life cycle stages of the same fungus, sexual (teleomorph) and asexual (anamorph), respectively. Hence, they proposed the use of *Scedosporium* as unique name for the genus, as it is the oldest term.

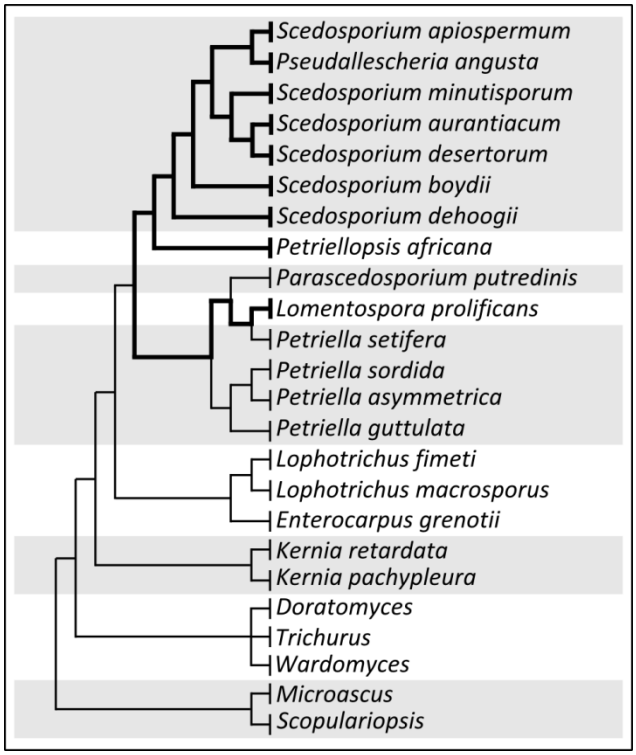


Figure 2. Phylogenetic scheme of *Microascaceae* based on ITS sequences of ribosomal DNA. Wide lines highlight the distance between *Scedosporium* spp. and the single-species genus *Lomentospora*. Adapted from Lackner *et al.*, 2014.

Moreover, the authors pointed out the significant differences between *S. prolificans* and the other species of the genus, at all morphological, genetic, and clinical levels, as discussed afterwards. Therefore, this species was excluded from the genus *Scedosporium* and was renamed as *Lomentospora prolificans*, forming a single-species genus.

2. Clinical relevance and epidemiology

2.1. Clinical manifestations of *Lomentospora prolificans* infections

Infections caused by fungi belonging to *Scedosporium* or *Lomentospora* may present a wide variety of symptoms, since these species manifest their pathogenicity in several ways. Therefore, fungus-host interactions at this level may be classified as colonization, localized infection, or systemic infection.

Firstly, when the fungus is occasionally isolated from a patient, both immunocompetent and immunocompromised, but no tissue damage is observed, the interaction is termed as

colonization. In these cases, bronchi, sinuses, ear, or skin are the main areas in which fungal material may be established. Depending on the underlying condition of the patient, this colonization can be transient or chronic. In this sense, patients without severe underlying conditions would be able to eliminate the fungus if the exposure to fungal material stopped. However, in those patients suffering from, for example, Cystic Fibrosis (CF), the colonization might become permanent, the fungus staying as resident mycobiota in the respiratory tract. In addition, some of these patients may suffer from allergic bronchopulmonary *Scedosporium* pneumonia (ABSP), presenting similar features as in the case of the one caused by *Aspergillus*: asthma, raised IgE levels, cutaneous reactivity to the etiologic agent, etc. (Guarro *et al.*, 2006; Chowdhary *et al.*, 2014).

Secondly, localized infections include those in which the fungus clearly damages host tissue, being very rare among immunocompetent population. These infections may be sub-classified into sinopulmonary and extrapulmonary infections (Cortez *et al.*, 2008). On the one hand, the first occurs more likely as the most common route used by *L. prolificans* to enter to the host is the respiratory tract. Once inside the body, conidia are able to germinate and invade airways tissues, producing pneumonia, being pulmonary involvement the second most abundant infection pattern of this fungus (Rodriguez-Tudela *et al.*, 2009). On the other hand, extrapulmonary infections result from the local inoculation of fungal material into cutaneous or subcutaneous tissues. In this sense, *L. prolificans* is able to infect eyes, skin, or joints. These infections have been associated to better survival rates than others. However, in some cases it is necessary to amputate the infected zone, including eye enucleation in the case of ocular infections.

Finally, both sinopulmonary and extrapulmonary infections may be the beginning of a disseminated involvement due to the ability of the fungus to rapidly spread through the bloodstream. The *L. prolificans* capacity to easily disseminate is mainly due to being able to produce conidia inside the host, unlike other fungi as *Aspergillus*. In fact, this is the most common infection pattern (44.4%), being also the one with the highest mortality rate (87.5%), as previously reported (Rodriguez-Tudela *et al.*, 2009). In addition, these infections are predominant among patients with haematological malignancies and transplant recipients. Interestingly, *L. prolificans* ability to disseminate through the bloodstream is

higher than that showed by *S. apiospermum*, as evidenced in an Australian study in which disseminated infections represented the 36% for *L. prolificans,* while only the 6% for *S. apiospermum* (Cooley *et al.*, 2007). The occurrence of fungal dissemination is characterized by the appearance of novel symptoms in different body areas, depending on the organ in which it establishes (Cortez *et al.*, 2008). Skin lesions, muscular pain or endocarditis (Fernandez Guerrero *et al.*, 2011), are lesions commonly reported. However, central nervous system (CNS) is, above all, the most frequently infected area (Rodriguez-Tudela *et al.*, 2009), both *Scedosporium* spp. and *L. prolificans* being termed as neurotropic fungi because of their tendency to it (Marco de Lucas *et al.*, 2006; Kantarcioglu *et al.*, 2008). Among CNS infections, although meningitis may occur (Uno *et al.*, 2014), fungal brain or cerebellar abscesses are more commonly found, being associated with neurological symptoms and deterioration.

2.2. Population risk groups for *Lomentospora prolificans* infections

Fungal infections associated with *Scedosporium* spp. and, specially, *L. prolificans* are mainly characterized by being opportunistic. This means that hosts are affected by an underlying condition which facilitates fungal colonization and infection. Precisely, immunocompromised patients are the main group suffering from these infections, being neutropenia the most important risk factor in the case of *L. prolificans* (Rodriguez-Tudela *et al.*, 2009). Therefore, patients undergoing antineoplasic or immunosuppressive therapy (e.g. transplant patients), with advanced human immunodeficiency virus (HIV) infection, primary immunodeficiencies (such as chronic granulomatous disease and Job's syndrome), or malignancies, are more susceptible to infections by these species (Cortez *et al.*, 2008). In the Table 1 the most relevant clinical characteristics for developing *L. prolificans* infections are summarized.

Table 1. Main clinical characteristics of patients suffering from *Lomentospora prolificans* infections (adapted from Rodriguez-Tudela *et al.*, 2009).

Characteristics	Number of patients (%)	Mortality rate
No underlying condition at time of infection		
Trauma	18 (11.1)	0
Surgery	5 (3.1)	0
None	11 (6.8)	0
Underlying condition at time of infection		
Malignancy	74 (45.7)	85.1
Cystic fibrosis	19 (11.7)	0
Solid organ transplantation	14 (8.6)	50
AIDS	8 (4.9)	37.5
Other*	13 (8)	23.1

*Including: immunological disorders, chronic obstructive pulmonary disease, injection drug use, bronchiectasis, pulmonary fibrosis, diabetes, prematurity, and myocardial infarction.

In spite of the fact that immunocompromised status is the main pathogenic hallmark to develop these mycoses, immunocompetent patients may also be infected by these fungi if they have been previously subjected to surgery or have suffered some kind of physical trauma (e.g. near drowning syndrome). In these cases, symptoms and disease progression are less severe in comparison with patients suffering from any underlying disease (Rodriguez-Tudela *et al.*, 2009).

Furthermore, there are some diseases unrelated with immune system impairment that make patients prone to develop *L. prolificans* infections. In this sense, CF is one of the most relevant. This genetic disorder, also known as mucoviscidosis, is the most frequent inherited autosomal recessive disease found in European caucasian population, appearing in 1-2 per 5,000 newborns (Quintana-Gallego *et al.*, 2015). These patients present a mutated variant in the *CFTR* gene (Cystic Fibrosis Transmembrane conductance Regulator) that produces an alteration in the chloride and sodium ions transport of epithelial cells. In the lungs, the mutated CFTR protein produces increased ion absorption by epithelial cells, which lead to dehydration and mucus accumulation.

In addition to these pathological effects, opportunistic pathogens are promoted in CF patients due to unbalanced normal lung microbiota. Bacteria, such as *Pseudomonas*

aeruginosa, and fungi, such as *Candida* or *Aspergillus*, are the most prevalent opportunistic pathogens in this kind of patients (Lipuma, 2010). The isolation of these bacteria is clearly associated with worse outcomes, however, although prevalence of filamentous fungi is rising in the last years, their role in the disease is not yet completely understood (Sudfeld *et al.*, 2010; Middleton *et al.*, 2013; Chotirmall and McElvaney, 2014). Regarding the species belonging to *Scedosporium*/*Lomentospora*, respiratory tract colonization percentages and species distribution are highly variable, mainly depending on geography and methodology used when processing CF samples (Borman *et al.*, 2010). These filamentous fungi are recovered from up to 17.4% of CF patients, being *S. apiospermum* and *S. boydii* the most prevalent species in European (Zouhair *et al.*, 2013; Nielsen *et al.*, 2014; Sedlacek *et al.*, 2015), and *S. aurantiacum* and *L. prolificans* in Australian clinical settings (Blyth, Harun, *et al.*, 2010; Blyth, Middleton, *et al.*, 2010).

2.3. Environmental distribution of *Scedosporium*/*Lomentospora*

The natural habitat of *Scedosporium*/*Lomentospora* species is not very well characterized, being only a few of reports focused on this topic. However, it has been described that they are widely distributed, preferentially in regions with temperate climates, being also able to tolerate high salt concentrations and temperatures, and to grow under low oxygen partial pressures (Cortez *et al.*, 2008; Lackner *et al.*, 2010). More interestingly, they have been associated to polluted ecosystems characterized by great amounts of organic compounds. In fact, *Scedosporium*/*Lomentospora* have been found tightly associated to human impacted environments, such as industrial areas or agricultural lands, playgrounds, potted plants in hospitals and homes of patients, or parks. Conversely, the presence of these fungi in natural or less-impacted areas is quite rare (Rougeron *et al.*, 2014), being completely absent in other territories, as reported elsewhere (Kaltseis *et al.*, 2009).

In spite of these common features, their prevalence in different geographic areas clearly depends on each species. Up to these days, three major ecological studies have been performed describing *Scedosporium*/*Lomentospora* species distribution in different niches. The first one was performed in Austria and The Netherlands, the second in the greater Sydney area, in Australia, and the third one in Northwestern France. The three studies were

mainly focused on terrestrial environments, and applied semi-selective culture media, such as SceSel+ (Rainer *et al.*, 2008) or Scedo-Select III (Pham *et al.*, 2015), to partially eliminate other fungal and microbial groups. As abovementioned, the abundance of these fungi in natural or less human-impacted environments was very low or, even, none, as reported in the Austrian/Dutch study. This can be observed more clearly in the Australian report, in which decreasing abundance of these fungi is found when analyzing less human-occupied areas, being the higher abundance reported in urban samples, followed by suburban samples, and finally rural samples.

Regarding species distribution, varying percentages of abundance were found depending on the country and the type of area analyzed. In the Austrian/Dutch study (Kaltseis *et al.*, 2009), the most prevalent species among the genus was by far *S. apiospermum* with abundance percentages between 58.7-77.7%, depending on the ecosystem. The second most relevant species was *S. dehoogii* (13.8-28.8%), which is rarely found as etiological agent of *Scedosporium* infections. The remaining species were present in lower percentages, being found *S. aurantiacum* (4.8-7.7%), *P. minustispora* (5.8%), and *P. boydii* (1.9-2.3%). Similar results were obtained in the French study in 2014 (Rougeron *et al.*, 2014), but in this case the most abundant species was *S. dehoogii* (39.38%), followed by *S. aurantiacum* (21.62%), *S. apiospermum* (18.91%), and *S. boydii* (19.39%). Minimal presence for *L. prolificans* and *P. minutispora* was found.

From these two European studies two points should be highlighted. First, environmental distribution is more or less correlated with the clinical situation of all the species, with the exception of *S. dehoogii*. As previously highlighted, while this species is quite abundant in the environment, it is rarely found in clinical settings. Surprisingly, it has been described as highly pathogenic in both immunocompetent and immunocompromised mice (Gilgado *et al.*, 2009), and as able to degrade complement molecules of the immune system (Rainer *et al.*, 2011). Second, *L. prolificans* was completely absent in the Austrian/Dutch study, and in very low proportion in the French one (only 3 isolates, out of 368). This fact is consistent with the low clinical prevalence of this species in these countries, but it contrasts with its clinical epidemiology in close countries such as Spain, as discussed later on.

Completely different species environmental distribution was found by Harun and co-workers in Eastern Australian areas (Harun, Gilgado, *et al.*, 2010), in which the commonest species was *S. aurantiacum*, representing the 54.64% of all isolates, followed by *L. prolificans* (43.09%). Remarkably, very low abundances of *S. boydii* (1.97%) and *S. dehoogii* (0.28%) were found in this country. Then, great differences were found between European and Autralian studies, highlighting the varying environmental epidemiology presented by these fungal species.

2.4. Clinical epidemiology

Fungal pathogens are a leading cause of disease in humans. Although superficial infections of the skin and nails remain as the most common fungal diseases (Havlickova *et al.*, 2008), in the last decades the importance of invasive fungal infections (IFI) has dramatically raised. This fact may be the consequence of two factors. First, since fungi causing this kind of infections are mostly opportunistic, there has been an increment in the number of clinical cases due mainly to the increasing number of susceptible population, such as immunosuppressed individuals (e.g. oncologic patients), transplant recipients (also partially or deeply immunosuppressed), elderly people, etc. Therefore, since the amount of susceptible population is anticipated to continue to rise, it is presumable that the incidence of IFIs may be greater in the near future. Second, and more importantly, IFIs are life-threatening diseases that are associated to very high mortality rates, which depend on socioeconomic conditions, fungal species, etc. In this way, fungal pathogens are responsible for about two millions of deaths every year worldwide (Brown *et al.*, 2012).

Epidemiological studies concerning IFIs clearly show that only a few of yeast and mold genera are the main cause of deaths worldwide, as *Candida*, *Cryptococcus*, or *Aspergillus*. However, the epidemiology of fungal infections is continuously varying. The use of prophylactic antifungal compounds against *Candida* or *Aspergillus* infections may produce a selective pressure, promoting emerging fungal pathogens and cryptic species, which traditionally have not been found as clinically relevant etiologic agents of disease. In this way, fungal groups or genera such as Mucorales, *Fusarium*, or *Scedosporium*/*Lomentospora*

have acquired relevance in the last decades, and have drawn attention of researchers since they present new diagnostic and therapeutic challenges.

Particularly, *L. prolificans*, *S. apiospermum*, *S. boydii*, and *S. aurantiacum* are the main etiologic agents of their lineage inside the family *Microascaceae*. Unfortunately, epidemiological studies concerning these pathogens are few and, frequently, are focused on certain risk groups, a single hospital centre, or a particular geographical region. Specifically, *L. prolificans* epidemiological history has been short, but quite relevant. Only four years after its description as a causative agent of infection, it was reported as an emerging pathogen (Salkin *et al.*, 1988). Later on, in 1991 the first disseminated and fatal *L. prolificans* infection in Europe was reported in Spain (Marin *et al.*, 1991), being highlighted the difficulties for treating and eliminating the fungus.

Nowadays, *Scedosporium*/*Lomentospora* are the second cause of non-*Aspergillus* filamentous fungal infections both in Spain (Alastruey-Izquierdo *et al.*, 2013) and Australia (Slavin *et al.*, 2015), representing the 31.91% and 33.3%, respectively. In spite of *S. apiospermum* or *S. boydii* remaining as the main cause of *Scedosporium* spp. infections worldwide, in both of abovementioned countries *L. prolificans* and *S. aurantiacum* are on the rise in comparison with other regions. In fact, Heath and co-workers reported in Australia a greater number of clinical cases related to *L. prolificans* (40.8%) than to *S. apiospermum* (35%) or *S. aurantiacum* (24.2%) (Heath *et al.*, 2009). In this sense, *L. prolificans* was the most frequently isolated filamentous fungus from blood cultures in a hospital in Northern Spain during the 1990 decade (Idigoras *et al.*, 2001). On the contrary, other regions of the globe are less predisposed to suffer *L. prolificans* infections, as Germany (Tintelnot *et al.*, 2009) or Finland (Issakainen *et al.*, 2010), in which few or none clinical cases were reported in long periods of time.

3. Diagnosis of *Scedosporium*/*Lomentospora* infections

Diagnosis of infections caused by these filamentous fungi is very challenging nowadays, mainly due to unspecific symptoms and clinical manifestations, which may be confused with *Aspergillus* or *Fusarium* infections. In addition, diagnostic tools remain having low specificity and sensitivity. This situation usually leads to a delay in diagnosis and, as a result, in the antifungal treatment, which increases morbidity and mortality rates. In recent years, novel diagnostic strategies have been developed, presenting increased sensitivity and specificity, but usually lacking of correct validation and standardization between laboratories (Quindós *et al.*, 2012; Arvanitis *et al.*, 2014; Teles and Seixas, 2015).

In the specific cases in which *Scedosporium*/*Lomentospora* may be the cause of the mycoses, identification at species level is very important due to the intrinsic heterogeneity among species concerning virulence (Gilgado *et al.*, 2009; Harun, Serena, *et al.*, 2010), or antifungal susceptibility (Lackner, De Hoog, *et al.*, 2012). In this sense, researchers and clinicians have focused their efforts on developing several methodologies in order to determine the etiologic agent causing disease.

3.1. Conventional diagnostic strategies

Nowadays, diagnosis of *Scedosporium*/*Lomentospora* infections is mainly performed by conventional microbiological methods, such as direct examination of samples or culture features. In fact, this set of techniques are the ones with the highest strength of recommendation given by the group of ESCMID and ECMM experts, as they are well-standardized and available in most clinical settings (Table 2). Moreover, other general recommendations should be taken into account when trying to identify etiological agents causing these and other mycoses (Ayats *et al.*, 2011). Among them, the type of sample is critical, being the most relevant for invasive infections biopsy or tissue aspiration, blood, cerebrospinal fluid, or bronchoalveolar lavage (BAL). Thus, samples should be rapidly submitted to the laboratory, and processed as soon as possible.

Table 2. Main recommendations of the ESCMID and ECMM for the diagnosis of *Scedosporium/Lomentospora* infections (Tortorano *et al.*, 2014).

Test	SoR*	QoE#	Comments
Direct microscopy	A	III	Essential investigation
Culture	A	III	Essential investigation. Use of selective media
Molecular-based identification methods	C	III	Accurate species assignment is important for guiding clinical management
Histopathology	A	III	Hyaline thin-walled septate hyphae, 2-5 µm wide similar to those seen with aspergilosis, but with irregular branching
Pan-fungal PCR	C	III	To be used in combination with conventional tests
Multiplex PCR	C	III	To be used in combination with conventional tests
In situ hybridization	C	III	Low sensitivity. Not yet validated
Species identification (MALDI-TOF and PCR)	C	III	Not yet validated
Physiological typing	C	III	In case of outbreak

*Strength of recommendation. #Quality of evidence.

It is worth highlighting that culture techniques are currently one of the most important method during diagnosis of microbial infections, including mycoses. In fact, recovery of *Scedosporium*/*Lomentospora* from a patient sample using culture media under sterile conditions, and supported with radiological or clinical abnormal findings, is a criterion for a proven IFI (De Pauw *et al.*, 2008). When the fungus is recovered, analysis for its identification, antifungal susceptibility, or epidemiological typing can be performed. In order to isolate *Scedosporium*/*Lomentospora* strains, routine culture media are used, such as blood agar, chocolate agar, or brain-heart infusion agar, and, more specifically for fungi, Sabouraud agar. Interestingly, in the last years several semi-selective culture media have been developed, containing one or more compounds that avoid or delay rapidly growing bacteria and fungi, such as *Aspergillus*, *Candida*, or Mucorales (Summerbell, 1993; Rainer *et al.*, 2008; Pham *et al.*, 2015). Therefore, patient samples, as BAL, can be directly cultured on these media in order to isolate more efficiently *Scedosporium*/*Lomentospora*. In addition, other features, such as culture temperature, may be useful to discriminate between fungal groups, or even *Scedosporium*/*Lomentospora* species.

After fungus isolation, morphological analysis can be performed, both at macroscopic and microscopic levels, in order to identify the species. As abovementioned, *L. prolificans* is characterized by certain morphological features that facilitate its identification, such as flask-shaped conidiogenous cells, absence of teleomorph, or dark grey to olive-green

colonies. On the contrary, *Scedosporium* spp. form white to pale-grey colonies and develop cylindrical conidiogenous cells, being in some cases present yellow diffusible pigments or teleomorphs. Conidiospores shape and size is another important taxonomic feature. However, differences are subtle and routine mycologists should have a lot of experience to appreciate them (Table 3). On the other hand, physiological and biochemical tests may also be performed to determine the species, but prolonged experimental times and standardization are needed (Gilgado *et al.*, 2008).

Table 3. Main morphological and physiological characteristics of *Lomentospora prolificans* and *Scedosporium* spp. (adapted from Gilgado *et al.*, 2008; Lackner *et al.*, 2014).

Species	Growth at		Yellow diffusible pigments	Anamorph		Teleomorph
	40ºC	45ºC		Conidiogenous cells	Sessile conidia	
L. prolificans	Yes	Variable	No	Flask-shaped	Globose to subglobose, thick-walled	No
S. apiospermum	Yes	No	Variable	Cylindrical	Globose to subglobose, thick-walled	Yes
S. boydii	Yes	No	Variable	Cylindrical	Globose to subglobose, thick-walled	Yes
S. aurantiacum	Yes	Yes	Yes	Cylindrical or slightly flask-shaped	Mostly obovoid, thick-walled	Yes
S. minutisporum	Yes	No	No	Cylindrical	Ellipsoidal to obovoid, thin-walled	Yes
S. dehoogii	No	No	No	Cylindrical or slightly flask-shaped	Mostly obovoid, thick-walled	No

Histopathological examination of biopsies may also be used in order to diagnose these mycoses. When tissue observation using KOH treatment or histological staining is performed, fungal structures and tissue inflammation can be analyzed. However, it is difficult to distinguish *Scedosporium*/*Lomentospora*-infected tissues from those by *Aspergillus*, *Fusarium*, or *Petriella*. All of these species present hyaline hyphae (excluding *L. prolificans* that may present highly melanised hyphae), regular hyphal septation, and dichotomous branching when observed in tissue. One slight difference is that *Aspergillus* displays a regular branching pattern, while *Scedosporium*/*Lomentospora* may present a more irregular pattern (Cortez *et al.*, 2008). Therefore, although these techniques are widespread through clinical laboratories and are highly recommended (Table 2), pathologists have to be very well-prepared in order to correctly determine the cause of infection. The use of fluorescent dyes on fungal material in tissue, such as calcofluor white,

facilitates the observation of fungal structures. Moreover, several polyclonal (Jackson *et al.*, 1983) and monoclonal antibodies to *Scedosporium*/*Lomentospora* have been developed and used in order to detect and discriminate these species. Of high relevance in this field is the contribution of Thornton and co-workers that have developed specific monoclonal antibodies to *S. apiospermum*/*S. boydii* (Thornton, 2009) and, more recently, to *L. prolificans* (Thornton *et al.*, 2014).

Finally, detection of antigens, fungal byproducts, or structural components has been an important subject in which researchers have focused on. The structural polymer 1,3-β-D-glucan is present in most fungi, excluding the large group Mucorales. In this sense, it is used as a panfungal biomarker of fungal infection, lacking of the ability to discriminate among specific etiologic agents as *Candida*, *Aspergillus*, *Fusarium*, or *Scedosporium*/*Lomentospora* infections. Detection of secondary metabolites may also be targeted for further research, since some promising results have been recently obtained detecting N(α)-methyl coprogen B, a siderophore, in CF patients sputa (Bertrand *et al.*, 2010). On the other hand, only a few studies have focused on the development of serological methods to detect *Scedosporium*/*Lomentospora*-specific antibodies, and discriminate them from other pathogenic fungi. In this way, counterimmunoelectrophoresis (Cimon *et al.*, 2000) has been applied for the diagnosis of *S. apiospermum* in CF patients. However, cross-reactivity with *Aspergillus* antigens was observed. Interestingly, a peptidorhamnomannan (Pinto *et al.*, 2001) and mycelial catalase A1 (Mina *et al.*, 2015) from *S. boydii* have been proposed to be used in diagnosis, but further research should be carried out in the future to determine their suitability.

3.2. Novel diagnostic methodologies

Molecular biology has revolutionized scientific knowledge in many ways, being mycological diagnostic strategies highly influenced. In this sense, PCR is currently one of the most widely used techniques in the development of diagnostic tools. This technique, coupled with nucleic acid sequencing, is very useful to determine the presence of a gene and, then, study its sequence. Several genomic regions have been used to identify and discriminate fungal groups and species, as ribosomal DNA, Internal Transcribed Spacer (ITS), calmodulin, or β-

tubulin (regions TUB and BT2), among others (Gilgado *et al.*, 2005; Gilgado *et al.*, 2008; Schoch *et al.*, 2012). However, ITS have been proved to be the most useful sequences since they can be used as universal or barcoding markers in the entire kingdom Fungi. Using the obtained sequences, and matching them to public databases such as the GenBank of the NCBI, it is possible to determine the species by sequence similarity. Moreover, dendrograms or phylogenetic trees may be constructed to determine the evolutionary relation among groups or species.

Several methods have been developed and applied in the last decades in order to diagnose, identify, and typify *Scedosporium* spp. and *L. prolificans* strains. Many of them are based on the study of nucleic acids as Random Amplified Polymorphic DNA (RAPD) (San Millán *et al.*, 1997; Zouhair *et al.*, 2001; Defontaine *et al.*, 2002), amplification of Intergenic Spacer Region (IGS PCR) (Williamson *et al.*, 2001), amplification of microsatellite regions or Inter Simple Sequence Repeats (ISSR PCR) (Sole *et al.*, 2003), *in situ* Hybridization (ISH) (Hayden *et al.*, 2003), Restriction Fragment Length Polymorphisms (RFLP) (Delhaes *et al.*, 2008; Lackner, Klaassen, *et al.*, 2012), or Multi Locus Sequence Typing (MLST) (Defontaine *et al.*, 2002; Harun *et al.*, 2009; Bernhardt *et al.*, 2013). In addition to these, proteomic profiles obtained by MALDI-TOF mass spectrometry are also useful to discriminate between these fungal species (Coulibaly *et al.*, 2011; Del Chierico *et al.*, 2012; Sitterlé *et al.*, 2014).

More recently, fungal culture-free techniques have been developed in order to detect fungal material, mainly DNA, directly on patient samples. In this sense, Castelli and co-workers designed two real-time PCR (RT-PCR) assays to specifically detect *S. apiospermum* and *L. prolificans* (Castelli *et al.*, 2008). These assays presented great sensitivity (detection of up to 10 fg of fungal DNA), and their validation in a murine infection model was performed with very promising results, especially for lung samples. Similarly, a multiplex PCR assay targeting ITS sequences has been recently developed to discriminate among several *Scedosporium* species and *L. prolificans*, being validated in CF patient sputum samples with high specificity but only 62.1% of sensitivity (Harun *et al.*, 2011). On the other hand, detection of *Scedosporium*/*Lomentospora* has been included in several wide-spectrum fungal diagnostic methods. In this way, applying techniques, such as panfungal PCR (Adam *et al.*, 2004; Lau *et al.*, 2007; Lau *et al.*, 2008) or DNA microarray (Spiess *et al.*, 2007;

Bouchara *et al.*, 2009; Boch *et al.*, 2015), directly on patient samples, it is possible to detect and discriminate several etiologic agents at the same time.

Despite this set of techniques seems to be very promising, the lack of standardization and evaluation in routine laboratories, low reproducibility, and the absence of public databases, limit their use at reference centres or clinical settings (Tortorano *et al.*, 2014). Moreover, all of these techniques usually require fungal culture before their application.

4. Treatment and outcomes of *Lomentospora prolificans* infections

The increasing number of fungal infections during the last decades created the need for developing antifungal drugs effective against them. While antibiotic drugs started to be used in early 20th century, the application of therapies directed to fungi did not begin until mid-1950s (Figure 3), being the number and availability of the latter compounds lower in comparison with the former.

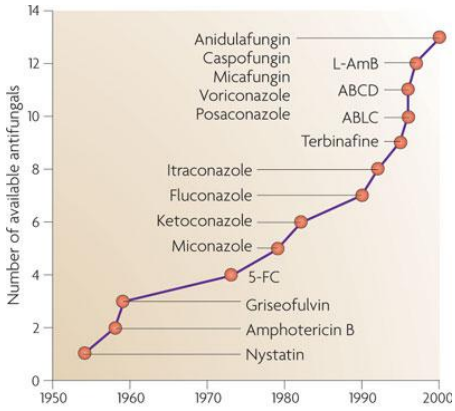


Figure 3. Timeline of systemic antifungal drug development. The graph shows the year of first use of each systemic antifungal drug, and the number of available compound at each time. 5-FC, flucytosine; ABCD, amphotericin B colloidal dispersion; ABLC, amphotericin B lipid complex; L-Amb, Liposomal amphotericin B. Extracted from Ostrosky-Zeichner *et al.*, 2010.

The first problem to overcome when antifungal drugs are developed is the fungal biology itself. Since fungi are eukaryotic microorganisms, they share many metabolic processes and cellular structures with mammals, offering only a few fungus-specific targets. Consequently, many antifungal drugs may cross-react with host cells, causing toxicity when used

therapeutically (Odds *et al.*, 2003). One of the most promising organelle for the development of novel antifungal drugs is the fungal cell wall, since it is in contact with the environment and, thus available to therapeutic agents, and also presents a unique structure, with a very special composition that is completely absent in mammalian cells.

Additionally, many other factors should be taken into account when evaluating an antifungal drug, concerning the compound itself, the host, and the fungus (Figure 4). Thus, immune status of the host, differential susceptibility between fungal species, even strains, or antifungal compound bioavailability are factors determining therapeutic success.

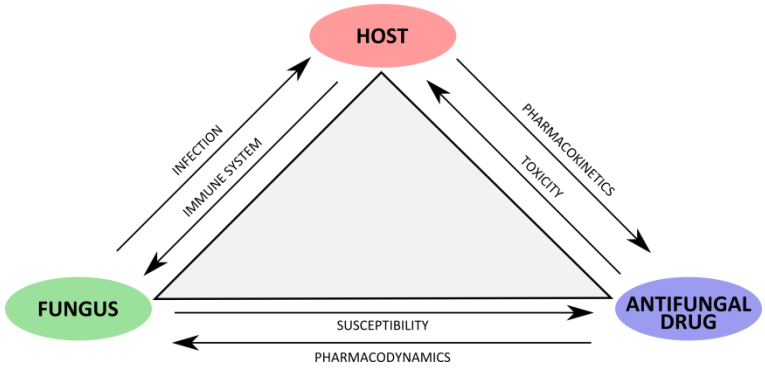


Figure 4. Davis triangle depicting the interactions between host, fungus, and antifungal drugs.

4.1. Main classes of antifungal drugs

Up to now, three main classes of systemic antifungal drugs are available and licensed to be used therapeutically, namely polyenes, azoles, and echinocandins (Figure 5). Although there exist other kinds of drugs, such as sordarins, or the chitin synthase-inhibitor nikkomycin Z, with promising activity and without reports of evident secondary effects, no advanced clinical trials have been developed at the moment (Ostrosky-Zeichner *et al.*, 2010). More recently, a novel class of antifungal compounds has been released which targets the biosynthesis pathway of fungal sphingolipids, in more detail, glucosylceramide (GlcCer) (Mor *et al.*, 2015). Interestingly, by inhibiting GlcCer synthesis promising results of fungicidal activity, both *in vitro* and *in vivo*, have been obtained, since these compounds produce cellular aberrations in fungi, including intracellular vesicle accumulation.

Among the abovementioned antifungal drugs, polyenes were the first to be used systemically, being compounds as nystatin or amphotericin B (AmB) members of this class. These molecules strongly bind to ergosterol, which is the most abundant sterol in fungal cell membranes. Then, ergosterol-drug aggregates form pores in the membranes, leading to intracellular content leakage. Due to this mechanism of action, polyenes show a broad-spectrum of activity, being used against both yeasts and filamentous fungi. Moreover, although structural similarities between ergosterol and mammalian cholesterol make these drugs toxic to host cells, several chemical modifications, including lipidic formulations, show less toxicity, being possible to give higher doses to patients.

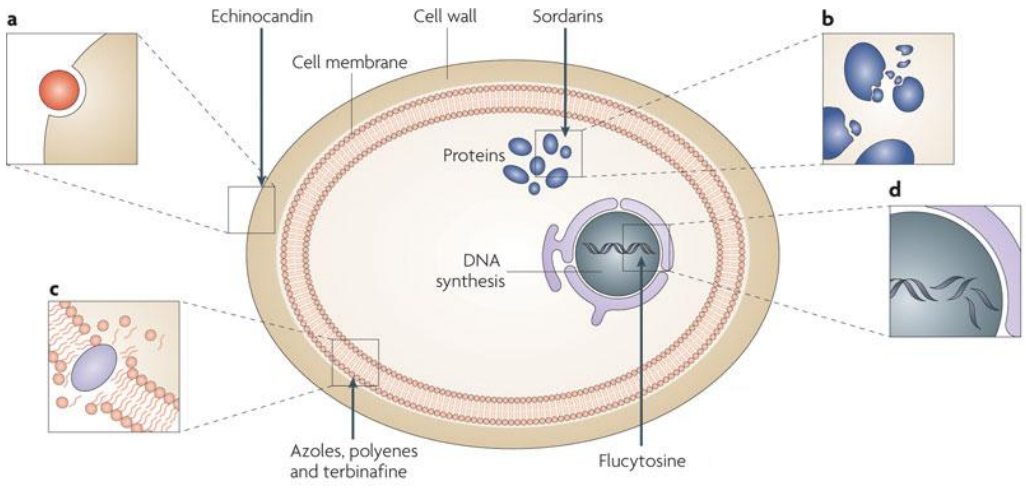


Figure 5. Mechanisms of action of main antifungal drugs. A) Echinocandins and nikkomycin Z inhibit the synthesis of fungal cell wall components, disturbing cell wall instability. B) Sordarins interfere with protein synthesis. C) Azoles and polyenes interact with fungal cell membrane. D) Flucytosine disturbs RNA and DNA synthesis. Extracted from Ostrosky-Zeichner *et al.*, 2010.

On the other hand, azoles are synthetic molecules that act by inhibiting the fungal enzyme lanosterol 14-α demethylase, which is involved in the ergosterol biosynthetic pathway. Therefore, these drugs induce sterol intermediates accumulation in fungal cells, avoiding cell membrane constructions and, consequently, fungal growth. Since there are numerous different azole compounds, two main groups can be formed, namely imidazoles and triazoles. The first are mostly used topically, since they present high toxicity and poor bioavailability. Triazoles as fluconazole (FLZ), posaconazole (POS), or voriconazole (VRC), may be used for systemic fungal diseases since they present more specificity for the abovementioned enzyme.

The most recently commercialized group, echinocandins, such as micafungin (MICA) or caspofungin (CAS), are lipopeptides that inhibit proteins involved in the synthesis of β-1,3 glucans, which are main components of the fungal cell wall. When applied to fungal cells, it is not possible for them to properly construct their wall, being sensible to osmotic shock. Since the target of this class of antifungal drugs is not present in mammal cells, their toxicity level is very low.

Concerning *L. prolificans*, it has been described low antifungal activities for almost all the drugs available at the moment. As discussed later on, this represents a major problem when managing *Scedosporium*/*Lomentospora* infections, especially those caused by *L. prolificans*.

4.2. Treatment strategies for the therapy-refractory fungus *Lomentospora prolificans*

One of the main features showed by *L. prolificans* is its inherent resistance to virtually all antifungals available nowadays in clinical settings, being the outcomes of its infections particularly poor (Rodriguez-Tudela *et al.*, 2009). Conversely, this resistance to multiple antifungals is observed in a less extent in *Scedosporium* spp. (Lackner, De Hoog, *et al.*, 2012). Cortez and co-workers reported in a nice review that *L. prolificans* showed high MICs for all antifungal drugs tested *in vitro* (above 8 μg/ml for most of them), although strain-dependent differences were observed (Cortez *et al.*, 2008). The triazole drug VRC presented the best results, but its median MIC_{50} (4 μg/ml) is rarely achieved as free drug in patients. This fact could be the reason explaining that, while 64% patients suffering from *S. apiospermum* infections responded to VRC treatment, only 44% of patients infected with *L. prolificans* experienced positive results (Troke *et al.*, 2008).

Interestingly, promising results have been obtained applying combinations of available systemic antifungals. Among them, one of the most remarkable is the synergistic interaction between terbinafine (TRB) (allylamine) and the azole compounds VRC, itraconazole (ITZ), or miconazole (MCZ). Using this combination, which inhibits ergosterol biosynthesis pathways on several steps, more than 85% of the strains tested presented lower MICs, allowing to

achieve proper concentrations in patient's plasma (Meletiadis *et al.*, 2000; Meletiadis *et al.*, 2003). In addition to this, synergistic interactions have been described for MICA with VRC or AmB (Heyn *et al.*, 2005; Yustes and Guarro, 2005), and for ravuconazole (RVZ) with CAS (Cuenca-Estrella *et al.*, 2008), but in a lower number of isolates.

Regarding to *in vivo* data, antifungal drugs, either alone or in combination, have been used to determine their efficacy in experimental *L. prolificans* infections. The use of the liposomal formulation of AmB was proved to be active against the fungus in immunosuppressed murine models (Ortoneda, Capilla, *et al.*, 2002; Bocanegra *et al.*, 2005), reducing the presence of the fungus in organs, and increasing mice survival rates and times. Similarly, Rodriguez and co-workers showed the efficacy of MICA, and double or triple combinations with VRC and AmB on increasing mice survival, even though the strain used in the study shown *in vitro* resistance to the antifungals (Rodríguez *et al.*, 2009). In addition, using immunocompetent rabbits, albaconazole (ALB) was shown to increase survival rates and completely eliminate the fungus from spleen, liver, and lungs (Capilla *et al.*, 2003). However, drug doses were higher than those applied against other fungi, which may produce toxicity or side effects.

As previously highlighted, patient immune system status is crucial during *L. prolificans* infections. In this sense, the administration of molecules which enhance immune response, such as cytokines, has been a subject of interest for researchers. Thus, the use of granulocyte macrophage colony-stimulating factor (GM-CSF) or interferon-γ increased *ex vivo* polymorphonuclear lymphocytes (PMN) damage to *L. prolificans* hyphae, being even higher when used combined (Gil-Lamaignere *et al.*, 2005). Similarly to the former, interleukin-15 enhanced hyphal damage and promoted cytokine release in PMNs exposed to *L. prolificans* (Winn *et al.*, 2005). Moreover, combination of PMNs and antifungals, such as AmB lipid complex, VRC, ITZ, or POS, have been shown to enhance damage in *L. prolificans* cells in comparison to when used alone (Gil-Lamaignere, Roilides, Mosquera, *et al.*, 2002; Gil-Lamaignere, Roilides, Maloukou, *et al.*, 2002). Consistent with these results, the combination of AmB, ITZ, and GM-CSF was successfully used to treat a neutropenic patient (Bouza *et al.*, 1996).

In the last few years, several novel compounds have been tested showing any antifungal activity against *L. prolificans*, some of them displaying new mechanisms of action different from conventional drugs. Among them, inhibitors of glycosyl-phosphatidyl-inositol (GPI) biosynthesis pathway (Castanheira *et al.*, 2012), the antibacterial compound colistin (Schemuth *et al.*, 2013), *N*-chlorotaurine (Lackner *et al.*, 2015), or drugs altering the heat shock protein 90-calcineurin axis (Lamoth *et al.*, 2015) are very promising. However, in spite of these interesting results, further research has to be performed (*in vivo* or clinical assays) to deeply determine their efficacy, toxicity, bioavailability, etc. Because of that, in a recent report of the group of ESCMID and ECMM experts only surgical debridement of infected tissue and treatment with VRC was highly recommended, also highlighting the importance of restoring immune system in the case of immunocompromised patients (Tortorano *et al.*, 2014).

5. Antifungal immunity and host-pathogen interactions: the emerging pathogen *Lomentospora prolificans*

Immune response to fungal infections in mammalian hosts is a complex network consisting on cellular and molecular mechanisms. Since most of fungi are ubiquitous in the environment, humans are usually exposed to their cells or propagules (such as conidiospores). In this sense, fungal cells have developed strategies to interact with mammals and other hosts, being able to completely reprogram their biology to overcome stressful environmental conditions encountered inside hosts, such as varying temperatures, nutrient starvation, etc. (Cooney and Klein, 2008). In addition, those which are pathogenic have developed mechanisms to evade immune responses in several ways, including masking their recognizable molecular structures, expressing potent enzymatic machinery, etc.

Regarding to *L. prolificans*, while it has been shown as one of the most virulent species of the genera *Scedosporium*/*Lomentospora* (Ortoneda, Pastor, *et al.*, 2002; Harun, Serena, *et al.*, 2010), only a few reports have focused on this species pathobiology, being very little what it is known about its virulence factors. These studies are mainly focused on some molecules suspected to have roles on *L. prolificans* pathogenesis, being worth highlighting cell wall peptidorhamnomannan, which may be related to fungal virulence and adhesion

(Barreto-Bergter *et al.*, 2008; Gorin *et al.*, 2010), and on host invasion-related enzymes, such as proteases, ecto-phosphatases, catalases, superoxide dismutases (Lima *et al.*, 2007; Santos *et al.*, 2009), and complement-degrading enzymes (Rainer *et al.*, 2011). Moreover, *L. prolificans* is equipped with melanin in its cell wall that, besides its activity as a scavenger molecule to absorb oxidative intermediates (Romani, 2004), has been described to take part during fungal immune evasion by masking PAMPs and to interfere in phagolysosomal maturation (Jahn *et al.*, 2002; Langfelder *et al.*, 2003).

5.1. First lines of defence and recognition of fungal cells by the immune system

The first lines of defence are host areas exposed to microorganisms, including the skin, and the surface of the respiratory, genito-urinary, and gastrointestinal tracts. Here, host and fungal pathogens directly interact, taking part mechanisms such as epithelial cell barrier, defensins, or antagonism with resident microbiota, to difficult fungal infection.

During these interactions, it is also necessary for the host immune system to be able to recognize pathogens or discriminate between infective and non-infective fungal morphs. In this sense, pattern recognition receptors (PRR) are a wide repertoire of molecules that allows the immune system to respond to different fungi by recognizing conserved structures, namely pathogen-associated molecular patterns (PAMP), such as mannans or glucans. The fungal cell wall is very important in this process, since it is in contact with the extracellular environment. This organelle is mainly formed (≈ 90%) by a complex matrix of carbohydrates that constructs a plastic, but strong, structure, crucial for fungal viability (Latgé, 2007). Although its composition varies among fungal groups, species, and even during the cell cycle or morphological switching, the fungal cell skeleton wall is mainly composed by β-1,3-glucans, covalently linked to β-1,6-glucans, and chitin (polymer of *N*-acetylglucosamine). In addition, this skeleton is decorated with different carbohydrates, as mannans (both *O*- and *N*-linked). Cell wall proteins (CWP) are also relevant for both interaction with the environment and cell wall remodelling, and are usually highly glycosylated. CWPs may be directly linked to polysaccharides (Pir-CWP) or anchored through GPI. Particularly, peptidorhamnomannans, rhamnomannans, and α-glucans are of special

importance in *Scedosporium*/*Lomentospora*, existing structural and compositional differences among species (Lopes *et al.*, 2011).

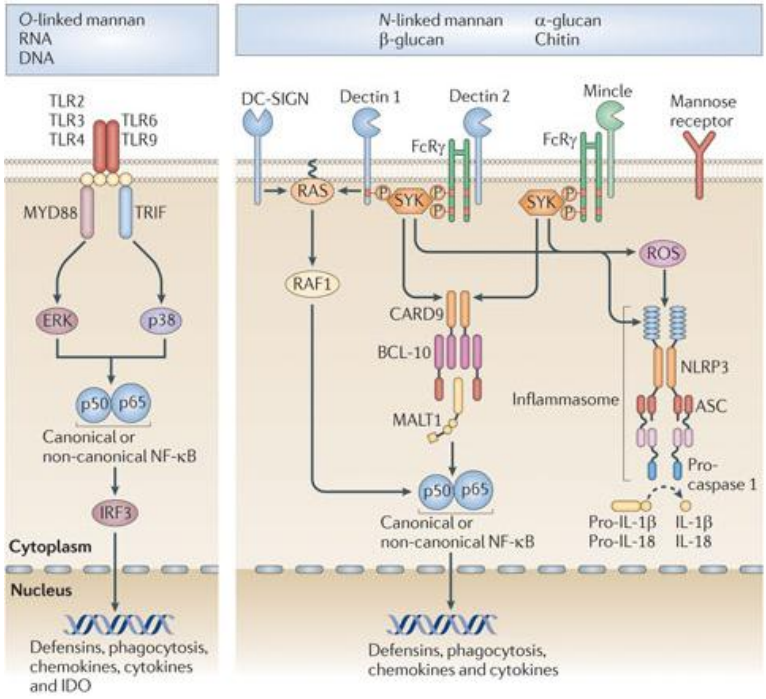


Figure 6. Fungal cell recognition by pattern recognition receptors (PRR). Schematic representation of the main PRR and fungal molecules involved in recognizing fungi by host cells. Adapted from Romani, 2011.

Host cells express these PRR, which may be classified in three main groups: C-type lectin receptors (CLR), Toll-like receptors (TLR), and Nod-like receptors (NLR) (Figure 6) (Netea *et al.*, 2008; Romani, 2011). The first group, CLRs, is essential for fungal recognition, since genetic mutations in genes encoding for these receptors or for molecules involved in their signalling pathways produce high susceptibility to fungal infections in hosts. CLRs include, among others, the receptors dectin-1 (for β-glucans recognition), dectin-2 (for high-mannose structures), mannose receptor, and DC-SIGN (both for *N*-linked mannans). Among the TLRs, TLR2, TLR4, and TLR9, are the most relevant for fungal infections, sensing phospholipomannan, *O*-linked mannans, and DNA, respectively. Similarly to CLRs, polymorphisms in genes encoding these receptors are associated to enhanced susceptibility to *Candida* and *Aspergillus* infections, but in a less extent (reviewed in Wójtowicz and Bochud, 2014). Finally, NLRs, as NLRP3, sense fungi and induce the formation of inflammasomes which will trigger the production of pro-inflammatory cytokines. While each receptor has been associated to be specific to a determined PAMP, the final immune

response developed by the host will depend on stimulus intensity and receptor cooperativity, which, for example, have been described between dectin-1 and TLR2 (Gantner *et al.*, 2003). In this sense, the presence of various PAMPs, and their relative abundance, will determine a pro- or anti-inflammatory response, being these events the basis of resistance or tolerance to fungal cells (Romani, 2011).

5.2. Role of phagocytes during fungal infections

Antifungal activity may occur in the first stages of the infection by phagocytes, including macrophages, neutrophils, and dendritic cells (DC) (Brakhage *et al.*, 2010). Moreover, other cells with phagocytic capacity may collaborate in these processes, such as epithelial cells. After recognition by PRR, these cells promote fungal cell death and delay fungal growth by activating oxidative and non-oxidative mechanisms.

Macrophages are the most prominent phagocytic cells, contributing to fungal clearance and triggering pro-inflammatory responses to attract other cell types. Once conidia are ingested, macrophages fuse vesicles to the one containing the fungal threat to form phagolysosomes. In these organelles extreme conditions are achieved, including low pH and the presence of hydrolytic enzymes. Moreover, macrophages are able to produce both ROS and RNS, which interact with fungal cells producing oxidative stress in proteins, lipids, and nucleic acids.

Furthermore, neutrophils are recruited to the site of infection by sensing cytokines. These cells are also able to recognize fungal cells by PRR, phagocytize them, and produce oxidative molecules. However, their most remarkable role is the formation of neutrophil extracellular traps (NET). If fungal cells germinate, neutrophils phagocytic capacity dramatically decreases due to the size of hyphae. Therefore, these cells start to degranulate, releasing their content to the extracellular matrix and trapping fungal cells in a matrix mainly composed by DNA and proteins with antimicrobial activity. More than a fungicidal activity, NETs are fungistatic, preventing fungal growth and dissemination, and retaining fungal cells while other immune cells are recruited (Brakhage *et al.*, 2010).

Finally, although DCs have also phagocytic capacity, their main functions are not related to fungal killing, but to antigen presentation and activation of T cell-mediated responses, which will be discussed later on. Upon fungal activation, DCs become mature by suffering a complex reprogramming in response to different fungal stimuli. Then, DCs are able to promote the differentiation of naïve T cells into effector (T helper cells, T_H; T cytotoxic cells, T_c) or regulatory (T_{reg}) T cells, depending on the received stimulus and the PRR involved in the process (reviewed in Romani, 2011).

5.3. Long-term antifungal response: T and B cell subsets

During fungal infections, antigen-presenting cells (mainly DCs) internalize fungal cells or components, digest them, and present potential antigens to naïve T cells using major histocompatibility complex (MHC) class I or II (Figure 7). As previously explained, depending on the stimulus or the PRR utilized, different pathways are activated. In this way, the connection between "innate" and "adaptive" immunity is achieved.

Regarding to antifungal T_H cell-mediated responses, these are mainly carried out by three cell subclasses, T_H1, T_H2, and T_H17 (reviewed in Romani, 2011; Verma *et al.*, 2012; Borghi *et al.*, 2014). The first, T_H1, promotes antifungal protective immunity and release of pro-inflammatory cytokines as interferon-γ, or tumour necrosis factor-α. In this way, this response produces enhanced phagocytic capacity and ROS release by macrophages, antigen processing and presentation, or antibody class switching to IgG2a in B cells. T_H2-mediated responses are considered negative for hosts during fungal infections, since they favour an alternative activation of macrophages (mediated by interleukins-(IL) 4, or 13), which interferes with phagocytosis and pathogen clearance. Moreover, they are associated with higher levels of IgE, inducing allergic responses in CF patients, for example. However, some T_H2-related responses have been proved to be efficient against some fungal species, as *A. fumigatus* or *Pneumocystis murina*. Furthermore, T_H17 subset is identified by the expression of IL-17A, IL-17F, and IL-22, which have been involved in protective antifungal responses. Among others, recruitment of neutrophils, or promotion of antimicrobial peptides release (e.g. defensins or histatins) are mechanisms that have been related to T_H17 responses.

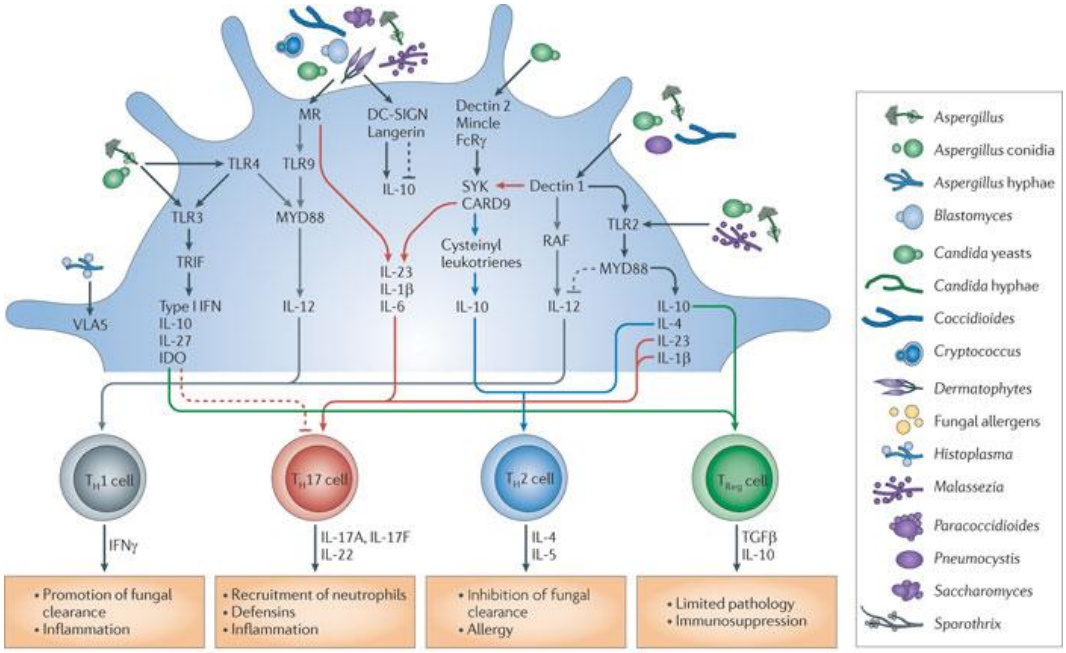
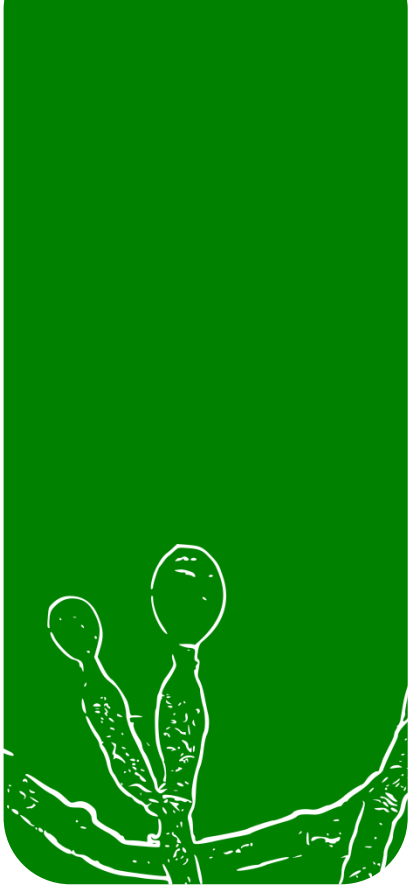


Figure 7. Dendritic cell (DC) activation upon fungal recognition. DCs are professional antigen-presenting cells which induce a variety of immune responses depending on their stimulation. Adapted from Romani, 2011.

Concerning T_{reg} antifungal response, their main role is to limit pro-inflammatory immune responses in order to avoid an excessive inflammation which may produce damage in surrounding tissues. By producing a sort of cytokines and other molecules, T_{reg} not only has immunosuppressive functions, but promote durable antifungal immunity. Finally, the role of T_c cells is poorly known, since they are mostly involved in antiviral and antitumor immunity. However, they have been shown to be critical for immunity induced by vaccines. This fact should, then, be taken into account in order to develop this kind of therapies that may protect immunodepressed individuals, the main target of IFIs.

Finally, B cells are also activated through T cells, starting to maturate and to produce essential molecules to immune response against any kind of infection, namely antibodies. Although the role of antibody-mediated immunity to fungal infections was not very clear for a long time (Casadevall, 1995), during the last two decades an increasing number of studies have proved their importance. In fact, proteomics-based analyses have been applied in order to determine a wide number of antigenic proteins recognized by IgA, IgG, or IgE of both healthy and infected individuals. The presence of such variety of antigenic proteins means that a great number of antibodies to fungi are developed in hosts, being probably some of them protective in some way. In this sense, monoclonal antibody (mAb) production

technology has provided evidence on the role of individual antibodies recognizing well-known antigens. Interestingly, variable effects have been obtained using these mAbs, being protective, indifferent, or detrimental. Among the first, which could be interesting as therapeutic strategies, a wide range of antifungal activities, both direct and indirect, have been described, including inhibition of biofilm formation or germination, enhanced phagocytosis and complement activation, etc. (reviewed in Casadevall and Pirofski, 2012).



Aim of the study

Aim of the study

The findings reported and knowledge acquired in the last decades concerning *Lomentospora prolificans* pathobiology and infections, point out that there is an emerging need for novel targets which, in the future, could contribute to the development of fully effective therapies, and specific diagnostic tools that facilitate the management of these mycoses. In this regard, omics techniques, including proteomics-based technologies, have lead to outstanding and innovative therapeutic and diagnostic approaches in medical mycology, especially for the clinically relevant genera *Candida* and *Aspergillus*.

With this concern, we proposed as a **general aim** a multifaceted study of *L. prolificans* pathobiology, focusing on fungal virulence factors and host-mediated antifungal immunity, to deepen into the understanding of the infections caused by this species, and to contribute to the development of novel therapies.

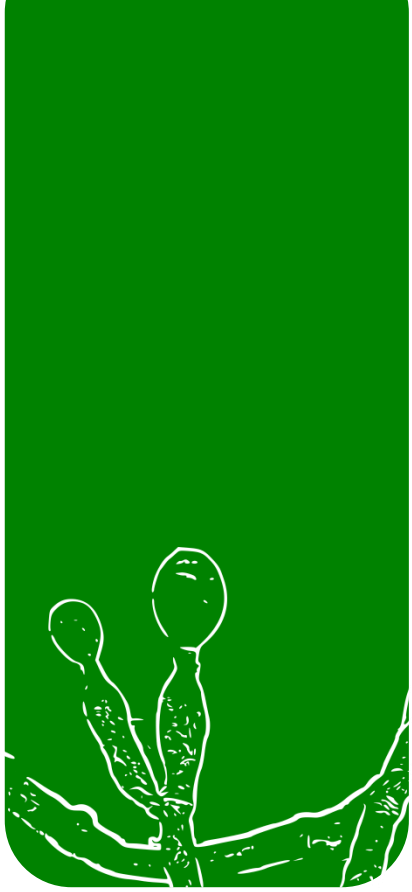
In this sense, the **specific objectives** of this study were the following:

1. To characterize the immunomes of *L. prolificans* conidia and hyphae recognized by salivary immunoglobulin A of immunocompetent humans.

2. To analyze the antigenicity of *L. prolificans* conidial and hyphal proteins against serum immunoglobulin G, determining the seroprevalence of immunodominant antigens among immunocompetent individuals.

3. To study cellular and molecular modifications occurred in *L. prolificans* cells in response to the antifungal drug voriconazole.

4. To characterize the *in vitro* interactions between microglia and *L. prolificans* in order to elucidate immune cell responses and fungal virulence factors.



Chapter 1

Lomentospora prolificans immunomes against human salivary Immunoglobulin A

Results published in:

Pellon, A., Ramirez-Garcia, A., Antoran, A., Fernandez-Molina, J.V., Abad-Diaz-de-Cerio, A., Montañez, D., et al. (2014) *Scedosporium prolificans* immunomes against human salivary immunoglobulin A. Fungal Biol 118: 94–105.

1. Introduction

Lomentospora prolificans is capable of causing infections both in immunocompetent and immunocompromised individuals, but infection severity and patient survival are clearly determined by their immune status. This suggests that a competent immunity is necessary to effectively eliminate the fungus, even without using an antifungal treatment. In fact, the activity of antifungal compounds against *L. prolificans* is increased by a synergistic relationship between them and the host response (Gil-Lamaignere *et al.*, 2002).

Hence, to increase the survival of immunocompromised patients, there is an urgent need for new therapeutic strategies to treat *L. prolificans* infections, giving priority to restoring immune system integrity. With this objective, immunotherapy-based treatments, such as leukocyte transfusion, immunomodulatory therapies, and monoclonal antibody therapy should be the target of further research. Among these, there has been increasing interest in recent years in the use of protective antifungal antibodies against pathogenic fungi due to their promising results against, among others, *Candida albicans*, *Cryptococcus neoformans*, and *Histoplasma capsulatum* (Casadevall, 1995; Matthews and Burnie, 2001; Sevilla *et al.*, 2006; Brena *et al.*, 2007; Guimaraes *et al.*, 2009; Guimaraes *et al.*, 2011; Antachopoulos and Walsh, 2012). These data suggest that it may be possible to harness these antibodies, alone or in combination with conventional antifungal agents, for novel therapies. They may also evolve into safe, broad-spectrum agents for prophylaxis in high-risk patients with an impaired immune response. However, since no proteomics-based research has been carried out on *L. prolificans*, the major antigens of this fungus have yet to be identified and, therefore, protective antifungal monoclonal antibodies against this fungus have not so far been developed.

The first barriers that our immune system presents against organisms, such as *L. prolificans*, entering through the respiratory tract are the mucosa and their secretions. These contain a variety of protective proteins that play a key role in maintaining airway defences and protecting respiratory and oral tissues. One of these is salivary secretory Immunoglobulin A (sIgA), which provides protection by inhibiting pathogen adhesion to and penetration into mucosal tissues and by promoting phagocytosis. Since *Scedosporium*/*Lomentospora* are

widely distributed and it is not unusual to find them in humanized environments (Harun *et al.*, 2010), the daily protection given by sIgA to healthy individuals may contribute to an effective defence against these filamentous fungi. Therefore, proteomics-based studies of the salivary sIgA-recognized antigens of *L. prolificans* and their immunogenic potential could be an important step in identifying new molecular targets.

Due to the absence of a completely annotated genome, free-gel-based proteomic techniques are not feasible for *L. prolificans*. However, gel-based proteomics have permitted the study of the proteome of some other fungi in the same status (DaSilva *et al.*, 2012; Zhang *et al.*, 2012), and other medically important fungi such as *Aspergillus* (Kniemeyer, 2011) and *Candida* (Pitarch *et al.*, 1999; Hernando *et al.*, 2007; Calcedo *et al.*, 2012). In the present work, we developed an improved workflow for protein extraction from conidiospores and hyphal cells of *L. prolificans*, optimizing growing conditions and the cell disruption method. In addition, we analyzed the antigenicity of both morphs against immunocompetent human salivary sIgA by indirect immunofluorescence and two-dimensional Western blot.

2. Materials & Methods

2.1. Strain, culture conditions, and germination assays

Lomentospora prolificans strain CECT 20842 (a clinical isolate from a patient with disseminated infection in the Hospital Marqués de Valdecilla, Santander, Spain) was cryopreserved at -80°C and subcultured on PDA (Pronadisa, Madrid, Spain) at 37°C as required. To harvest conidiospores, the fungus was grown on potato dextrose agar at 37°C for 7 days and, then, cultured plates were washed twice with sterile saline (0.9% [w/v] NaCl). Several germination assays were performed so as to optimize the growth conditions for obtaining hyphae. Seven culture media were assessed: potato dextrose broth (PDB) (Pronadisa), Sabouraud glucose broth (SAB) (Panreac, Barcelona, Spain), RPMI-MOPS (Sigma-Aldrich, St Louis, MO, USA), RPMI-MOPS containing 10% (w/v) glucose, RPMI-MOPS containing 10% (v/v) FBS (Sigma-Aldrich), Lee's medium, and antibiotic medium 3 (Oxoid). All germination assays were carried out by inoculation of $5x10^5$ conidiospores/ml and subsequent incubation at 37°C in a rotary shaker (120 rpm). Samples of 200 μl were collected at hourly intervals and fixed with 20 μl of 10% formaldehyde to calculate germination indices. Each culture medium was assayed in triplicate to ensure statistical significance.

2.2. Human saliva sample collection and preparation

Saliva samples were collected from a total of five healthy immunocompetent individuals in order to perform the immunofluorescence and Western blot analyses described below. After extraction, saliva samples were centrifuged to eliminate cell debris and, then, supernatants were collected and pooled to minimize the effect of interindividual variations. Pooled saliva was aliquoted and stored at -20°C until use. The methods for human sample collection and manipulation were approved by the Ethics Committee of the University of the Basque Country (UPV/EHU).

2.3. Protein extraction

Three commonly used methods for fungal cell disruption were assessed in order to optimize the protocol to obtain high quality protein extracts from *L. prolificans*: grinding in a cold mortar in the presence of liquid nitrogen (Kniemeyer *et al.*, 2006), sonication and beating with zirconium beads using the Mini BeadBeater (Biospec, Bartlesville, OK, USA) (Klimek-Ochab *et al.*, 2011). After cell disruption, crude extracts were centrifuged for 5 min at 14,100 g to collect supernatants. Then, proteins were precipitated at -20°C for 1 h in four volumes of acetone, 10% (w/v) TCA, 0.07% (v/v) 2-mercaptoethanol, and centrifuged for 15 min at 14,100 g. After that, pellets were washed twice with acetone, 0.07% (v/v) 2-mercaptoethanol and completely air-dried. Finally, proteins were resuspended in rehydration buffer (7 M urea, 2 M thiourea, 4% [w/v] CHAPS, 2% [v/v] ampholytes, 2% [v/v] 2-mercaptoethanol, 0.002% [w/v] bromophenol blue). Protein concentration was quantified by using RC/DC assays (Bio-Rad, Hercules, CA, USA) and protein extract quality was analyzed by SDS-PAGE.

2.4. Two-dimensional electrophoresis, protein visualization, and image analysis

Firstly, IEF was carried out in 7-cm-long Immobiline Drystrip immobilized linear pH 4-7 and 6-11 gradient gels (GE Healthcare, Freiburg, Germany) using the Ettan IPGphor system (GE Healthcare). The protocol for IEF differed depending on the pH range: 30 min at 1,000 V, 1:30 h at 5,000 V and 30 min at 5,000 V for 4-7 strips; and 1:30 h at 5,000 V and 30 min at 5,000 V for 6-11 strips. Then, strips were incubated twice for 15 min in equilibration buffer (6 M urea, 75 mM Tris-HCl pH 6.8, 25.5% [v/v] glycerol, 2% [w/v] SDS, 0.002% [w/v] bromophenol blue), firstly with 1% (w/v) DTT, and secondly with 2.5% (w/v) IAA. The second dimension was performed in the Mini-PROTEAN II (Bio-Rad) system at 70 mA. Broad range Precision Plus Protein™ Standards (Bio-Rad) were used as molecular weight markers. Proteins were visualized by CBB G-250 (Sigma-Aldrich) staining as described elsewhere (Dyballa and Metzger, 2009), but omitting the first Milli-Q water washes before staining step to prevent protein leakage from the gels. Gel images were digitalized by using ImageScanner III (GE Healthcare) and analyzed using ImageMaster 2D Platinum Software, Version 5.0 (GE Healthcare).

2.5. Human salivary sIgA-recognized antigen detection

Protein extracts separated in 2-DE gels were electrotransferred to Hybond-P PVDF membranes (GE Healthcare). Then, proteins recognized by sIgA from human saliva were detected by Western blot. Firstly, membranes were blocked for 1 h in TBS (50mM Tris-HCl pH 7.5, 150 mM NaCl) containing 7% (w/v) low-fat dried milk (TBSM). After that, membranes were incubated with human saliva supplemented with 7% (w/v) low-fat dried milk and 0.25% (v/v) Tween 20, followed by three washes in TBS (for 10 min each time). Then, a HRP-labelled anti-human IgA diluted 1/100,000 in TBSM containing 0.25% (v/v) Tween 20, was added as the secondary antibody. Finally, membranes were washed again three times with TBS (10 min each), and immunoreactive proteins were detected using the enhanced chemiluminiscence kit ECL Prime system (GE Healthcare) in a G:BOX Chemi system (Syngene, Cambridge, United Kingdom). All incubations were carried out at room temperature. Western blot membranes were analyzed by image analysis using ImageMaster™ 2D Platinum Software in order to detect and quantify the intensity of all the antigenic spots. Spots which had a relative intensity of more than 2% were classified as immunodominant and selected for further analysis.

2.6. Immunogenic protein identification

Immunodominant antigens, selected according to the aforementioned relative intensity criterion, were manually excised from CBB-stained gels and identified by PMF in the proteomics facility of the National Centre for Biotechnology-CSIC, Madrid, Spain. Briefly, antigenic spots were in-gel digested with trypsin for 6 h at 37°C. Tryptic peptides were analyzed in an ABi 4800 MALDI TOF/TOF mass spectrometer (AB SCIEX, Foster City, CA). Spectra obtained were compared to those in the non-redundant NCBI protein database using MASCOT software v.2.3.02 (Matrix Science, London, UK). The confidence interval for protein identification was set to ≥ 95% ($p < 0.05$) and only peptides with an individual ion score above the identity threshold were considered correctly identified. Due to the *L. prolificans* genome being incompletely annotated, protein identification was performed by comparison with orthologous proteins from other fungi whose genomes have been already annotated.

For the functional study, the UniProtKB/Swiss-Prot database (http://www.expasy.org/) was searched for each antigen identified. Functional groups were then created depending on the involvement of each protein in given biological processes.

2.7. Indirect immunofluorescence

Cell suspensions, both conidia and hyphae, were air-dried on slides overnight and then fixed in acetone at room temperature for 20 min to perform indirect immunofluorescence (IIF) staining. After that, slides were air-dried and incubated with non-diluted human saliva, 1% (w/v) BSA (Sigma-Aldrich) at 37°C for 15 min. Then, slides were washed three times with PBS (pH 7.5), 10 min each. Incubation with an anti-human IgA labelled with FITC (Sigma-Aldrich) diluted 1:50 in PBS, 0.025% (w/v) Evans Blue, and 0.05% (v/v) Tween 20 was carried out at 37°C for 20 min. After incubation, slides were washed three times for 10 min each with PBS, and then coverslips were placed using Fluoromount Aqueous Mounting Medium (Sigma-Aldrich). A negative control was also performed without incubation with human saliva. Cells were visualized under a confocal microscope FluoView FV500 (Olympus, Tokyo, Japan).

3. Results

3.1. Optimization of the workflow for protein extraction from *Lomentospora prolificans*

Since no proteomic approaches had been applied previously to analyze this species, we decided to optimize several parameters, including fungal growth conditions and cell disruption method. First of all, germination assays were carried out in different culture media in order to evaluate the most appropriate for hyphal growth (Figure 1 A). Of all those assessed, PDB was found to be the most appropriate medium for obtaining these cells, higher percentages of germination being obtained in shorter times, compared to the results with other culture media evaluated. In addition, in the other media new conidiation was observed during the germination experiments, which made it impossible to achieve high percentages of germination, while new conidia were not produced in PDB in the times assessed. Given our results, culturing in PDB and for 9 h were the conditions selected to obtain hyphae for further experiments.

To perform proteomics-based studies, it was also necessary to establish the most efficient method for *L. prolificans* cell disruption. To this end, we assessed several cell lysis protocols including bead beating, sonication, and grinding in a cold mortar in the presence of liquid nitrogen. Protein extracts obtained with each method were analyzed by SDS-PAGE to evaluate their quality (Figure 1 B) and their protein concentrations were measured. Cell disruption using bead beating yielded more concentrated and higher quality protein extracts. Having selected this method as the best for *L. prolificans* cell disruption, we also evaluated several beating times. It was clearly observed that over the first 20 min, the more beating time applied the more protein amount was obtained, with no apparent protein degradation (Figure 1 C). However, no significant increases were found with longer beating times.

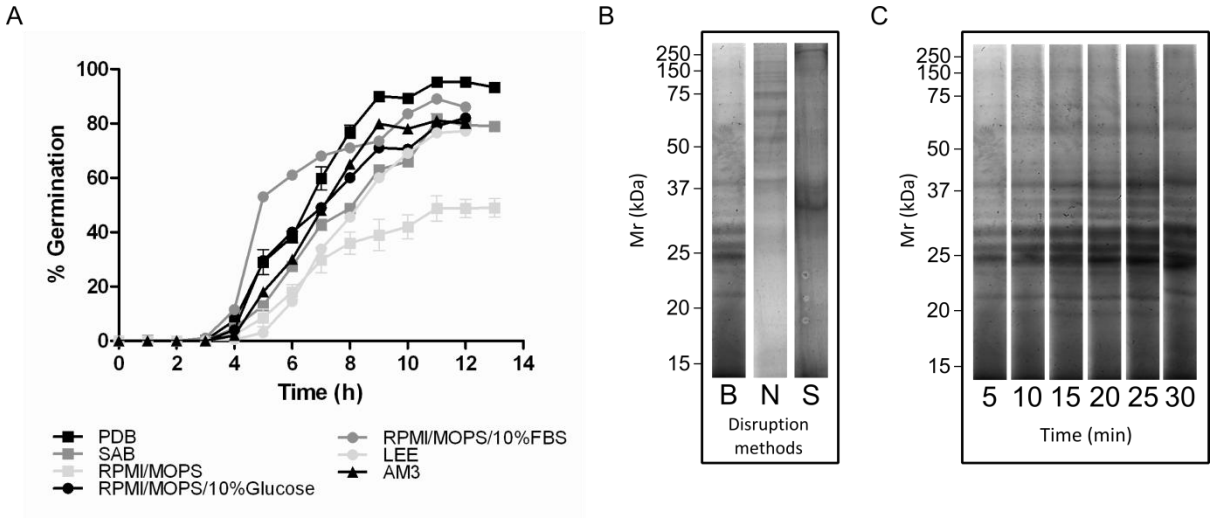


Figure 1. Improvement of protein extraction workflow from *Lomentospora prolificans*. A) Fresh conidia were inoculated in seven different culture media to optimize their germination in order to obtain hyphal protein extracts. LEE: Lee's medium; AM3: antibiotic medium 3. B) Electrophoresis showing protein extracts obtained by the three fungal cell disruption methods assessed: bead beating (B); sonication (S); and grinding in a cold mortar in the presence of liquid nitrogen (N). C) Electrophoresis of proteins extracts during a time-course experiment using bead beating to disrupt *L. prolificans* cells.

3.2. Differential recognition of conidial and hyphal cells by indirect immunofluorescence

In order to improve our understanding of the humoral recognition of *L. prolificans* by saliva from immunocompetent individuals, we conducted IIF. Fungal antigens reacting with human salivary sIgA were detected by incubating the cells with human saliva and an anti-human IgA labelled with FITC. Figure 2 shows the results of a representative immunofluorescence assay at starting time of incubation (0 h) to visualize conidia, and after 9 h to detect recognition of hyphae by human saliva. In addition, the third row shows the negative control in which no incubation with human saliva was performed. IFF assays clearly showed that conidia were strongly recognized by human salivary sIgA, while hyphal cells were weakly recognized. On the other hand, we observed a weak autofluorescence signal in hyphae which also appeared in the negative control.

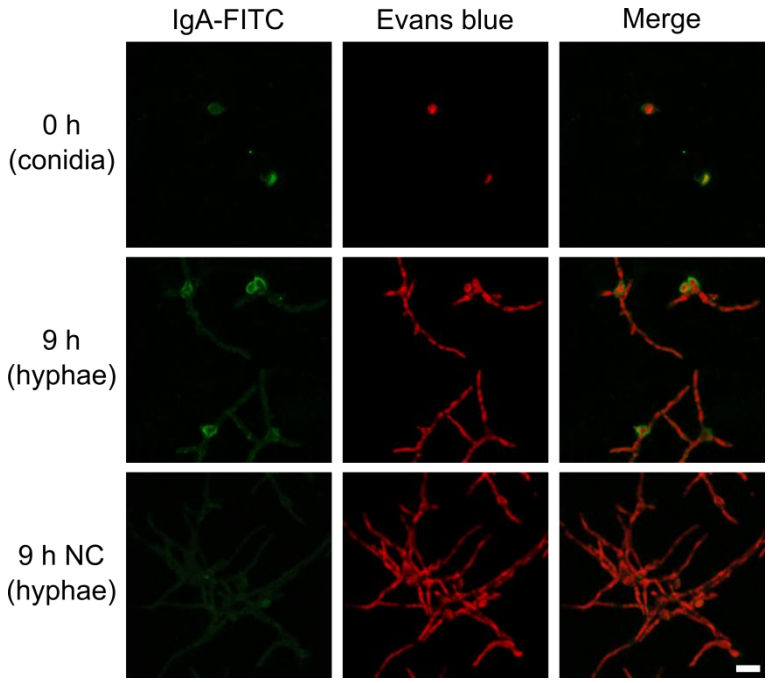


Figure 2. Indirect immunofluorescence staining to detect sIgA-recognized antigens. *Lomentospora prolificans* cells were visualized by confocal microscopy at 0 h, to observe conidia (first row), and after 9 h of growth, for hyphae (second row). A negative control (NC), without incubation with human saliva, was also performed (third row). In columns, cells were stained with anti-human IgA-FITC and Evans blue, detected at 495 and 680 nm, respectively. Scale bar = 10 µm.

3.3. *Lomentospora prolificans* immunomes reacting with human salivary sIgA

Conidial and hyphal protein extracts from *L. prolificans* were separated by 2-DE SDS-PAGE in two distinct pH ranges in order to better visualize their proteome profiles. Pooled saliva samples from five immunocompetent individuals were used to detect specific antigens in three independent Western blot experiments. In parallel to these assays, CBB-stained gels representing each protein pattern were analyzed by the ImageMaster 2D Platinum Software in order to estimate the p*I* and Mr of the immunogenic spots. Up to 250 and 396 protein spots were detected in conidial or hyphal proteomic profiles, respectively. Of these, Western blot results showed 101 immunoreactive protein spots in conidial (Figure 3), and 123 in hyphal protein extracts (Figure 4). That is, 40.4% of protein spots in conidia and 31% in mycelium reacted against sIgA present in human saliva, being, on the whole, widely distributed across the proteomic profiles. Moreover, it was empirically observed that global signal intensity was higher in conidial Western blot membranes than in hyphal ones.

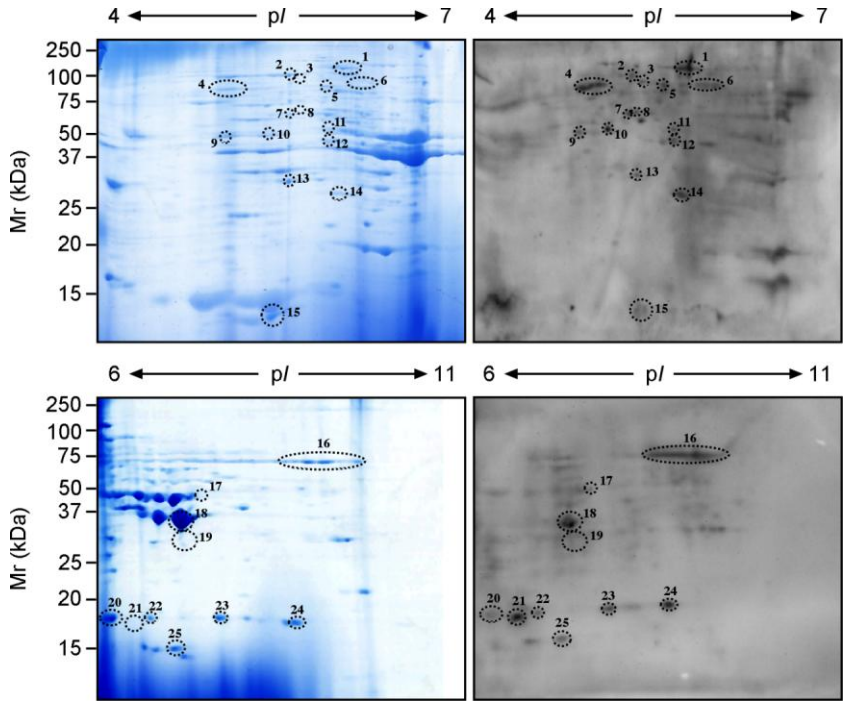


Figure 3. Two-dimensional analysis of *Lomentospora prolificans* conidial proteome and immunome. Protein extracts were separated in two different pH ranges, 4-7 (top) and 6-11 (bottom). Then, proteins were stained with CBB (left) or human salivary sIgA-recognized antigens were detected by Western blot (right). Broken circles depict the immunodominant antigens identified by mass spectrometry (spot numbers refer to those found in Table 1).

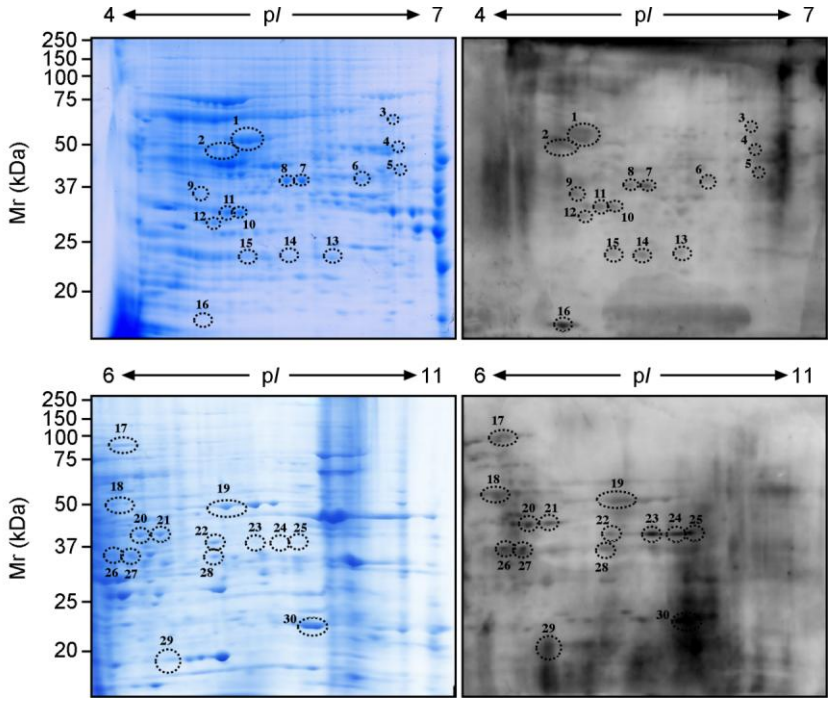


Figure 4. Two-dimensional analysis of *Lomentospora prolificans* hyphal proteome and immunome. Protein extracts were separated in two different pH ranges, 4-7 (top) and 6-11 (bottom). Then, proteins were stained with CBB (left) or human salivary sIgA-recognized antigens were detected by Western blot (right). Broken circles depict the immunodominant antigens identified by mass spectrometry (spot numbers refer to those found in Table 2).

3.4. Identification of human salivary sIgA-recognized antigens of *Lomentospora prolificans*

To focus the analysis on the most relevant antigens, protein spots with a relative intensity of over 2% in the Western blot membranes, according to image analysis, were selected. Protein identification results are given in terms of orthologous proteins belonging to other fungal species the genomes of which have been sequenced (Table 1 and 2), because *L. prolificans* genome has not yet been completely annotated. Thus, 25 and 30 immunoreactive spots, from conidial and hyphal protein extracts respectively, were manually excised from CBB-stained gels and identified by PMF. These protein spots corresponded to only 22 and 23 different proteins, in conidia (Table 1) and hyphae (Table 2), respectively. Hence, some spots were identified as the same protein with nearly the same Mr, but different p*I*. This suggests that they were different protein species of a single protein, such as 40S ribosomal protein S0, superoxide dismutase, and cyclophilins. In addition, two distinct subunits of ATPase were detected as antigens.

Several antigenic proteins were more relevant in terms of their signal intensity. In the case of conidia, they were catalase, putative glyceronetransferase, translation elongation factor-1α, serine/threonine protein kinase, putative superoxide dismutase, putative mitochondrial cyclophilin 1, and peptidyl-prolyl *cis-trans* isomerase. On the other hand, the most immunogenic proteins in hyphal extracts were putative heat shock protein (Hsp) 60, ATP synthase β chain, 40S ribosomal protein S0, citrate synthase, and putative ATP synthase.

Table 1. Identification of proteins from conidiospores that reacted with human salivary sIgA and were identified by PMF. Antigens identified in both morphs are presented in bold text.

Spot No.	Accession No.	Protein name	Orthologous to	Matching peptides (No.)	Sequence coverage (%)	MASCOT score	Theor. pI/Mr (kDa)	Exper. pI/Mr (kDa)
1	gi|322698427	catalase	*Metarhizium acridum* CQMa 102	15	20	166	5.78/78.59	6.19/122.46
2	gi|171694998	hypothetical protein (nucleotidyltransferase activity)	*Podospora anserina* S mat+	13	21	291	5.39/52.74	5.70/100.24
3	**gi|425774732**	**Heat shock 70 kDa protein**	***Penicillium digitatum* PHI26**	**13**	**19**	**223**	**5.04/69.86**	**5.79/96.60**
4	**gi|322705285**	**Heat shock protein 60 (Antigen HIS-62)**	***Metarhizium anisopliae* ARSEF 23**	**19**	**26**	**214**	**5.91/61.53**	**5.34/77.43**
	*gi|121704598	dihydrolipoamide succinyltransferase, putative	*Aspergillus clavatus* NRRL 1	7	14	89	8.20/50.83	
5	gi|398403590	hypothetical protein MYCGRDRAFT_71522 (Rab GDP-dissociation inhibitor activity)	*Zymoseptoria tritici* IPO323	8	14	112	5.80/52.41	5.99/81.23
6	gi|380472222	aldehyde dehydrogenase, partial	*Colletotrichum higginsianum*	9	15	117	5.36/45.16	6.36/77.43
	*gi|336472095	hypothetical protein NEUTE1DRAFT_115658 (metalloexopeptidase activity)	*Neurospora tetrasperma* FGSC 2508	7	20	85	5.96/47.71	
7	gi|116198005	actin	*Chaetomium globosum* CBS 148.51	16	34	546	5.47/40.56	5.68/52.20
8	gi|302423320	mannitol-1-phosphate 5-dehydrogenase	*Verticillium albo-atrum* VaMs.102	14	21	445	5.30/43.83	5.74/53.57
9	**gi|119186907**	**40S ribosomal protein S0**	***Coccidioides immitis* RS**	**11**	**25**	**333**	**4.87/31.49**	**5.24/44.15**
10	gi|358379322	hypothetical protein TRIVIDRAFT_80466 (sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate glyceronetransferase activity)	*Trichoderma virens* Gv29-8	11	27	155	5.23/35.44	5.49/44.15
11	gi|340923536	fructose-bisphosphate aldolase-like protein	*Chaetomium thermophilum var. thermophilum* DSM 1495	5	17	265	5.57/37.22	6.07/44.44
12	gi|402086834	malate dehydrogenase	*Gaeumannomyces graminis var. tritici* R3-111a-1	13	27	419	5.87/34.56	6.07/38.48
13	gi|400597495	triosephosphate isomerase	*Beauveria bassiana* ARSEF 2860	7	21	93	6.04/27.19	5.76/29.06
14	gi|299739616	1,4-benzoquinone reductase	*Coprinopsis cinerea* okayama7#130	6	25	222	9.15/23.17	6.13/24.71
15	gi|380481040	peptidyl-prolyl cis-trans isomerase fkr-3	*Colletotrichum higginsianum*	7	57	124	6.26/12.15	5.77/12.32
16	**gi|38603415**	**translation elongation factor-1α**	***Syncephalis depressa***	**16**	**30**	**209**	**8.84/43.63**	**8.63/78.33**
17	gi|261196333	alcohol dehydrogenase	*Ajellomyces dermatitidis* SLH14081	19	15	84	6.85/36.89	7.53/45.68
18	gi|343425195	related to serine/threonine protein kinase	*Sporisorium reilianum* SRZ2	8	22	82	9.22/83.45	7.28/32.37
19	gi|336463095	formyltetrahydrofolate deformylase	*Neurospora tetrasperma* FGSC 2508	15	25	194	6.56/32.08	7.37/28.56
20	gi|367021508	hypothetical protein MYCTH_2297816 (superoxide dismutase activity)	*Myceliophthora thermophila* ATCC 42464	9	32	324	6.03/16.02	6.33/17.80
21	gi|367021508	hypothetical protein MYCTH_2297816 (superoxide dismutase activity)	*Myceliophthora thermophila* ATCC 42464	9	47	327	6.03/16.02	6.67/17.70
22	gi|10179991	cyclophilin	*Magnaporthe grisea*	9	43	183	8.67/17.89	6.93/18.10
23	gi|33357681	putative mitochondrial cyclophilin 1	*Botryotinia fuckeliana*	9	25	190	9.14/24.26	7.79/18.71
24	gi|154320536	peptidyl-prolyl cis-trans isomerase	*Botryotinia fuckeliana* B05.10	8	24	197	6.30/16.61	8.53/19.13
25	gi|151941479	nucleoside diphosphate kinase	*Saccharomyces cerevisiae* YJM789	6	36	90	8.65/17.22	7.22/15.94

*Correct identification result, but barely significant.

Table 2. Identification of proteins from hyphae that reacted with human salivary s-IgA and were identified by PMF. Antigens identified in both morphs are presented in bold text.

Spot No.	Accession No.	Protein name (putative activity)	Orthologous to	Matching peptides (No.)	Sequence coverage (%)	MASCOT score	Theor. pI/Mr (kDa)	Exper. pI/Mr (kDa)
1	**gi|358399658**	**hypothetical protein TRIATDRAFT_297734 (protein folding; Hsp60 family)**	***Trichoderma atroviride* IMI 206040**	**19**	**28**	**351**	**5.48/61.43**	**4.66/54.03**
2	gi|116204743	ATP synthase β chain, mitochondrial precursor	*Chaetomium globosum* CBS 148.51	22	36	412	5.10/55.68	4.48/50.43
3	gi|322695916	inosine-5'-monophosphate dehydrogenase IMD2	*Metarhizium acridum* CQMa 102	8	11	342	6.68/57.53	6.28/55.45
4	**gi|53830852**	**translation elongation factor-1α**	***Beauveria bassiana***	**13**	**28**	**259**	**8.79/46.86**	**6.30/46.37**
5	gi|378729766	phosphoglycerate kinase	*Exophiala dermatitidis* NIH/UT8656	11	24	200	6.63/44.90	6.33/39.29
(mixture)	**gi|380091217**	**unnamed protein product (protein folding; Hsp70 family)**	***Sordaria macrospora* k-hell**	**11**	**17**	**95**	**5.83/70.13**	
6	gi|407916927	Acetohydroxy acid isomeroreductase	*Macrophomina phaseolina* MS6	15	28	730	9.00/44.71	5.88/37.28
7	**gi|85082069**	**40S ribosomal protein S0**	***Neurospora crassa* OR74A**	**8**	**24**	**99**	**4.80/31.70**	**5.28/36.25**
8	gi|398393150	60S ribosomal protein L4	*Zymoseptoria tritici* IPO323	7	13	103	10.63/39.82	5.13/36.50
9	**gi|119186907**	**40S ribosomal protein S0**	***Coccidioides immitis* RS**	**15**	**35**	**495**	**4.87/31.49**	**4.61/35.05**
10	gi|340959617	40S ribosomal protein S3-like protein	*Chaetomium thermophilum* var. thermophilum DSM 1495	15	48	413	9.06/28.00	4.95/31.88
11	gi|302884265	predicted protein (pyruvate dehydrogenase activity)	*Nectria haematococca* mpVI 77-13-4	9	13	227	5.83/41.73	4.83/31.88
(mixture)	gi|367019060	hypothetical protein MYCTH_2295083 (belongs to 14-3-3 family)	*Myceliophthora thermophila* ATCC 42464	6	25	143	4.89/29.61	
12	gi|115399950	electron transfer flavoprotein α-subunit, mitochondrial precursor	*Aspergillus terreus* NIH2624	16	25	128	8.07/65.67	4.69/30.09
13	gi|346972135	1-Cys peroxiredoxin B	*Verticillium dahliae* VdLs.17	7	15	183	5.67/22.88	5.56/23.88
14	gi|322704573	ATP synthase β chain	*Metarhizium anisopliae* ARSEF 23	9	21	183	4.82/44.74	5.21/23.88
15	gi|347831683	similar to mitochondrial ATP synthase β subunit	*Botryotinia fuckeliana*	10	19	124	5.44/54.56	4.92/23.88
16	**gi|363752599**	**hypothetical protein Ecym_4678 (protein folding; Hsp70 family)**	***Eremothecium cymbalariae* DBVPG#7215**	**12**	**16**	**135**	**5.03/70.29**	**4.44/16.91**
17	gi|302895561	predicted protein (positive regulation of translational elongation; belongs to elongation factor 2 family)	*Nectria haematococca* mpVI 77-13-4	16	14	234	6.24/93.18	6.64/210.85
18	gi|322695916	inosine-5'-monophosphate dehydrogenase IMD2	*Metarhizium acridum* CQMa 102	10	15	345	6.68/57.53	6.52/65.80
19	gi|358400735	mitochondrial F1 ATPase subunit α	*Trichoderma atroviride* IMI 206040	20	38	411	9.06/59.66	8.20/63.27
20	gi|302418130	citrate synthase	*Verticillium albo-atrum* VaMs.102	15	23	492	7.68/52.05	7.00/50.24
21	gi|302418130	citrate synthase	*Verticillium albo-atrum* VaMs.102	15	23	488	7.68/52.05	7.30/50.65
22	gi|320587473	mRNA-binding post-transcriptional regulator	*Grosmannia clavigera* kw1407	15	24	399	7.12/42.78	8.16/45.70
23	gi|403215038	hypothetical protein (ATP synthesis-related activity)	*Kazachstania naganishii* CBS 8797	15	26	119	9.16/59.58	8.76/46.58
24	gi|85068355	26S protease regulatory subunit 8	*Neurospora crassa* OR74A	17	41	194	8.52/43.72	9.10/46.58
25	gi|384489780	ATP synthase subunit α	*Rhizopus delemar* RA 99-880	14	26	140	9.03/59.55	9.28/47.41
26	gi|225557718	septin	*Ajellomyces capsulatus* G186AR	10	30	226	8.19/39.32	6.66/36.58
	*gi|440631848	branched-chain amino acid aminotransferase	*Geomyces destructans* 20631-21	4	10	122	8.10/45.29	
27	gi|261188270	cell division control protein 10	*Ajellomyces dermatitidis* SLH14081	8	26	131	8.20/39.49	6.94/36.58
28	gi|380490447	zinc-binding dehydrogenase (alcohol dehydrogenase activity)	*Colletotrichum higginsianum*	6	18	171	7.01/39.338	8.08/37.66
29	gi|304557442	HEX1 (translation elongation factor activity)	*Trichoderma harzianum*	12	25	197	6.75/24.43	7.34/19.65
30	gi|340517423	predicted protein (voltage-gated anion channel activity)	*Trichoderma reesei* QM6a	4	11	185	8.80/29.93	9.26/22.64

*Correct identification result, but barely significant.

3.5. Human salivary-sIgA target eminent fungal cell processes

Immunogenic protein identification results led us to perform a functional analysis by exploring orthologous protein profiles in the UniProtKB/Swiss-Prot database. Five different functional groups were created to include conidial reactive proteins: metabolism, protein fate, oxidative-stress response, translation, and miscellaneous. Another functional group, energy production, was added for hyphal antigens (Figure 5). On the whole, enzymes participating in ordinary metabolism and protein fate-related processes were the most abundant in conidia, accounting for 44% and 24% of the total, respectively. On the other hand, hyphal antigens were grouped mostly as metabolism- (24%), translation- (30%) or energy production-related (23%) enzymes. Thereby, several functional differences were found between the immunogenic proteins of the two morphs. There was a rise in the number of translation- and energy production-related antigens from conidia to hyphal cells. However, the numbers of protein fate- and oxidative stress-related proteins decreased markedly, as did metabolic enzymes to a lesser extent. No variation in the number of antigens was observed in miscellaneous group.

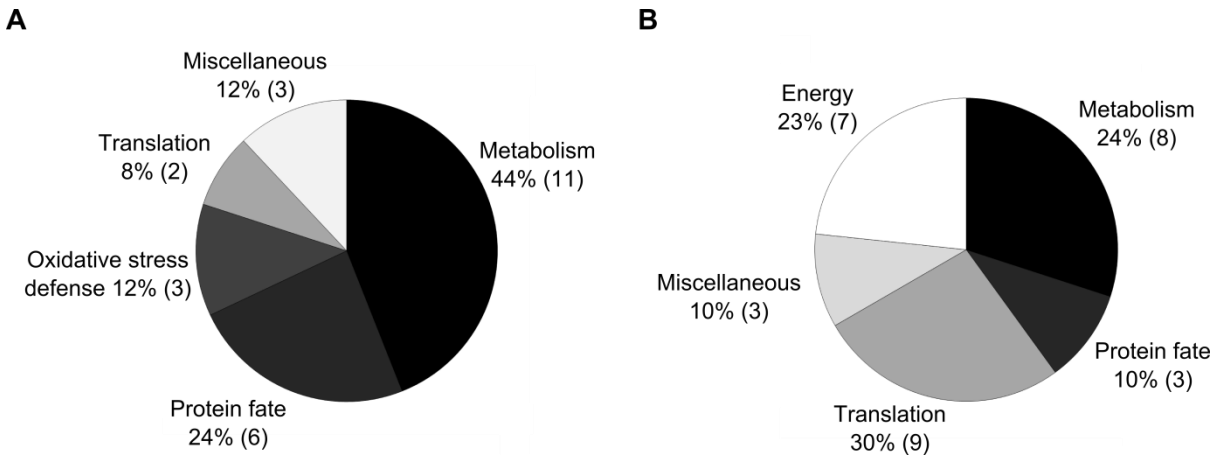


Figure 5. Functional distribution of immunodominant antigens recognized by human salivary sIgA. Immunogenic proteins from conidia (A) and form hyphae (B) were grouped by searching orthologous proteins against UniProtKB/Swiss-Prot database.

4. Discussion

In order to analyze in detail *Lomentospora prolificans* immunomes, growth and germination parameters were evaluated in different culture media. It was found that growing this fungal species on PDB was the best option to obtain the highest percentage of germination in the shortest period of time and with no further conidiation. Additionally, the protein extraction protocol was optimized so as to obtain protein extracts of a high quality and concentration. Although sonication and grinding in a mortar have been widely used for cell disruption in both yeasts (Ramirez-Garcia *et al.*, 2011) and moulds (Kniemeyer *et al.*, 2006), they did not produce suitable results with *L. prolificans*, and this may be attributable to the greater strength of its cell wall (Bowman and Free, 2006). Overall, it was observed that bead beating was the most effective method.

In spite of *L. prolificans* being able to enter the host by several routes, as a traumatic inoculation, invasion through airways is the most probable scenario. Mucosal surfaces, including those in airways, are considered the first line of defence, both physical and immunological, of the body surfaces in contact with the environment. For that reason, mucosae are provided with a wide repertoire of proteins that are secreted into the lumen. Among them, sIgA is the most important immunoglobulin in secretions, and plays two roles by specifically recognizing airway-invading pathogens: i) immune exclusion, which means that sIgA binds to important structures of the pathogen impeding their adhesion to and colonization of the airway epithelium, and ii) initiation of immune responses such as phagocytosis of IgA-opsonized microbes, production of pro-inflammatory compounds and ROS, among others (Pilette *et al.*, 2001).

Given this, in this study we analyzed the sIgA-mediated recognition of this fungal pathogen in human saliva. For this, we first performed IIF to detect antigenic structures in *L. prolificans* cells, being observed that conidia were strongly recognized by human saliva, while hyphae were not. It is conceivable that *Scedosporium*/*Lomentospora* cells may be present inside human hosts, as these fungi are ubiquitous and numerous in humanized environments (Kaltseis *et al.*, 2009; Harun *et al.*, 2010). However, during a hypothetical fungal airway invasion, conidia, but rarely hyphae, are inhaled by the host. This could

explain our results since healthy donors whose saliva samples were used in this study would rarely have been exposed to germinated hyphae.

To delve into the immunogenic properties of *L. prolificans*, immunomic analysis was performed with human salivary sIgA. As already indicated, the complete annotation of the *L. prolificans* genome is not yet available. This hampers progress in the study of this fungus when using genomic, transcriptomic, or proteomic approaches. Accordingly, protein identification strategies have usually been focused on finding orthologous proteins belonging to related fungi. Overall, 101 and 123 antigenic spots were found to be recognized by human sIgA in conidia and in hyphae, respectively, scattered across the entire ranges of Mr and p*I*. The presence of several protein species and/or subunits of the same protein was evidenced by the fact that a total of 22 different proteins from 25 spots were identified in conidiospores (Table 1), and 23 from 30 antigenic spots in hyphal protein extracts (Table 2). This phenomenon may also be related to the presence of different post-translational modifications, such as phosphorylation, methylation or acetylation, which may slightly modify a protein Mr or p*I*.

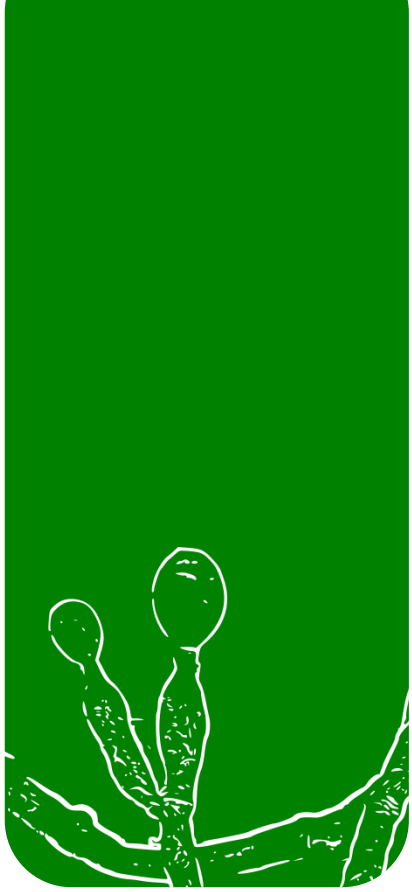
It is worth noting that, consistent with the data from IIF, the intensity of the antigenic spots corresponding to conidial protein extracts was higher than that from hyphal cells. Despite slight, almost none, sIgA-mediated recognition being observed in the latter by IIF, a large number of antigenic protein spots were detected by Western blot. Hence, it is reasonable to suppose that all or most of the proteins found to be antigens in hyphae are not present on the surface of the cell wall, as they are not available for salivary sIgA. Otherwise, if they were to be wall-linked proteins, their abundance must be very low. Several hypotheses could explain these results. Some of the antigens found are housekeeping enzymes with highly conserved domains and/or epitopes showing cross-reactions to similar proteins of other fungi or microorganisms. Other antigens could be released from hyphal cells inside the host as a result of uncontrollable damage leading to their lysis or as a part of *L. prolificans* secretome. In relation to the latter, it is well known that fungi, especially moulds, produce great diversity and quantity of secreted proteins, most of them enzymes that are related to virulence (reviewed in Girard *et al.*, 2013).

Remarkable functional differences were found between the immunomes of the two cell morphs. Although metabolic enzymes were similarly represented, protein fate-, energy production- and translation-related enzymes change dramatically with the morphological transformation. As hyphae are metabolically more active cells, it makes sense that antigens whose functions are related to these groups become more relevant than in conidia. Conversely, in conidial cells protein fate-related antigens were more abundant than in hyphae. It is worth highlighting that enzymes whose main function is to prevent oxygen-produced damage in the cells were identified as main antigens in conidia, these being structures known to be related to dispersion and resistance to environmental stress.

Interestingly, metabolic enzymes were found to be the most abundant *L. prolificans* inducers of sIgA-mediated immune response, both in conidia and hyphae. It is, though, common that these kinds of enzymes are reported as immunogens, such as fructose bisphosphate aldolase and phosphoglycerate kinase in both *Candida albicans* (Pitarch *et al.*, 2004; Calcedo *et al.*, 2012) and *Aspergillus fumigatus* (Asif *et al.*, 2010). The abundance of metabolic enzymes is not surprising since a large proportion of the proteins of the cell are related to metabolism. It could similarly happen in the case of the other groups described in this paragraph. However, it does not lessen the importance of the role of these enzymes as immunogens and targets of our immune system. As well as metabolic enzymes, we identified several antigenic proteins with functions related to protein fate, including heat shock proteins (HSP) in both morphologies, and cyclophilins in conidiospores. HSPs (Hsp60 and Hsp70) have been widely described as antigens (reviewed in Zugel and Kaufmann, 1999), and as relevant for pathogenesis in other fungi, as in the case of *Histoplasma capsulatum* (Guimaraes *et al.*, 2009; Guimaraes *et al.*, 2011). In addition to these, cyclophilins have been found as IgE-recognized proteins in several fungi, for example *A. fumigatus*, so they may initiate an allergic response (Fluckiger *et al.*, 2002). Further, some ribosomal proteins and translation elongation factors identified in this study have also been described as antigens in several microorganisms, including the pathogenic yeast *C. albicans* (Pitarch *et al.*, 2004), and may be relevant in triggering host immune response (Crowe *et al.*, 2003). Among the most important groups, we also found enzymes related to energy production, such as ATPases, which are present in all organisms, but have been found to be immunogenic in several fungal pathogens (Pitarch *et al.*, 2004; Asif *et al.*, 2010).

During a fungal invasion, host immune cells produce several types of ROS so as to kill fungal cells, as previously shown for *L. prolificans* (Gil-Lamaignere *et al.*, 2001). However, pathogenic fungi have efficient enzymatic machinery, such as catalase and superoxide dismutase identified in this study, able to eliminate ROS, this machinery being considered an important virulence factor. In addition, several immunodominant antigens have diverse functions in the cell related to key processes for fungal viability, and these have been classified into the Miscellaneous functional group, representing 12% and 10% of all the antigens identified in conidial and mycelial cells respectively.

To conclude, it has been presented a description of the immunomic profiles of *L. prolificans* in response to human salivary sIgA, and the identification of the immunodominant antigens. To our knowledge, this is the first study concerning *L. prolificans* antigen identification using proteomics-based techniques. More than 50 *L. prolificans* immunogenic proteins have been identified that might be major elicitors of specific immune responses, and could be useful for establishing prognosis and developing new diagnostic tools or even treatments for *L. prolificans* infections. It is possible that the use of antifungal monoclonal antibodies targeting some of these candidate antigens to fight against *L. prolificans* infections, both alone or combined with other antifungal agents, could provide long-awaited innovative treatments to fight these therapy-refractory fungal infections.



Chapter 2

Immunoproteomics-based analysis of the immuno-competent serological response to *Lomentospora prolificans*

1. Introduction

The absence of a truly effective therapy against *Lomentospora prolificans* infections intensifies the need for in-depth knowledge and understanding of the ecology, immunopathology, and virulence mechanisms of this species to find an effective alternative to currently available antifungal compounds. Until now, data from ecological studies have pointed out that distribution of *L. prolificans* is directly related to human activity, being widely distributed in human-impacted ecosystems, whereas it is absent in soils from natural environments (Harun *et al.*, 2010). Precisely for that reason, it is very remarkable that diseases caused by *L. prolificans* are almost exclusively developed in immunocompromised patients, despite humans being usually in contact with the fungus. Therefore, we can conclude that in the immunocompetent population our immune system provides successful protection against *L. prolificans*, in the same way as it does against most fungal pathogens.

The immune response against fungi comprises many mechanisms, both innate and adaptive (Romani, 2004). Initial defences are mechanical and chemical barriers of the skin and mucosae, however, a response based on Pattern Recognition Receptors (PRR)-mediated inflammation and several cell types such as macrophages, neutrophils, or T lymphocytes may be activated. Nevertheless, in this study attention is focused on the humoral response, as protective antibodies may also play an important role during *L. prolificans* infections.

The urge for new therapeutic strategies against *L. prolificans* that increase the survival of immunocompromised patients, requires delving into other approaches unrelated to conventional antifungal compounds, giving special relevance to restoring integrity of the immune system. In this area of research, there are certain promising immunotherapy-based treatments, such as leukocyte transfusion or immunomodulatory therapies, which should be the target of further research. In this way, there has been increasing interest in recent years in the use of protective antifungal antibodies against pathogenic fungi (Casadevall and Pirofski, 2012; Elluru *et al.*, 2015). However, there is no evidence of protective antibodies against *L. prolificans* reported in literature at the present time. Interesting results have been obtained suggesting that the use of specific antibodies, alone or in combination with conventional antifungal agents, may evolve into safe, broad-spectrum agents for

prophylaxis in high-risk patients with impaired immune response. In addition to an inherent antifungal effect, antibody-based therapy may protect by promoting Th1 immunity, or altering fungal gene expression and intracellular trafficking. Moreover, the efficacy of certain vaccines indicates that antibody responses may provide a crucial contribution to antifungal immunity (Borghi *et al.*, 2014).

Proteomics-based research offers the opportunity to identify and characterize the major antigens of microorganisms, thereby providing the gateway to using them as therapeutic targets for new drugs, developing vaccines, or producing specific protective antibodies for passive immunotherapy. However, in the case of *L. prolificans*, proteomics studies have to tackle the problem of the absence of the completely annotated genome. In spite of this, gel-based proteomics have enabled the study of the proteome of some other fungi in the same status by comparison with orthologous proteins from other fungi whose genomes have been already annotated (DaSilva *et al.*, 2012; Zhang *et al.*, 2012). In fact, our research group recently used these techniques to identify the major antigens of *L. prolificans* recognized by immunoglobulins A (IgA) from the saliva of immunocompetent people (Pellon *et al.*, 2014).

In this work, we have analyzed the antigenicity of *L. prolificans* conidial and hyphal protein extracts against immunocompetent human sera using proteomics-based techniques. Thanks to which, the main antigens recognized by IgG from sera in both morphs were identified, and their prevalence in the population studied. Moreover, it was studied the functionality and localization, both bioinformatically and experimentally, of the immunodominant antigens identified.

2. Materials & Methods

2.1. Microorganism

The clinical isolate *Lomentospora prolificans* CECT 20842 was used in this study. Fungal spores and hyphae were obtained as previously described (Pellon *et al.*, 2014). Briefly, conidiospores were released by washing with sterile saline (0.9% [w/v] NaCl) 7-day old *L. prolificans* cultures on potato dextrose agar (Pronadisa, Madrid, Spain) and, then, sieved through gauze to remove hyphae and other debris. Conidiospores were pelleted by centrifugation for 5 min at 4,500 g, and inoculated into potato dextrose broth (Pronadisa) at 37°C for 9 h to obtain hyphae. Both conidiospores and hyphae were washed twice with sterile saline prior to protein extraction.

2.2. Human samples and ethics statement

Ten immunocompetent volunteers, 50% female and 50% male, with ages ranging 24-50 years old, donated blood and saliva samples. All donors were born and resident in the Basque Country, Spain. Immediately after extraction, saliva samples were centrifuged, and serum samples were coagulated and centrifuged in 5 ml glass tubes (Vacutainer; BD Biosciences, Madrid, Spain). After being cleaned, both types of samples were stored at -80°C until use. All procedures and methodologies used to obtain or manipulate human samples were approved and supervised by the Ethics Committee of the University of the Basque Country (UPV/EHU).

2.3. Indirect immunofluorescence

To analyze the localization of human IgG- or IgA-reactive antigens at cell level, indirect immunofluorescence (IIF) was performed. Fungal cell suspensions were obtained as previously mentioned, air-dried on slides overnight, and fixed with acetone for 20 min. After which, slides were air-dried and blocked with PBS, 5% (w/v) BSA at room temperature for 30 min. Pooled human sera or saliva were then diluted 1:50 in PBS, 5% (w/v) BSA, and slides incubated with it at 4°C overnight. Next, slides were washed three times with PBS, 10 min

each, and incubated at room temperature for 30 min with FITC-labelled anti-human IgG or anti-human IgA antibodies (Sigma-Aldrich, St Louis, MO, USA) diluted 1:100 in PBS, 0.025% (w/v) Evans blue, 0.05% (v/v) Tween 20. After incubation, slides were washed as mentioned above and, then, cover slips were placed using Fluoromount Aqueous Mounting Medium (Sigma-Aldrich). A negative control was also performed without incubation with human samples. Cells were visualized under a confocal microscope FluoView FV500 (Olympus, Tokyo, Japan).

2.4. Protein extraction

After obtaining fungal cells, these were pelleted by centrifugation and re-suspended in PBS containing 1% (v/v) 2-mercaptoethanol and 1% (v/v) ampholytes (GE Healthcare, Freiburg, Germany) to facilitate cell disruption. Total protein extracts were obtained according to the previously standardized workflow (Pellon *et al.*, 2014). Briefly, glass beads were added and cells disrupted using the MillMix 20 bead beater (Tehtnica, Slovenia) for 20 min at 30 Hz. Cell debris was removed by centrifugation and supernatant was stored at -20°C until use.

To analyze cell surface-associated proteins (CSP), conidiospores and hyphae were boiled in extraction buffer (50 mM Tris-HCl pH 8.0, 0.1 M EDTA, 2% [w/v] SDS, 10 mM DTT) for 10 min, as previously reported (Pitarch *et al.*, 2002). Next, suspensions were centrifuged 5 min at 4,500 g, and the supernatant stored at -20°C until used. To verify there was no cytoplasmic protein leakage and cell integrity was maintained, cells were pelleted after CSP extraction, washed three times with PBS, then stained with propidium iodide (1 mM) and calcofluor white (40 ng/ml) for 30 min at room temperature and protected from light. Since propidium iodide only is able to enter dead cells, fungal cell integrity was assessed by observing cells only stained with calcofluor white. Autoclaved cell suspensions (15 min, 121°C) were used as positive control for cell breakage.

2.5. Two-dimensional electrophoresis and image acquisition

Fungal proteins were precipitated in four volumes of acetone, 10% (w/v) TCA, 0.07% (v/v) 2-mercaptoethanol, at -20°C for 1 h, and pelleted by centrifugation for 15 min at 14,100 g.

Cleaned protein extracts were re-suspended in rehydration buffer (7 M urea, 2 M thiourea, 4% [w/v] CHAPS, 2% [v/v] ampholytes, 2% [v/v] 2-mercaptoethanol) and incubated at room temperature for 1 h for correct protein dissolution. Protein concentration was measured using the Pierce 660 nm Protein Assay Reagent (Thermo Scientific, Rockford, IL, USA).

Four hundred µg of protein were loaded onto 18-cm-long Immobiline DryStrip pH 3-10 (GE Healthcare) for IEF, after adding 1% (v/v) 2-mercaptoethanol, 1% (v/v) ampholytes, and 0.002% (w/v) bromophenol blue to the samples. Strips were left to rehydrate overnight, then followed by IEF at 500 V for 2,000 Vhr, 1,000 V for 9,000 Vhr, 8,000 V for 20,000 Vhr, and 8,000 V for 100,000 Vhr; 50 µA per strip. Next, proteins were reduced and alkylated for 15 min each in equilibration buffer (6 M urea, 75 mM Tris-HCl pH 6.8, 25.5% [v/v] glycerol, 2% [w/v] SDS, 0.002% [w/v] bromophenol blue) with 1% (w/v) DTT and 2.5% (w/v) IAA, respectively. The SDS-PAGE was performed in the PROTEAN II xl Cell (Bio-Rad, Hercules, CA, USA) at 20 mA per gel, under refrigerated conditions. Broad range Precision Plus Protein™ Standards (Bio-Rad) were used as molecular weight markers. Gels were stained with CBB as previously described (Dyballa and Metzger, 2009) and, then, digitalized using ImageScanner III (GE Healthcare).

2.6. Immunoblot using human sera

Proteins were electrotransferred following 2-DE to Hybond-P PVDF membranes (GE Healthcare) at 150 mA for 20 h. Correct transference was assessed by Ponceau red stain (0.2% [w/v] Ponceau red, 1% [v/v] acetic acid). To detect antigenic proteins, immunoblot experiments were performed using human sera, both pooled and individually. All incubations were carried out at room temperature, except when noted. First, membranes were blocked for 2 h in TBS (50 mM Tris-HCl pH 7.5, 150 mM NaCl) containing 5% (w/v) low-fat dried milk and 0.1% (v/v) Tween 20 (TBSM). Next, membranes were incubated with human sera diluted 1:100 in TBSM at 4°C overnight. Afterwards, antibody excess was removed by washing 3 times with TBS, 5 min each, and an HRP-labelled anti-human IgG diluted 1/100,000 in TBSM added as a secondary antibody. Immunogenic proteins were detected using the enhanced chemiluminiscence kit ECL Prime system (GE Healthcare) following manufacturer's instructions in a G:BOX Chemi system (Syngene, Cambridge,

United Kingdom).

2.7. Bioinformatic image analysis and heat map construction

Gel and Western blot images were analyzed using ImageMaster 2D Platinum Software (GE Healthcare) to detect protein and antigenic spots, respectively. To do that, automatic spot detection was used, applying the following parameters: smooth, 4; min. area, 5; and saliency, 3. Then, the results were manually reviewed and corrected, modifying them as little as possible so as to ensure reproducibility. The Western blots of the pooled sera were carried out in triplicate and analyzed to obtain a reference pattern. Then, the prevalence and relative volume of the antigens of each individual serum was calculated by comparison with the pattern of the pool.

After obtaining relative volume data, heat maps for both conidial and hyphal antigenic proteins were created using TM4 free available software (Saeed *et al.*, 2003).

2.8. Identification of seroprevalent antigens by mass spectrometry

For protein identification, antigenic spots recognized on Western blots by over 70% of the sera tested were excised manually from fresh CBB-stained gels, and subjected to in-gel digestion with trypsin. Gel pieces were swollen in digestion buffer containing 50 mM NH_4HCO_3 and 12.5 ng/µl proteomics grade trypsin (Roche, Basel, Switzerland), and the digestion processed at 37°C overnight. The supernatant was recovered and peptides were extracted twice: first, with 25 mM NH_4HCO_3 and acetonitrile (ACN), and then with 0.1% (v/v) trifluoroacetic acid and ACN. The recovered supernatants and extracted peptides were pooled, dried in a SpeedVac (Thermo Electron, Waltham, MA, USA) dissolved in 10 µl of 0.1% (v/v) formic acid (FA) and sonicated for 5 min.

LC-MS/MS spectra were obtained using a SYNAPT HDMS mass spectrometer (Waters, Milford, MA, USA) interfaced with a nanoAcquity UPLC System (Waters). An aliquot (8 µl) of each sample was loaded onto a Symmetry 300 C18, 180 µm x 20 mm precolumn (Waters) and washed with 0.1% (v/v) FA for 3 min at a flow rate of 5 µl/min. The precolumn was

connected to a BEH130 C18, 75 µm x 200 mm, 1.7 µm (Waters), equilibrated in 3% (v/v) ACN and 0.1% (v/v) FA. Peptides were eluted with a 30 min linear gradient of 3–60% (v/v) ACN directly onto a NanoEase Emitter (Waters). Capillary voltage was set to 3,500 V and data-dependent MS/MS acquisitions performed on precursors with charge states of 2, 3, or 4 over a survey m/z range of 350–1990.

Obtained spectra were processed using VEMS (Matthiesen *et al.*, 2005), and searched for against the NCBI non-redundant (nr) database restricted to Fungi (version 20150309) using the online MASCOT server (Matrix Science Ltd., London; http://www.matrixscience.com). Protein identification was carried out by adopting the carbamidomethylation of cysteines as a fixed modification and, oxidation of methionines as a variable modification. Up to one missed cleavage site was allowed, and values of 50 ppm and 0.1 Da were set for peptide and fragment mass tolerances, respectively.

Due to the *L. prolificans* genome sequence being incompletely annotated as yet, antigenic protein identification was performed by comparison with orthologous proteins from other fungi whose genomes were already available in the NCBInr database. Mass spectrometric analyses were performed in the Proteomics Facility of the SGIker, University of the Basque Country (UPV/EHU).

2.9. *In silico* immunogenic protein analyses

Several online available bioinformatic tools were used in this study to characterize the antigens identified. First, we analyzed protein functionalities inferred from homology using the PEDANT web server (http://pedant.gsf.de) (Walter *et al.*, 2009), which predicts cellular roles and functions, and categorizes proteins according to the Functional Catalogue (FunCat) (Ruepp *et al.*, 2004). Protein localization was also predicted by TargetP (http://www.cbs.dtu.dk/services/TargetP/) which detects the presence of signal peptides for several destinations, including secretion and mitochondria. As this tool is based on canonical signal peptides, we applied another available online application, SecretomeP 2.0 (http://www.cbs.dtu.dk/services/SecretomeP/), which predicts protein secretion via non-conventional pathways. In both predictive methods, a value is assigned per protein ranging

from 0 to 1, with a score of ≥ 0.5 considered positive for a given situation. Finally, we analyzed the possible adhesin-like properties of each antigen identified using the Faapred web server (Ramana and Gupta, 2010), in which a score threshold of -0.8 was established.

3. Results

3.1. Recognition of conidia and hyphae given by saliva and serum samples from immunocompetent individuals at cell level

Since conidia is the morph which mainly penetrates via airways and hyphae the morph used to invade tissues, it is likely that the recognition patterns given by saliva and sera will be different on both conidia and hyphae morphs.

Therefore, to determine whether sera and saliva of human immunocompetent population display distinct humoral recognition of conidia and hyphae of *L. prolificans*, we performed IIF using pooled sera samples and compared the results obtained with pooled saliva samples. Interestingly, whereas IgAs from saliva were able to almost exclusively detect the conidial morph, the IgG from sera recognize both conidia and hyphae (Figure 1).

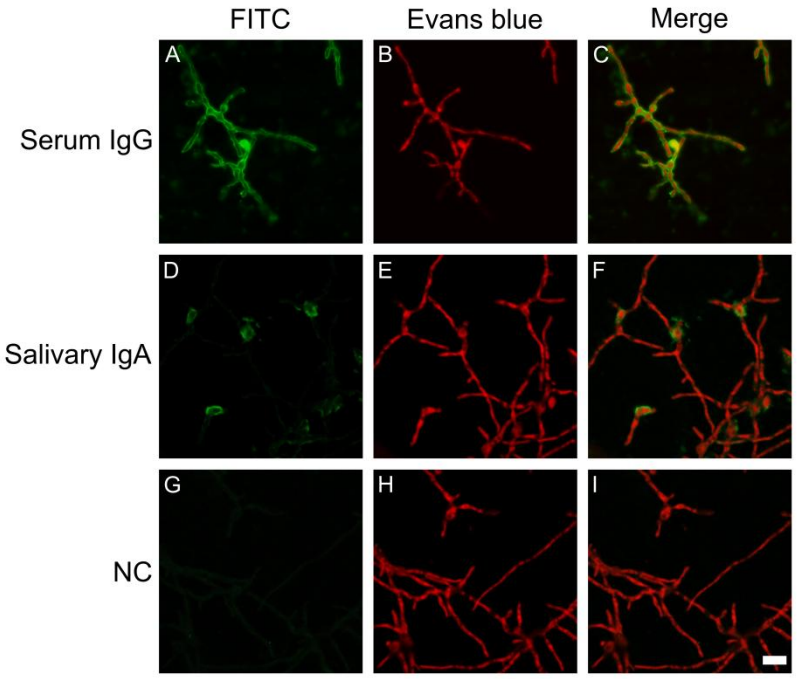


Figure 1. Indirect immunofluorescence staining to detect IgG- and IgA-reactive *Lomentospora prolificans* cells. Fungal cells were incubated with human serum (A-C), saliva (D-F), or without any sample (Negative Control, NC, G-I). Antigens were detected using FITC-labelled secondary antibodies (first column) and Evans blue was used for contrast staining (second column). Overlaid images are shown in the third column. Scale bar = 10 µm.

3.2. Humoral response to *Lomentospora prolificans* proteomes and seroprevalence of immunodominant antigens in immunocompetent individuals

With the purpose of identifying the major antigens recognized by serum IgG in immunocompetent individuals, Western blot experiments using serum samples, both pooled and individually, were performed. Therefore, proteomes of *L. prolificans*, both conidial and hyphal, were resolved by 2-DE, and proteins electrotransferred to PVDF membranes to identify IgG-reactive antigens. Firstly, sera samples were pooled and used to analyze in triplicate whole immunomes of both morphs. Developed Western blot images were acquired and, later, analyzed using the ImageMaster 2D Platinum Software. In this way, estimations of p*I* and Mr of each immunogenic spot were carried out. In addition, relative volume values were calculated as a measurement of the protein spot antigenic capacity.

Interestingly, a large number of protein spots were found to react to serum IgG from healthy donors, with some being intensely recognized. Important differences were found between the recognition given by each volunteer, making it even more important to detect the most prevalent antigens among the healthy population (Supplementary figures 1 and 2). To be precise, of the whole conidial proteome (Figure 2 A), up to 169 antigenic spots were detected to be IgG-reactive, being scattered on the conidial immunome (Figure 2 B), with Mr ranging from 18.3 to 196.9 kDa, and p*I* from 3.8 to 9.4. On the other hand, hyphal whole proteomes (Figure 3 A) presented up to 102 reactive protein spots (Figure 3 B). Although hyphal antigens were found scattering all the Mr (from 17.8 to 190.4 kDa) and p*I* (from 4.1 to 7.9) spectra, the majority and most intense were present on the acidic side of the immunome.

Using image analysis, frequency and relative volume of each antigenic protein spot were quantified by comparing reactivity given by sera samples from the ten donors separately with immunomes obtained using pooled samples (Supplementary material 1 and 2). Thus, seroprevalence values were obtained, and identification of the conidial and hyphal proteins forming those antigenic spots present in at least the 70% of the human serum samples was performed by LC-MS/MS. A total of 26 protein spots were excised from fresh CBB-stained

gels, 13 belonging to conidia and the other 13 to hyphae, being, then, subjected to mass spectrometric analyses. All immunoreactive spots were correctly identified corresponding to different 12 and 9 conidial and hyphal proteins, respectively.

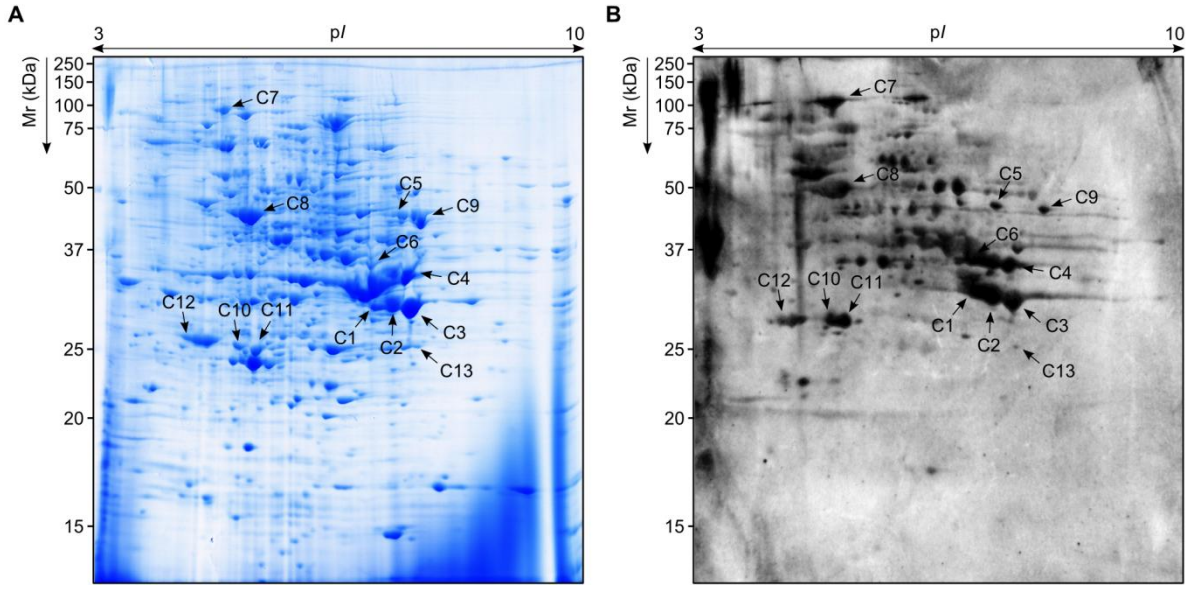


Figure 2. Representative proteome and immunome of *Lomentospora prolificans* conidia. Conidial total protein extracts resolved by 2-DE and CBB-stained (A), or electrotransferred to PVDF membranes to detect serum IgG-reactive proteins using Western blot (B). Depicted arrows point to the most prevalent antigens identified by LC-MS/MS (refer to Table 1).

Regarding the identified conidial antigens (Table 1), G-protein β subunit-like protein, malate dehydrogenase (Mdh) (present in two antigenic spots), and DHN1 were found to be the most prevalent immunogenic proteins, the first being recognized by 100% of the individuals tested, and the latter by 90%.

Concerning hyphal immunodominant antigens (Table 2), heat shock protein (Hsp) 70-like protein was found to be the most seroprevalent antigen, recognized by 100% of the healthy human sera samples. Other immunodominant hyphal antigens recognized by 90% of the individuals were: another Hsp70, Hsp90, putative ATP synthase β subunit protein, and glyceraldehyde-3-phosphate dehydrogenase (Gapdh). Interestingly, Hsp70-like proteins, Mdh, and G-protein β subunit-like proteins were detected in both morphs among the immunodominant antigens identified in this study but with varying seroprevalence values. Unfortunately, two protein spots, one per morph, turned out to be composed by a mixture of proteins (C9 and H7).

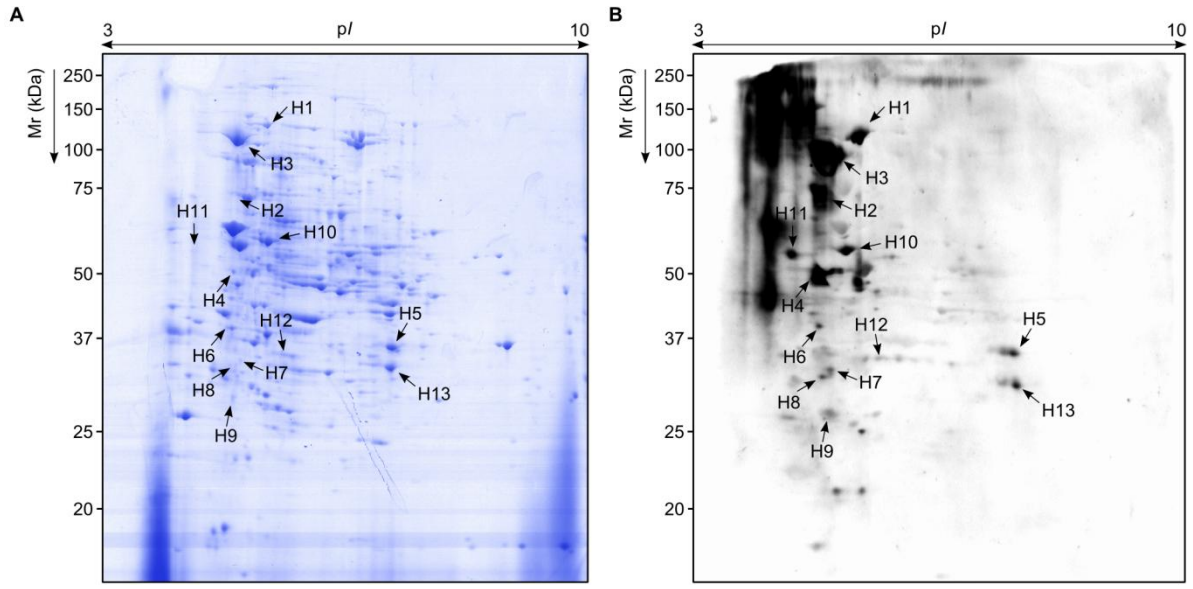


Figure 3. Representative proteome and immunome of *Lomentospora prolificans* hyphae. Hyphal total protein extracts resolved by 2-DE and CBB-stained (A), or electrotransferred to PVDF membranes to detect serum IgG-reactive proteins using Western blot (B). Depicted arrows point to the most prevalent antigens identified by LC-MS/MS (refer to Table 2).

In addition to these data, the presence of several protein species of some of the identified antigens has been confirmed, presenting different Mr and/or p*I*. In the case of conidia, Mdh was present in C2 and C3 spots, both being recognized by 90% of the tested sera. On the other hand, hyphal Hsp70 identified in both H1 and H2 spots, was found to react against 100 and 90% of the sera. In addition, spots: H4, H6 and H10 were formed by ATP synthase β subunit, and recognized by 90, 80 and 70% of the tested sera, respectively.

In-depth analysis of relative volumes of seroprevalent antigens was also performed using image analysis. Using this approach, it was observed that in both morphs, immunodominant conidial and hyphal antigens also presented the highest relative volume values in comparison to all the spots analyzed as pictured in the heat maps (Figure 4 A and C). Moreover, a relation between relative volume and prevalence in the most seroprevalent antigens was observed in both morphs (Figure 4 B and D).

Table 1. Identification and seroprevalence of immunodominant antigens from *Lomentospora prolificans* conidia reacting with human serum IgG.

Spot No.	% Prevalence	NCBI No.	Protein name	Orthologous to	Matching peptides	Sequence coverage (%)	MASCOT score	Theor. pI/Mr (kDa)	Exper. pI/Mr (kDa)
C1	**100**	**gi|629729816**	**G-protein β subunit-like protein (WD40 repeat 2)**	***Metarhizium robertsii* ARSEF 23**	**14**	**27**	**615**	**6.75/35.42**	**7.01/33.74**
C2	**90**	**gi|666869544**	**Malate dehydrogenase, mitochondrial[#]**	***Scedosporium apiospermum***	**12**	**29**	**510**	**7.67/35.08**	**7.28/32.75**
C3	**90**	**gi|576039574**	**Malate dehydrogenase-like protein[#]**	***Chaetomium thermophilum* var. thermophilum DSM 1495**	**5**	**18**	**320**	**8.57/35.60**	**7.54/32.33**
C4	90	gi|83638421	DHN1	*Tuber borchii*	1	2	63	6.64/3476	7.48/37.01
C5	80	gi|666862968	Cysteine transaminase	*Scedosporium apiospermum*	5	11	324	6.22/45.78	7.27/48.75
C6	80	gi|666864016	Fructose-bisphosphate aldolase-like protein (hypothetical protein SAPIO_CDS8665)[#]	*Scedosporium apiospermum*	1	4	110	5.52/39.68	6.95/37.95
C7	**70**	**gi|666864352**	**Heat shock protein70-like protein (hypothetical protein SAPIO_CDS6996)[#]**	***Scedosporium apiospermum***	**10**	**10**	**471**	**5.10/79.45**	**5.06/116.75**
C8	70	gi|666868085	Enolase	*Scedosporium apiospermum*	42	28	572	5.19/47.50	5.16/52.17
C9	70	gi|666863970	Poly(A)+ RNA export protein	*Scedosporium apiospermum*	3	8	186	7.12/39.94	7.92/48.13
		gi|666865792	Putative fumarate reductase protein	*Scedosporium apiospermum*	3	5	158	6.17/66.85	
		gi|380482569	Acetyl-CoA acetyltransferase	*Colletotrichum higginsianum*	2	5	126	6.35/41.77	
C10	70	gi|530461331	ABC-type transporter-like protein (hypothetical protein CGLO_16789)	*Colletotrichum gloeosporioides* Cg-14	6	11	118	5.86/32.73	5.14/29.52
C11	70	gi|584141564	Polyketide synthase-like protein (hypothetical protein FVEG_10010)	*Fusarium verticillioides* 7600	1	4	52	5.26/25.38	5.32/29.44
C12	70	gi|666863172	Had superfamily-like protein (hypothetical protein SAPIO_CDS8905)	*Scedosporium apiospermum*	3	8	106	4.87/26.50	4.49/29.29
C13	70	gi|302417738	Enoyl-CoA hydratase	*Verticillium alfalfae* VaMs.102	1	5	54	9.75/28.46	7.59/27.38

*Immunogenic proteins detected in both *L. prolificans* morphs are presented in bold letter.

[#]Antigens that have been also described to be detected by human IgA (Pellon *et al.*, 2014).

Table 2. Identification and seroprevalence of immunodominant antigens from *Lomentospora prolificans* hyphae reacting with human serum IgG.

Spot No.	% Prevalence	NCBI No.	Protein name	Orthologous to	Matching peptides	Sequence coverage (%)	MASCOT score	Theor. pI/Mr (kDa)	Exper. pI/Mr (kDa)
H1	**100**	**gi|666864352**	**Heat shock protein 70-like protein (hypothetical protein SAPIO_CDS6996)[#]**	***Scedosporium apiospermum***	**15**	**14**	**634**	**5.10/79.45**	**5.40/121.61**
H2	**90**	**gi|666870829**	**Heat shock 70 kDa protein[#]**	***Scedosporium apiospermum***	**19**	**31**	**887**	**5.10/71.25**	**4.76/70.52**
H3	90	gi|666870612	Heat shock protein 90-like protein	*Scedosporium apiospermum*	25	28	1157	4.92/80.16	4.89/97.72
H4	90	gi|631232067	Putative ATP synthase subunit β protein[#]	*Togninia minima* UCRPA7	22	42	1084	5.36/55.76	4.76/46.59
H5	90	gi|630042184	Glyceraldehyde-3-phosphate dehydrogenase	*Pestalotiopsis fici* W106-1	23	21	612	7.01/36.30	7.93/33.41
H6	80	gi|116204743	ATP synthase β chain, mitochondrial precursor[#]	*Chaetomium globosum* CBS 148.51	7	19	328	5.10/55.68	4.75/33.35
H7	**80**	**gi|685397755**	**Malate dehydrogenase[#]**	***Gaeumannomyces graminis* var. *tritici* R3-111a-1**	**7**	**26**	**392**	**5.87/34.67**	**4.93/31.25**
		gi|477529454	Pyridoxine biosynthesis protein	*Colletotrichum orbiculare* MAFF 240422	12	23	367	5.74/32.77	
H8	80	gi|666868259	14-3-3-like protein (hypothetical protein SAPIO_CDS3671)	*Scedosporium apiospermum*	17	40	617	4.92/30.90	4.80/30.29
H9	80	gi|666863507	40S ribosomal S1-like protein (hypothetical protein SAPIO_CDS8739)	*Scedosporium apiospermum*	5	14	163	10.10/29.46	4.93/26.01
H10	70	gi|34447120	Mitochondrial ATPase β-subunit[#]	*Zygosaccharomyces rouxii*	6	15	353	5.06/54.08	5.21/52.63
H11	70	gi|666868184	ATP synthase subunit α-like protein (hypothetical protein SAPIO_CDS3582) [#]	*Scedosporium apiospermum*	11	23	636	9.18/59.65	4.29/51.56
H12	70	gi|666864705	Transaldolase-like protein (hypothetical protein SAPIO_CDS7435)	*Scedosporium apiospermum*	19	29	611	5.78/35.14	5.72/32.65
H13	**70**	**gi|666863913**	**G-protein β subunit-like protein (hypothetical protein SAPIO_CDS8541)**	***Scedosporium apiospermum***	**36**	**46**	**808**	**6.55/35.37**	**7.96/29.29**

*Immunogenic proteins detected in both *L. prolificans* morphs are presented in bold letter.

[#]Antigens that have been also described to be detected by human IgA (Pellon *et al.*, 2014).

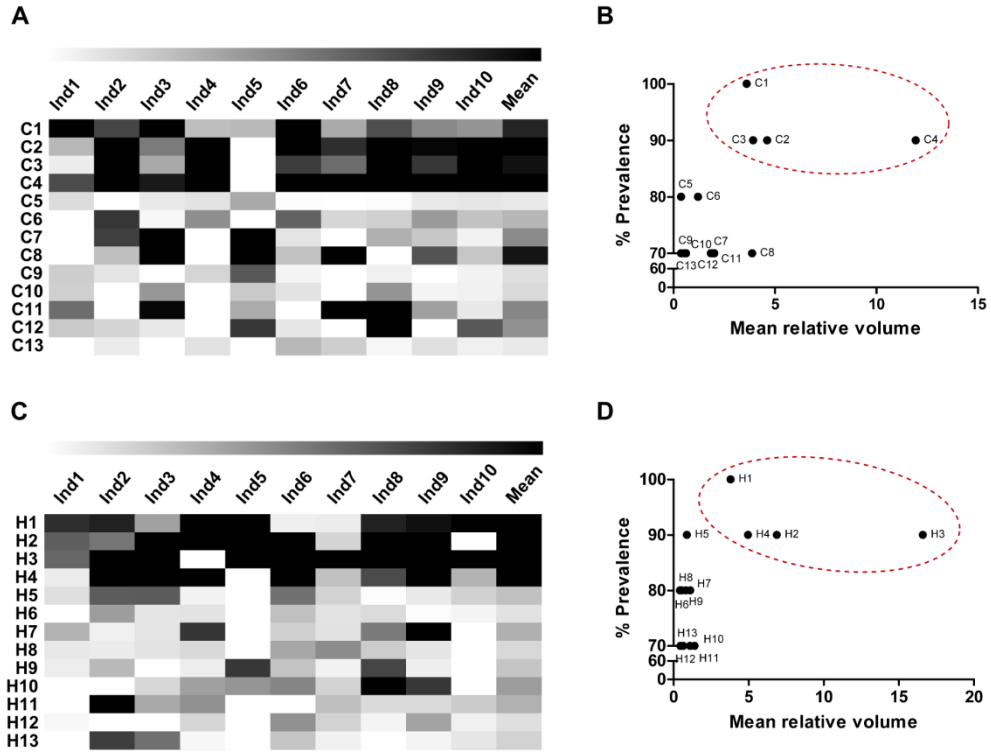


Figure 4. Prevalence and relative volume patterns of immunodominant *Lomentospora prolificans* antigens. Immunomic profiles given by recognition of individual serum samples from the ten immunocompetent donors were analyzed and relative volume values for the most prevalent antigens collected. Heat maps were created for conidial (A) and hyphal (C) antigens. The relation between prevalence and relative volume is also represented (B, D).

3.3. Study of the functionality and cellular location of the major antigens of *Lomentospora prolificans*

Proteins identified by LC-MS/MS were subjected to bioinformatic analyses to study their role and localization in fungal cells. To do so, we explored the orthologous protein profiles using several software and databases. First, we studied the functionality of each antigen by extracting data from FunCat database. Most of the proteins presented several functions in the cell, all of which were taken into account and displayed in Figure 5 A. Several differences were found among the *L. prolificans* morphs. The major conidial immunogenic proteins were found to be mainly related to primary metabolism and energy production, and less importantly to protein fate, and cell rescue, defence and virulence. However, energy production and cellular transport-related antigens were the most abundant in the hyphal immunome.

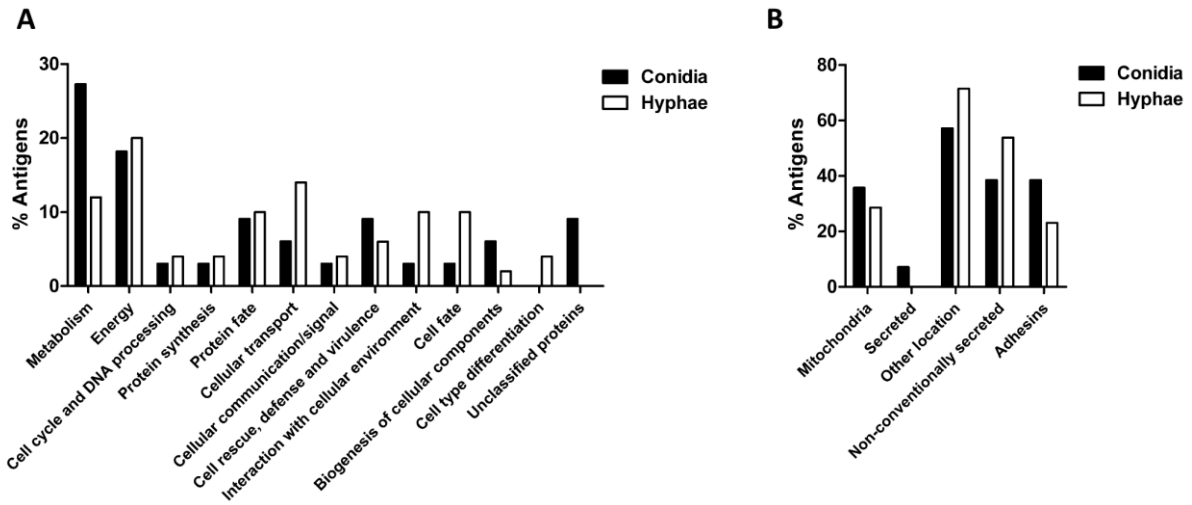


Figure 5. Classification of serum IgG-reactive immunodominant *Lomentospora prolificans* antigens by function and cellular localization. Functional Catalogue (FunCat) was searched to determine the functions carried out by the identified antigens in fungal cells, both conidia and hyphae (A). Similarly, bioinformatic tools were applied to obtain information about the cellular localization and adhesin-like properties of these proteins (B).

In addition, prediction of the identified antigens localization was performed using the TargetP web application (Figure 5 B). On the one hand, we observed that most antigens of both morphs were non-conventionally secreted or targeted to mitochondria. Among others, Mdh (present in C2 and C3 protein spots) and enoyl-CoA hydratase (spot C13) from conidia; and ATPase, both α and β subunits (H4, H6, H10 and H11 spots) from hyphae, were predicted to have mitochondrial signal peptides. On the other hand, among all the proteins identified, only one conidial antigen present in C9 spot was predicted to have secretion signal peptide. However, as already mentioned, this antigenic spot was found to be formed by a mixture of three proteins. Despite this, we analyzed all antigen sequences using the web application SecretomeP, which not only takes into account the presence of consensus signal peptides, but also the markers for non-conventional secretion pathways. Hence, we were able to determine that conidial C3, C5, C9, C12 and C13; and hyphal H4, H5, H6, H7, H9, H10 and H11 antigenic spots contained proteins predicted as likely to be secreted.

Finally, we performed a bioinformatic prediction of adhesin-like proteins using the Faapred web server. Several major antigens were found to have adhesin-like properties, representing 38.46% and 23.07% of the identified conidial and hyphal antigenic spots, respectively. These included the conidial G-protein β subunit-like protein, Mdh, DHN1, and fructose-bisphosphate aldolase (Fba)-like protein, likewise the hyphal Gapdh, Mdh, and G-

protein β subunit-like protein.

3.4. Detection of immunodominant antigens on *Lomentospora prolificans* cell surface

To determine whether antigenic proteins were also present on the surface of *L. prolificans* cells, antigenicity analysis of CSP was performed. To that end, we performed a chemical extraction of the proteins present in the surface of fungal cells, being surface subproteomes resolved by 2-DE. As abovementioned, Western blot using pooled human serum samples to detect IgG-reactive proteins in both conidial and hyphal surfaceomes.

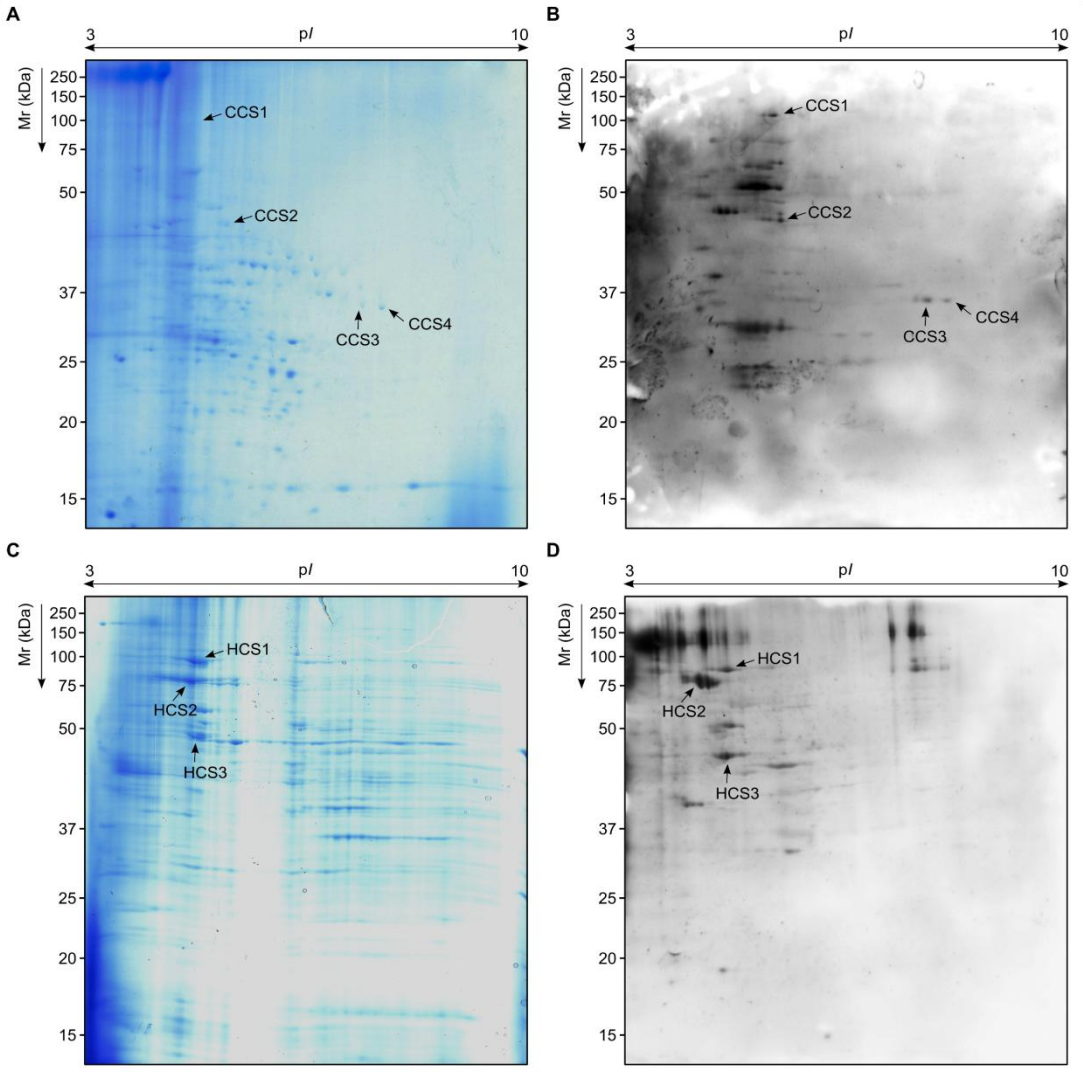


Figure 6. Representative immunoblots of *Lomentospora prolificans* cell surface proteins (CSP). Conidial (A) and hyphal (B) CSP extracts were obtained by chemically shaving entire cells and separated by 2-DE. Serum IgG-reactive proteins were detected using Western blot, being interesting spots identified by LC-MS/MS (refer to Table 3).

Interestingly, in both cases most of the antigenic spots were found on the acidic side of the immunome. To focus on the most relevant proteins, we compared whole immunomes with cell surface immunomes to find the antigenic spots with the higher percentages of prevalence in the population studied. Hence, we selected four and three protein spots in conidia (Figure 6 A) and hyphae (Figure 6 B), respectively, which had similar p*I* and Mr to those in whole immunomes. Interestingly, Hsp70 and Eno, in conidia, and Hsp70, Hsp90, and ATP synthase subunit β, in hyphae, were detected in the surfaceome of *L. prolificans* (Table 3). The other two protein spots form conidial cells, CCS3 and CCS4, returned different protein identification results from those expected, moreover, worse identification parameters were obtained due to small spot size.

Table 3. Identification of the most relevant antigenic proteins in the cell surface of *Lomentospora prolificans* cells.

Spot No.	Whole Immunome Spot No.[#]	NCBI No.	Protein name	Orthologous to	Matching peptides	Sequence coverage (%)	MASCOT score	Theor. pI/Mr (kDa)	Exper. pI/Mr (kDa)
Conidial Cell Surface Antigens									
CCS1	**C7**	gi|666870829	Heat shock 70 kDa protein	*Scedosporium apiospermum*	6	13	257	5.10/71.25	5.19/108.59
CCS2	**C8**	gi|74661880	Enolase	*Cryphonectria parasitica*	6	18	375	5.25/47.39	5.32/46.09
CCS3	**C2**	gi|16751297	Glyceraldehyde 3-phosphate dehydrogenase	*Aspergillus fumigatus*	2	16	82	5.45/18.49	8.03/35.57
CCS4	**C3**	gi|452846047	Heat shock protein 70-like protein (hypothetical protein DOTSEDRAFT_86336)	*Dothistroma septosporum* NZE10	2	3	99	5.02/71.36	8.41/35.68
Hyphal Cell Surface Antigens									
HCS1	**H1**	gi|666864352	Heat shock protein 70-like protein (hypothetical protein SAPIO_CDS6996)	*Scedosporium apiospermum*	7	8	270	5.10/79.45	4.94/90.86
HCS2	**H3**	gi|194716766	Heat shock protein 90	*Humicola fuscoatra*	20	28	857	4.90/79.59	4.54/80.46
HCS4	**H10**	gi|631232067	Putative ATP synthase subunit β protein	*Togninia minima* UCRPA7	33	51	1321	5.36/55.76	4.92/51.59

[#]Spots present in the whole immunome with similar p*I* and Mr.

4. Discussion

Lomentospora prolificans infections are dangerous, often lethal, mycoses mainly affecting immunocompromised patients. Although clinical cases concerning this fungus are not very common worldwide, the relevance of this pathogen and the related genus *Scedosporium* in countries like Spain or Australia is increasing. In fact, they are the cause of over 30% non-*Aspergillus* mould infections (Alastruey-Izquierdo *et al.*, 2013; Slavin *et al.*, 2015). In addition, the absence of fast diagnostic tools and effective treatments to fight these infections results in high mortality rates. As a result, in recent years research has focused on the characterization of new biomarkers that may be used as therapeutic or diagnostic targets (Mina *et al.*, 2015).

Thus, proteomics-based techniques have been widely used to study host-pathogen interaction-related proteins, such as antigens or adhesins (Thomas *et al.*, 2006; Doyle, 2011; Kniemeyer *et al.*, 2011; Del-Chierico *et al.*, 2014). Using these techniques, combined with immunoblot, our research group recently described a wide panel of novel *L. prolificans* antigens recognized by human salivary IgA (Pellon *et al.*, 2014). To understand the humoral response to this fungus in-depth, we performed here an analysis of serum IgG-mediated recognition of *L. prolificans* proteomes in immunocompetent individuals, who seem to be significantly less susceptible to these infections. The use of sera from healthy young people is of special relevance because, even though they are probably in contact with the fungus daily, they do not suffer from L. prolificans infection. This is due, above all, to the immune response they have developed and therefore the results obtained may give us significant information about the antigens targeted. The most prevalent of these molecules could be studied as therapeutic targets or to develop protective vaccines, and antibodies against them may be useful in passive immunotherapy.

Firstly, it was observed that immunocompetent human sera IgG were reactive to both conidia and hyphae using IIF, in contrast to the results obtained when analyzing IgA-mediated recognition in saliva samples, which mainly target conidia. Hence, it was determined the differential humoral recognition that distinct immunoglobulin isotypes display against *L. prolificans*. This fact is consistent with the biological scenario where

conidia represent the morph used by the fungus for dispersion and enter hosts primarily through airways, while hyphae are commonly considered the infective morph, with their formation being a virulent mechanism shared with many other pathogenic fungi (Brand, 2012).

Interestingly, a large number of protein spots were found to be reactive with healthy human serum IgG, many of which were intensely recognized. In fact, up to 169 and 102 antigenic spots were detected scattered over the entire conidial and hyphal immunomes, respectively. Thus, our data suggest there is a strong complex humoral response against *L. prolificans* among the immunocompetent individuals analyzed. This fact contrasts with studies concerning other pathogenic fungi against which very little (Pitarch *et al.*, 2014) or, even, no detectable antibody recognition in non-infected patient sera has been found (DaSilva *et al.*, 2012; Rodrigues *et al.*, 2015). However, this may be the result of different experimental procedures. Nevertheless, the results presented in our study are consistent with previously reported data concerning IgA-mediated humoral response in the mucosae of the airways (Pellon *et al.*, 2014), and other studies regarding *L. prolificans* and *Scedosporium* spp. environmental distribution, where the trend of these fungi to occupy human-impacted ecosystems has been highlighted (Harun *et al.*, 2010; Rougeron *et al.*, 2014). In this sense, the unique biogeography of *L. prolificans* is also remarkable with regard to its relation with human hosts, since all serum donors were resident in the Basque Country, in Northern Spain, where this fungus is becoming an increasingly relevant pathogen (Idigoras *et al.*, 2001). This fact contrasts with the situation in other European countries, like France (Rougeron *et al.*, 2014) or Austria (Kaltseis *et al.*, 2009), where little or no trace of the fungus has been found. It is also worth highlighting the cross-reactivity of some or all of the antigens identified may be occurring with proteins form other fungi or microorganisms. Therefore, more research may be necessary to identify common and specific epitopes.

However, among all the detected antigens, a total of 26 seroprevalent immunogenic spots, 13 from conidia and 13 from hyphae, were identified by LC-MS/MS. Despite the *L. prolificans* genome being sequenced albeit incompletely annotated and not publicly available (Thornton *et al.*, 2014), all antigens were successfully identified by comparison to other fungi as orthologous proteins, the majority of which belong to the closely related

fungus *S. apiospermum*, whose genome has recently been released (Vandeputte *et al.*, 2014).

With regard to conidial antigens, G-protein β subunit-like protein, Mdh (present in two different protein spots), and DHN1 were the most seroprevalent antigens since they were detected by at least 90% of the human sera used, with the first protein spot recognized by 100%. Among the other proteins found to be antigenic were Fba-like protein, Hsp70-like protein, and Eno, which have been widely described as antigens in many microorganisms. It should be highlighted that in some of the conidial protein identifications, precisely, DHN1, Fructose-bisphosphate aldolase, Polyketide synthase-like protein, and Enoyl-CoA hydratase, one single peptide was matched and MASCOT score, despite being statistically significant, is relatively low. In this sense, these identifications might be considered as not totally definite. The fact that in some of the conidial identifications, to be specific, DHN1, Fructose-bisphosphate aldolase, Polyketide synthase-like protein, and Enoyl-CoA hydratase, only one single peptide matched and the MASCOT score, despite being statistically significant, was relatively low must be recognized. In this sense, these identifications can be considered as not totally definitive.

Regarding hyphal antigens, Hsp70 was detected in H1 and H2 spots, and recognized by 100 and 90% of the tested sera, respectively. In addition, Hsp90, putative ATP synthase β subunit, and Gapdh were also targeted by 90% of the human sera. Interestingly, ATP synthase β subunit was detected in other two antigenic spots (H6 and H10), with variable humoral recognition. The presence of several protein species was evidenced in this study and, surprisingly, some of these proteins species exhibited different seroprevalence. Little variations among protein species, which may affect epitopes, might explain the fact that these antigens present distinct relevance for humoral response. Remarkably, a comparative analysis revealed that the most seroprevalent antigens (C1 to C4, and H1 to H4) also showed the highest relative volume values. This may indicate that, in addition to present high seroprevalence, these antigens are recognized by large amounts of serum IgG.

Interestingly, some of the immunogenic proteins detected in this study, namely ATP synthase α and β subunits, Fba, Mdh, and Hsp70, were previously identified by our research

group as reactive to human salivary IgA (Pellon *et al.*, 2014). This finding makes them especially relevant for the development of prophylactic therapies such as vaccines, since with the use of these antigens, patients may develop antifungal antibodies to protect them from *L. prolificans* at different infection levels.

Metabolism-related proteins represent one of the most important functional group in our study, gathering immunodominant antigens like: Mdh, Gapdh, Fba-like protein, or Eno, among others. Unsurprisingly, these proteins have been widely described as antigenic in many other microorganisms. Despite detecting G-protein β subunit-like proteins as antigens in both morphs (C1 and H13 spots), the immunogenicity of these proteins has been poorly described, only being detected in *A. fumigatus* immunoproteome (Shi *et al.*, 2012), in addition to being linked to its cell wall (Kubitschek-Barreira *et al.*, 2013). G-proteins are enzymes with GTPase activity involved in many cell signalling processes since they are usually connected to a wide variety of receptors (called G protein-coupled receptors). Interestingly, in pathogenic fungi these proteins have been related to important processes such as: cell growth, asexual and sexual development, or virulence (Bölker, 1998; Li *et al.*, 2007). Finally, several Hsp have been identified, in both conidial and hyphal immunomes. These proteins were first described during thermal shock experiments, where they were upregulated as their main function is to maintain other protein structures as chaperones. However, they are known to play more roles in fungal cells, being related for example to antifungal resistance or fungal biofilm dispersion (Cowen, 2008; Robbins *et al.*, 2011).

Cellular localization of immunogenic proteins is crucial to develop novel diagnostic or therapeutic strategies (Gozalbo *et al.*, 2004; Pitarch *et al.*, 2006; Moragues *et al.*, 2014). As already mentioned, most of the antigens identified participate in pivotal processes in the fungal cell. The immunodominant nature of these normally internal proteins may be given by their release from fungal cells via secretion or cell lysis, or their presence in the cell wall. In this sense, cell wall-linked proteins are very interesting since they represent available targets for diagnosis, or new therapeutic treatments, such as protective antifungal antibodies. To analyze this, we used two experimental approaches: firstly, *in silico* analyses of the identified antigens, and, secondly, by analyzing the cell surface subproteome.

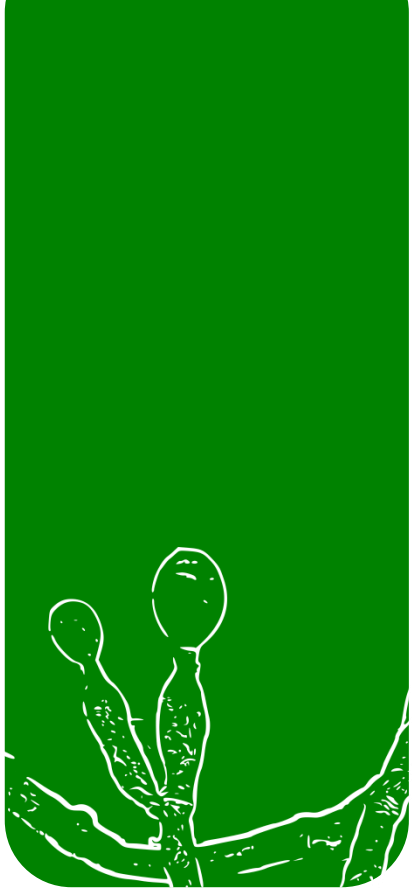
Bioinformatic analyses predicted that conidial antigens C3, C5, C9, C12, and C13; and hyphal antigens H4, H5, H6, H7, H9, H10, and H11 may be secreted or anchored to the fungal cell wall. However, this was not an unprecedented finding since many of these proteins were found to be secreted and/or attached to the fungal cell wall in other important pathogenic fungi (Pitarch *et al.*, 2006; Singh *et al.*, 2010; Vargas *et al.*, 2015). Additionally, many of the proteins, such as Gapdh or Mdh (and others identified in the immunomes, but with no positive results when using the predictive bioinformatic tools), have been widely related to unusual functions, such as: invasion of host tissues, biofilm formation, and adaptation to stress, known as moonlighting proteins (Karkowska-Kuleta and Kozik, 2014). Moreover, antigens C1, C2, C3, C4, C6, H5, H7, and H13 were predicted to have adhesin-like properties, which might indicate these proteins are important for *L. prolificans* virulence.

To detect empirically human serum IgG-reactive proteins in the cell wall of *L. prolificans*, we performed experiments to analyze cell surface subproteomes. When using this strategy, the presence of several fundamental proteins in *L. prolificans* cell wall, such as Eno, Gapdh, Hsp70, or Hsp90 were determined for the first time. Therefore, more research should be focused on these proteins since they may well represent promising novel targets for therapies or detection methods. In fact, Eno or Hsp90 are well-described useful antigens as vaccines (Moragues *et al.*, 2014) or as part of detection methods (Ardizzoni *et al.*, 2014; Pitarch *et al.*, 2014) applied to *C. albicans* invasive infections. More interestingly, recent studies targeting the Hsp90-calcineurin axis have proven the efficacy of specific inhibitors against *L. prolificans* (Lamoth *et al.*, 2015), and azole- and echinocandin-resistant *A. fumigatus* strains (Lamoth *et al.*, 2013).

In addition, profiling the serological response to *L. prolificans* proteomes in patients suffering from these fungal infections is a critical field of research for the future, as these data, combined with the study presented here, might lead to improving the knowledge on these proteins likewise determining which one may represent valuable molecular targets. It is worth highlighting the fact that many of the proteins identified in this study, among them Eno, Gapdh, Hsp70, and Hsp90, found on cell walls, have been also described as antigens in other fungi (Pitarch *et al.*, 2004; Asif *et al.*, 2010). Therefore, this suggests that the cross-reactivity between species may play a significant role in the detection of fungi, whose

clarification could be the aim of future research.

In conclusion, we have analyzed the immunomic profile of L. prolificans conidia and hyphae describing a panel of novel immunodominant antigenic proteins recognized by serum IgG of immunocompetent individuals using a proteomics-based approach. Moreover, the presence of some relevant antigens such as Eno, Hsp70, or Hsp90 on the surface of fungal cell walls has been described. In this sense, due to the limited number of antifungal compounds and inherent multidrug resistance of L. prolificans, these antigens could be an interesting subject for research, in order to develop novel diagnostic tools or therapies, such as compounds to inhibit their pathways, specific antifungal monoclonal antibodies, or protective vaccines.



Chapter 3

Changes in the cell wall architecture and subproteome of the fungus *Lomentospora prolificans* in response to voriconazole

1. Introduction

The emerging filamentous fungal pathogen *Lomentospora prolificans* is mainly characterized by its tendency to cause disseminated infections in immunocompromised patients (Rodriguez-Tudela *et al.*, 2009), and by its low susceptibility to all antifungal compounds currently available (Lackner *et al.*, 2012). In fact, due to its intrinsic resistance *L. prolificans* infections are very hard to eliminate, and, consequently, associated to high morbidity and mortality rates (Cortez *et al.*, 2008).

Among the main classes of systemic antifungal drugs, azoles, especially voriconazole (VRC), have been shown to be the most effective against *L. prolificans*. However, median MICs observed in *in vitro* studies are too high, being hard to achieve these concentrations in patients (Cortez *et al.*, 2008). Moreover, although clinical response to treatment with VRC is better in comparison to other drugs, such as polyenes or echinocandins, disease outcomes are still poor (Troke *et al.*, 2008; Rodriguez-Tudela *et al.*, 2009). Therefore, it is necessary to develop new approaches to manage *L. prolificans* infections.

In this sense, the fungal cell wall (CW) represents one of the most interesting organelle in fungal cells as a target for developing novel therapeutic strategies. On the one hand, its specific composition and structure, completely absent in mammalian cells, reduces drug-associated toxicity and side-effects to host cells. On the other hand, the direct contact of the CW with the environment make this structure available for antifungal compounds. Currently, approaches such as proteomics-based techniques are helpful in order to identify novel and specific targets, and to describe the molecular interactions between drugs and fungal cells, as previously reported for *Candida albicans* (Hoehamer *et al.*, 2010; Sorgo *et al.*, 2011), *Cryptococcus gattii* (Chong *et al.*, 2012), or *Aspergillus fumigatus* (Cagas *et al.*, 2011; Kniemeyer, 2011). In this sense, the identification of key proteins for drug resistance could be very useful to design novel therapies based on, for example, function-inhibiting compounds or protective monoclonal antibodies.

In this work, we performed an integrated study of the changes occurring in *L. prolificans* cells when exposed to VRC. More concretely, we studied changes in the CW architecture

and composition, focusing attention on the cell surface carbohydrates and subproteome. In addition, we attempted to produce null-mutant strains for some of the differentially expressed proteins to deepen into our understanding of their role in VRC resistance, since they may be novel targets for the treatment *L. prolificans* infections.

2. Materials & Methods

2.1. Fungal strain, culture conditions, and germination assays

In this work, *Lomentospora prolificans* strain CECT 20842, which showed MIC >16 for VRC in previous analysis in our laboratory, was utilized. Fungal conidiospores were cryopreserved at -80°C, being the strain recovered on potato dextrose agar (PDA) (Pronadisa, Madrid, Spain) and subcultured at 37°C, as required. In order to determine *L. prolificans* germination rates, conidia were obtained from 7-day old cultured PDA plates by scraping fungal cultures using an inoculation loop and sterile saline (0.9% [w/v] NaCl). Conidial suspensions were filtered through gauze to remove hyphae and other debris, and cell concentration determined using a Bürker counting chamber. Then, 10^8 spores were inoculated in 200 ml of potato dextrose broth (PDB) (Pronadisa) in the presence VRC (Sigma-Aldrich, St Louis, MO, USA), and incubated for a total of 9 h at 37°C, 120 rpm. In control flasks, DMSO was added since this was the solvent used for the antifungal drug. At hourly intervals, 200 μl samples were obtained and fixed with 20 μl of 10% formaldehyde. At least one hundred cells per experimental time and condition were counted, and their germination rates determined. Three assays with technical triplicates were performed to ensure statistical significance. To measure the rest of parameters considered in this study, *L. prolificans* cells were harvested after 9 h of culture by centrifugation (5 min, at 4,500 g), and extensively washed with PBS.

2.2. Indirect determination of chitin content by calcofluor white staining

Cell suspensions were air-dried on slides overnight at 37°C before cell fixation with acetone for 20 min. Next, fungal chitin was stained using calcofluor white (40 ng/ml) for 20 min, staining excess being washed with PBS three times. Finally, cover slips were placed using Fluoromount Aqueous Mounting Medium (Sigma-Aldrich), and cells were visualized by a Zeiss Axioskop fluorescence microscope (Oberkochen, Germany). Micrographs were analyzed by ImageJ software (http://imagej.nih.gov/ij/) to quantified emitted fluorescence, and cell morphological parameters. Three biological replicates were performed, being, at least, one hundred cells per sample examined under the microscope.

2.3. Transmission electron microscopy

Grown cells in the presence or absence of VRC were harvested, washed twice with Sorensen buffer (SB) (0.1 M, pH 7.4), and fixed with 2% glutaraldehyde in SB for 4 h. Samples were then washed with 6% sucrose in SB, 10 min each, and a post fixation step was performed in 1% OsO_4 in SB for 1 h at 4°C. Later, cells were washed three times with SB, 10 min each, and progressively dehydrated with acetone solutions increasingly concentrated (30%, 50%, 70%, 90%, 96%, and twice in 100% acetone), being incubated 15 min with each one. Next, samples were embedded using the Epoxy Embedding Medium kit (Sigma-Aldrich) by consecutive 1 h-long incubations with solutions of acetone:epoxy resin at 2:1, 1:1, 1:2, and, then, a final overnight open-air incubation with 100% epoxy resin to completely eliminate acetone residues. After two resin substitutions, embedded samples were polymerized for 48 h at 55°C. Resin blocks were sculpted to determine interesting areas, being semi-thin sections (1 μm) obtained by using a Leica Ultracut UCT microtome (Leica, Wetzlar, Germany), stained with toluidine blue, and viewed with a Leica Labovert FS light microscope. Next, ultrathin sections were sliced (70 nm), and contrasted with uranyl acetate and lead citrate staining, and micrographs were obtained using a transmission electron microscope Philips CM120 at 100 kV (Koninklijke Philips Electronics, Amsterdam, The Netherlands) in the Analytical And High-Resolution Microscopy Facility of SGIker, University of the Basque Country (UPV/EHU). All incubations were performed at room temperature, except when noted. After image acquisition, thickness measurements of chitin plus glucan and mannan layers were performed using the ImageJ software in at least 20 cells per condition, quantifying 10 areas of each cell, as previously described (Ene *et al.*, 2012).

2.4. Whole cell wall and cell surface carbohydrate composition

To determine VRC-induced variations in the composition of the CW, two approaches were carried out: analysis of the carbohydrate composition of the whole CW and of those linked to cell surface-associated proteins (CSP). On the one hand, to measure entire CW carbohydrates, cells were extensively washed three times with cold distilled H_2O, resuspended in 10 mM Tris-HCl (pH 8), and disrupted by using glass beads and a MillMix 20 bead beater (Tehtnica, Slovenia) for 20 min at 30 Hz, being cell disruption proved under the

light microscope. Supernatants were recovered, and glass beads completely cleaned with cold 10 mM Tris-HCl three times. After that, suspensions containing CWs were centrifuged 5 min at 3,800 g, and pellet washed five times with cold distilled H_2O. Next, acid-based degradation of CW polysaccharides was carried out, as previously reported (Dallies *et al.*, 1998). Briefly, 75 μl of 72% (v/v) H_2SO_4 per mg of CW were added, samples being left at room temperature for 3 h. Then, cold distilled H_2O was added little by little to achieve 2N H_2SO_4, being later samples transferred to sealed glass tubes, and incubated at 100°C for 3 h. Suspensions were cooled, neutralized using saturated BaOH, and centrifuged 5 min at 3,800 g to eliminate $BaSO_4$ precipitates.

On the other hand, to extract CSP intact cells were boiled in extraction buffer (50 mM Tris-HCl pH 8.0, 0.1 M EDTA, 2% [w/v] SDS, 10 mM DTT) for 10 min, as previously reported (Pitarch *et al.*, 2002). Then, H_2SO_4 was added to the samples to achieve an acid concentration of 2N, and followed the protocol abovementioned from this step.

After carbohydrate extraction, contents of rhamnose, arabinose, glucosamine, galactose, glucose, and mannose were determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) by using CarboPac PA-10 columns and the chromatography system Dionex-600 (Thermo Scientific, Rockford, IL, USA). These measurements were performed in the Phytotron Service of SGIker, University of the Basque Country (UPV/EHU).

2.5. Protein extraction from cell surface

Proteome and surfaceome of *L. prolificans* cells exposed to VRC were also scrutinized to identify proteins related to azole resistance. In order to obtain whole cell protein extracts, cells were harvested and resuspended in PBS containing 1% (v/v) 2-mercaptoethanol and 1% (v/v) ampholytes (GE Healthcare, Freiburg, Germany), and disrupted by bead beating, as abovementioned. In this case, cell debris were removed by centrifugation (5 min, at 4,500 g), and supernatants were kept frozen until use. Furthermore, for the surfaceomics study, CSP were released as mentioned in the previous section.

In order to clean protein extracts from both origins, fungal proteins were incubated in four volumes of acetone, 10% (w/v) TCA, 0.07% (v/v) 2-mercaptoethanol, at -20°C for 1 h, and, then, precipitated by centrifugation for 15 min at 14,100 g. After protein pellets being completely air-dried, rehydration buffer (7 M urea, 2 M thiourea, 4% [w/v] CHAPS, 2% [v/v] ampholytes, 2% [v/v] 2-mercaptoethanol) was added, and samples left for one hour at room temperature to entirely dissolve. Protein concentration was determined using the Pierce 660 nm Protein Assay Reagent (Thermo Scientific).

2.6. Proteomic profile analysis by two-dimensional electrophoresis

Proteomic profiles of both entire cells and CW surface were resolved by 2-DE. First, 400 µg of fungal protein 1% (v/v) 2-mercaptoethanol, 1% (v/v) ampholytes, and 0.002% (w/v) bromophenol blue were added, and samples loaded onto 18-cm-long Immobiline DryStrip pH 3-10 (GE Healthcare) for IEF. After an overnight rehydration step, IEF was performed as follows: 500 V for 2,000 Vhr, 1,000 V for 9,000 Vhr, 8,000 V for 20,000 Vhr, and 8,000 V for 100,000 Vhr; 50 µA per strip. Then, two 15 min-long incubation steps in equilibration buffer (6 M urea, 75 mM Tris-HCl pH 6.8, 25.5% [v/v] glycerol, 2% [w/v] SDS, 0.002% [w/v] bromophenol blue) were performed, one with 1% (w/v) DTT and the other with 2.5% (w/v) IAA to reduce and alkylate fungal proteins. Next, samples were separated by SDS-PAGE in a PROTEAN II xl Cell (Bio-Rad, Hercules, CA, USA) at 20 mA per gel, and using refrigerated conditions. To determine protein molecular weight, Broad range Precision Plus Protein Standards (Bio-Rad) were used. After that, fungal proteins were stained with CBB as previously described (Dyballa and Metzger, 2009) and, then, gel images acquired using the ImageScanner III (GE Healthcare).

2.7. Identification of differentially expressed proteins by mass spectrometry

The protein spots most differentially expressed between non-treated and VRC-treated cells were manually excised from fresh CBB-stained gels, and submitted to the Proteomics Facility of SGIker, University of the Basque Country (UPV/EHU) for protein identification by LC-MS/MS. First, proteins were in-gel digested with 12.5 ng/µl proteomics grade trypsin (Roche, Basel, Switzerland) in 50 mM NH_4HCO_3 at 37°C overnight. Then, peptides were

extracted twice: firstly, with 25 mM NH_4HCO_3 and acetonitrile (ACN); secondly, with 0.1% (v/v) trifluoroacetic acid and ACN. Supernatants were pooled, vacuum-dried in a SpeedVac (Thermo Electron, Waltham, MA, USA), and tryptic peptides resuspended in 10 μl of 0.1% (v/v) formic acid (FA) and sonicated for 5 min.

Next, a SYNAPT HDMS mass spectrometer interfaced with a nanoAcquity UPLC System (Waters, Milford, MA, USA) was used to obtain LC-MS/MS spectra. Briefly, 8 μl of each sample were loaded onto Symmetry 300 C18, 180 μm x 20 mm precolumns (Waters), being, then, washed with 0.1% (v/v) FA for 3 min at a flow rate of 5 μl/min. Precolumns were connected to BEH130 C18, 75 μm x 200 mm, 1.7 μm (Waters), being equilibrated with 3% (v/v) ACN and 0.1% (v/v) FA. Then, peptides were eluted with a 30 min linear gradient of 3–60% (v/v) ACN directly onto a NanoEase Emitter (Waters). Capillary voltage was set to 3,500 V, and data-dependent MS/MS acquisitions performed on precursors with charge states of 2, 3, or 4 over a survey m/z range of 350–1990.

MS/MS spectra were processed using VEMS 3.0 (Matthiesen *et al.*, 2005), and searched against the NCBI non-redundant (nr) database restricted to Fungi (version 20150309) using the online MASCOT server (Matrix Science Ltd., London, UK). Carbamidomethylation of cysteines and oxidation of methionines were set as fixed and variable modifications, respectively. In addition, up to one missed cleavage site was allowed, and values of 50 ppm for peptide mass tolerance and 0.1 Da for fragment mass tolerance were set. Since *L. prolificans* genome has been released, but is not publicly available yet (Thornton *et al.*, 2014), protein identification was performed by comparison with orthologous proteins from other fungi whose genomes were already available in the NCBInr database

2.8. Construction of cassettes for gene deletion

To produce null mutant strains for the differentially expressed proteins upon VRC exposure, directed gene deletion was performed to desired genes by using the double-joint PCR method (Yu *et al.*, 2004). Hence, genomic DNA from *L. prolificans* was extracted by the method previously described (Liu *et al.*, 2000), with certain modifications. Briefly, the fungus

was cultured for 3 days in 5 ml of PDB at 37°C, 120 rpm, harvested by centrifugation, and washed three times with sterile saline. Then, 1 ml of lysis buffer was added (400 mM Tris-HCl pH 8, 60 mM EDTA pH 8, 150 mM NaCl, 1% SDS) to a 1.5 ml-tube containing fungal material and 150-200 mg of glass beads. Samples were disrupted for 5 min in a MillMix 20 bead beater, being, then, 300 μl of potassium acetate buffer (60% 5 M potassium acetate pH 4.8, 0.115% [v/v] glacial acetic acid) added. Tubes were inverted several times, centrifuged twice for 5 min at 8,000 g, and supernatants recovered. One volume of cold isopropanol was added, being samples mixed by tube inversion, and centrifuged for 15 min at 14,000 g. Pellets were washed with 300 μl cold ethanol, centrifuged for 15 min at 14,000 g, and supernatants discarded. Finally, samples were air-dried, and pellets containing fungal DNA dissolved in TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) overnight at 4°C.

Deletion cassettes for each gene were constructed by fusing the flanking regions of the gene-of-interest and antibiotic resistance markers. First, primers were designed using gene sequences kindly provided by Dr. Christopher Thornton (Supplementary Material 3) (Thornton *et al.*, 2014), by using Primer3 web application (http://bioinfo.ut.ee/primer3-0.4.0/) and Multiple primer analyzer webtool (Thermo Scientific) to determine primer self- and cross dimerization, and melting temperatures (Supplementary Material 4). In addition hygromicin B (*hyg*) resistance gene was amplified from pCB1636 plasmid, kindly provide by Dr. Sandrine Giraud from the Institut de Biologie en Santé, Angers, France (Figure 1).

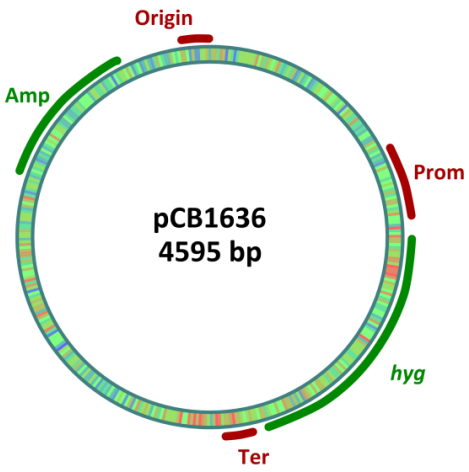


Figure 1. Schematic representation of the plasmid used in this study. Selective marker of antibiotic resistance used to select positive deletion mutants was obtained from pCB1636 (A), for hygromycin B (*hyg*). Prom, promoter; Ter, terminator; Amp, gene for bacterial resistance to ampicillin.

Next, to obtain desired amplicons of flanking regions and resistance genes (first round PCR), PCR was performed using Q5 high-fidelity polymerase (New England Biolabs, Ipswich, MA, USA) in a MJ Mini thermal cycler (Bio-Rad, Hercules, CA, USA). Then, amplicons were purified using the PCR Clean-up kit (Macherey-Nagel, Düren, Germany) before their fusion to construct deletion cassettes (second round PCR). After that, complete cassettes were amplified (third round PCR) to achieve proper transforming DNA quantity for fungal transformation. PCR protocols for each step are described in Table 1, having the third round PCR the same protocol as the first, but using nested primers.

Table 1. Description of PCR mixes and protocols carried out during deletion cassette construction. Volumes used for second round PCR showed as X, were dependent on amplicon concentration achieved.

	PCR mix		PCR protocol		
First Round PCR	Buffer 5X	5 μl	1. Denaturation	10 min, 95°C	
	dNTPs (10 mM)	0.5 μl	2. Denaturation	30 s, 95°C	X 35 cycles
	Q5 Pol.	0.25 μl	3. Annealing	30 s, 55°C	
	Primers (For.+ Rev.)	1 + 1 μl	4. Elongation	1 min, 72°C	
	Template DNA	1 μl (50 ng)	5. Elongation	7 min, 72°C	
	H_2O	To 25 μl			
	PCR mix		**PCR protocol**		
Second Round PCR	Upstream amplicon	X μl	1. Denaturation	2 min, 95°C	
	Downstream amplicon	X μl	2. Denaturation	30 s, 95°C	X 15 cycles
	Res. gene amplicon	X μl	3. Annealing	10 min, 58°C	
	dNTPs (10 mM)	0.5 μl	4. Elongation	5 min, 72°C	
	Q5 Pol.	0.25 μl	5. Elongation	10 min, 72°C	
	H_2O	To 25 μl			

2.9. Generation and validation of knock-out strains

To produce null-mutant strains, constructed cassettes were amplified by PCR to obtain 2-5 μg and, then, to transform *L. prolificans* cells. First, hyphae were obtained after incubating the fungus for 16 h at 37°C, 90 rpm in 100 ml of yeast peptone dextrose broth (YPD) at a concentration of $5x10^7$ conidia/ml. Then, cells were harvested by filtration through Miracloth (Merck, Darmstadt, Germany), and washed with sterile H_2O. Next, fungal CWs were enzymatically degraded using 0.48 g of lysing enzymes or Glucanex (Sigma-Aldrich) in 40 ml of OM buffer (pH 5.6; 1.2 M $MgSO_4$, 0.1 M NaH_2PO_4) for 4-5 h at 37°C, 90 rpm. After the incubation, cold ST buffer (0.6 M sorbitol, 0.1 M Tris-HCl pH 7.5) was added little by little, without letting the phases mix. Samples were centrifuged in a swinging bucket rotor

for 20 min at 4000 g and 4°C, being fungal protoplasts recovered from the OM/ST interface. A volume of cold STC buffer (1.2 M sorbitol, 0.01 M Tris-HCl pH 7.5, 0.05 M $CaCl_2$) was added to protoplasts suspension, and centrifuged for 20 min at 1500 g and 4°C. Protoplast pellet were gently resuspended in 10 ml of STC buffer, centrifuged again as mentioned before, and resuspended in 500 μl of STC buffer to count and adjust them to 10^8 protoplasts/ml microscopically.

Once the protoplast suspension was ready, 100 μl were gently mixed with 25 μl containing deletion cassettes or with STC for negative controls. Next, 200 μl of cold PTC buffer (0.1 ml of 1 M Tris-HCl pH 7.5, 0.1 ml of 1 M $CaCl_2$, 9.8 ml of PEG 60%) were added, mixed by slight inversion, and incubated 10 min in ice. After repeating this step one more time, 800 μl of temperate PTC buffer were added, mixed, and incubated 10 min at room temperature. Then, 675 μl of STC buffer were added to achieve a final volume of 2 ml. Finally, transformation reaction volume was distributed in four Petri dishes, adding 500 μl in each, containing molten agar (1 M sucrose, 0.2 % [w/v] yeast extract, 0.2 % [w/v] casaminoacids, 30% [w/v] agar). Cultures were incubated overnight at 37°C to let the fungus recover from the process, and, the next day, 150 μg/ml of hygromycin B were added to the plates diluted in molten agar. The growth of mutant colonies was monitored daily

Presumed mutant strains growing in transformation plates were isolated twice in YPD agar containing the corresponding antibiotic in order to prove the stability of the mutation. Strains that presented positive resistance to the antibiotic compound were grown and their DNA extracted as abovementioned in order to validate the correct insertion of the deletion cassette by PCR, which was assessed by different amplicon size in comparison with *L. prolificans* wildtype strain.

2.10. Statistical analyses

Statistical analysis was performed using the GraphPad Prism 5 software (GraphPad Software, CA, USA). Differences of the mean were analyzed by using the Student's paired or unpaired *t*-test.

3. Results

3.1. Voriconazole-induced modifications on *Lomentospora prolificans* growth and morphology

The inherent resistance to virtually all available antifungal drugs presented by *L. prolificans* makes these fungal infections difficult to manage and eliminate. Among these drugs, VRC has been proved to be the most active compound, even though the fungus is able to tolerate it. Since no in-depth studies of this inherent resistance have been performed in *L. prolificans*, in this work we analyzed the effects produced by VRC on fungal biology. To do this, we firstly determined the germination rate, hyphal morphology, and chitin content.

The presence of VRC did not affect *L. prolificans* germination rates, starting to germinate at 3 h after inoculation and reaching approximately 80% of germinated cells in less than 8 h, in all cases (Figure 2 A). In contrast, hyphal morphology was drastically modified, as visualized microscopically (Figure 2 B). In fact, image analysis showed that exposure to the antifungal drug promoted a significant cell thickening and a significant reduction on hyphal length, being these last changes dose-dependent (Figure 2 C and D). Consequently, in spite of the variation in length and width, the total area occupied by fungal cells was not significantly modified (Figure 2 E). Finally, calcofluor white staining was also used as an indirect measurement of chitin content by fluorescence emission. The results showed that VRC-treated cells emitted more fluorescence than control ones, being this parameter dependant on drug concentration, and reaching an increase of more than two fold when the higher concentration was used (Figure 2 F).

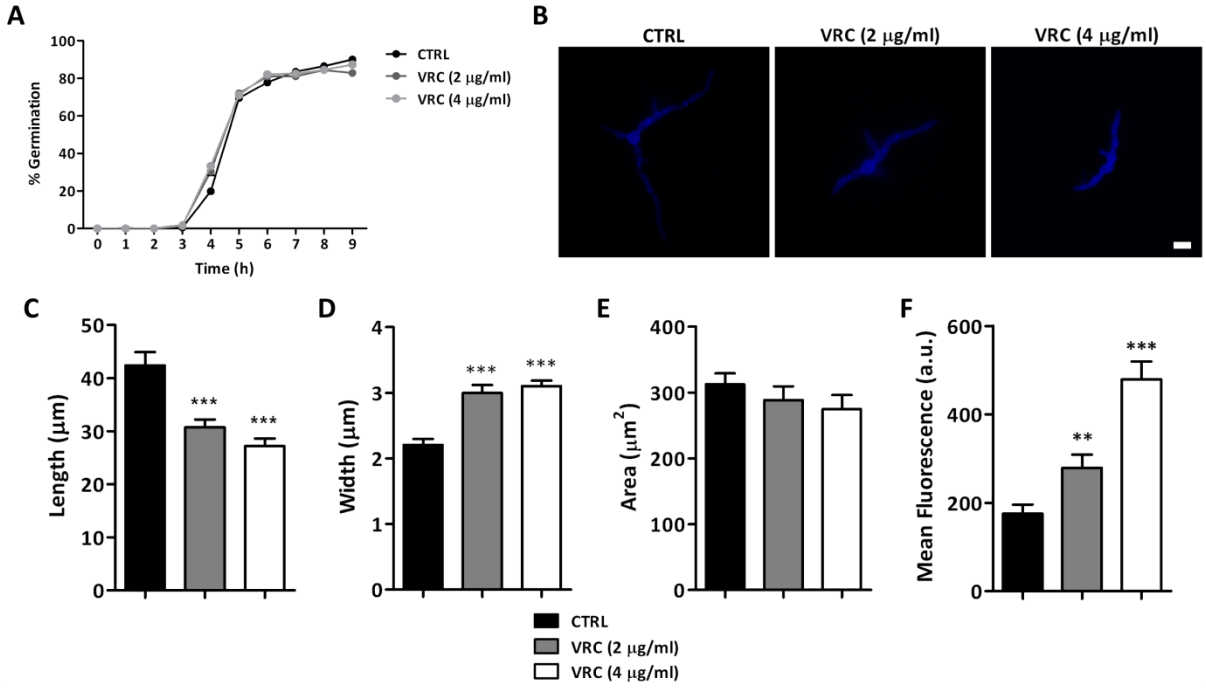


Figure 2. Morphological changes on *Lomentospora prolificans* cells caused by voriconazole (VRC) exposure. Germination assays (A) were performed to analyze the effect of VRC on fungal cells. After 9 h of incubation, cells were stained with calcofluor white and microscopically analyzed (B) to determine their length (C), width (D), occupied area (E), and emitted fluorescence (F). Scale bar = 5 μm. Results are shown as mean ± SEM, n=4. **p<0.01, ***p<0.0001 compared to non-treated cells. a.u., arbitrary units.

3.2. Ultrastructural and compositional changes on *Lomentospora prolificans* cells caused by voriconazole

To determine whether VRC induced structural changes in the fungal CW, cells under antifungal drug stress conditions were examined by transmission electron microscopy. Interestingly, it was observed that VRC-treated cells increased their CW thickness overall (Figure 3 A). This increase was mainly due to the remarkable increment on the outer fibrillar layer, mainly composed by mannans, being almost three times thicker. However, no significant changes were observed in the less-electrodense inner layer, which has been described to be composed by a mixture of chitin and β-glucans (Figure 3 B).

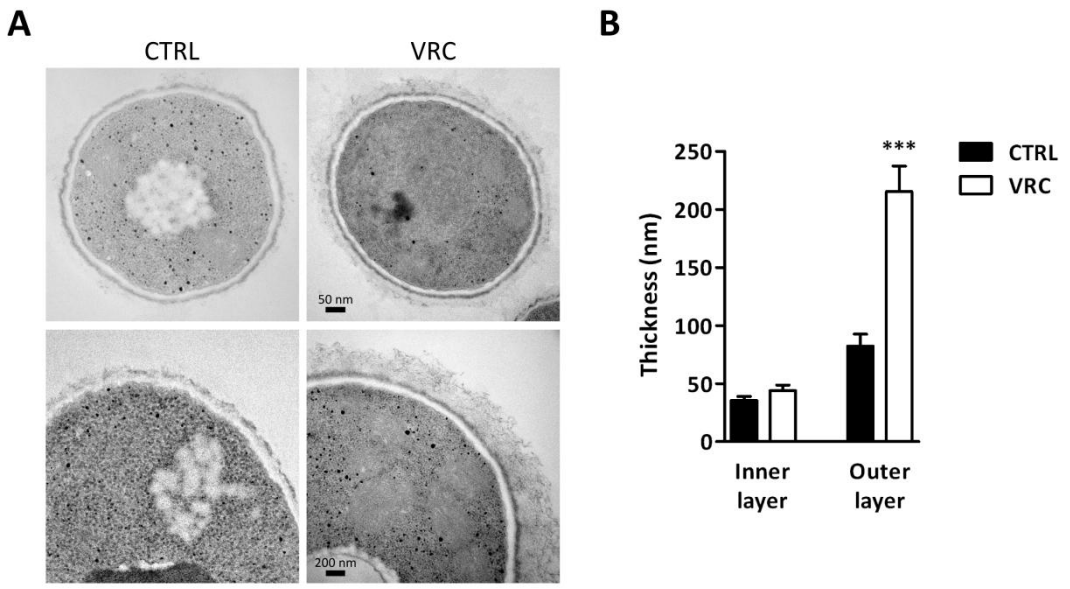


Figure 3. Ultrastructural analysis of voriconazole (VRC)-induced changes on *Lomentospora prolificans* cells. Transmission electron microscopy images (A) and measurements of cell wall thickness (B) of non-treated and 2 µg/ml VRC-treated fungal cells. Results are shown as mean ± SEM, n≥20 cells. ***p<0.0001 compared to non-treated cells.

In addition, a carbohydrate biochemical characterization of CW was carried out to determine the effect of VRC on its composition. Interestingly, VRC exposure induced changes in the composition of the CW backbone, significantly increasing the presence of rhamnose, glucosamine, glucose, and mannose. There were also detected low levels of arabinose and galactose, but their levels did not significantly change in treated cells (Figure 4 A).

Moreover, it was also analyzed the change in the presence of cell surface-associated carbohydrates upon VRC exposure. As Figure 4 B shows, there was a significant increase in the abundance of galactose, glucose, and mannose residues in VRC-treated cells in comparison with control cells. Therefore, it is worth highlighting that in both CW and cell surface there was an increase in the presence of glucose and mannose, being of especial relevance the changes regarding the first one.

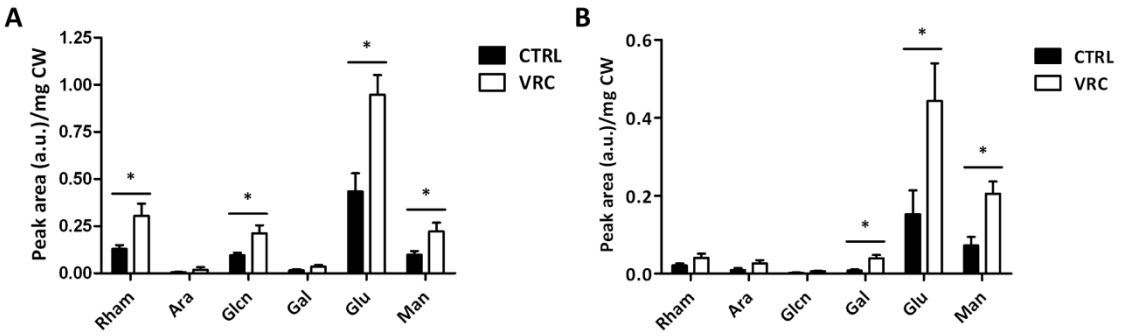


Figure 4. Biochemical characterization of carbohydrate composition of *Lomentospora prolificans* cell wall. Carbohydrate compositional analysis of whole cell wall (A) and cell wall surface (B) upon voriconazole exposure. Results are shown as mean ± SEM, n=3. *p<0.05 compared to non-treated cells.

3.3. Differential protein expression in the *Lomentospora prolificans* cell wall-associated proteins induced by voriconazole

To determine whether VRC was promoting changes in the quantitative or qualitative presence of proteins on the whole fungal cell or on the cell surface, we studied by 2-DE the proteomes and surfaceomes of *L. prolificans* cells upon antifungal drug exposure. Regarding the entire cell proteome, no significant changes, either quantitative or qualitative, were observed when comparing control and VRC-exposed cells (data not shown). Conversely, when surfaceomes were analyzed, up to 72 protein spots were found to be differentially expressed between treated and non-treated cell surfaceomes. Among them, 40 protein spots were underexpressed and 32 overexpressed in VRC-treated cells, being one of these present exclusively after VRC exposure (Figure 5).

Then, in order to focus on the most relevant protein spots that may be involved in *L. prolificans* resistance to VRC, 8 protein spots were subjected to mass spectrometric analyses by LC-MS/MS: the four most overexpressed (including the one solely present in VRC-treated surfaceomes), and the four most underexpressed in cells exposed to VRC in comparison to control cells (Table 2).

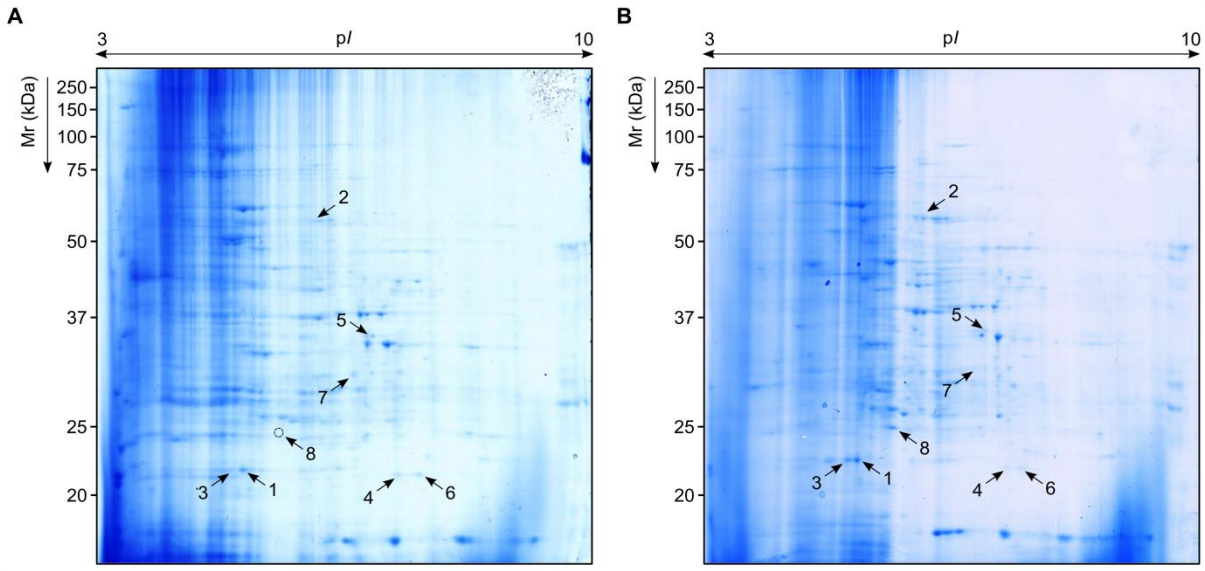


Figure 5. Effect of voriconazole (VRC) on proteomic profiles of *Lomentospora prolificans* cell surface subproteome. Fungal cells were grown in absence (A) or presence (B) of 2 µg/ml VRC, and their surfaceomes resolved by two-dimensional electrophoresis. Depicted arrows point to the most differentially expressed protein spots that were identified by LC-MS/MS (refer to Table 2).

Among the four protein spots most overexpressed by VRC-treated cells, two were identified as heat shock protein (Hsp) 70, one as a glutamate dehydrogenase-like protein, and another one as a Srp1 family-like protein (Serine-rich RNA polymerase I suppressor protein), being this last the protein spot only appearing when cells were exposed to VRC. Regarding to proteins underexpressed in the presence of the antifungal drug, two protein spots corresponded to 60S ribosomal protein-like protein, being in the other two identified an inconclusive mixture of several proteins, including glyceraldehyde-3-phosphate dehydrogenase or RNA recognition domain-containing protein.

Table 2. Identification the most differentially expressed proteins on *Lomentospora prolificans* cells exposed to voriconazole.

Spot No.	Fold Change	NCBI No.	Protein name	Orthologous to	Matching peptides	Sequence coverage (%)	MASCOT score
1	3.365 (↑VRC)	gi|666870829	Heat shock 70 kDa protein	*Scedosporium apiospermum*	24	18	941
2	2.504 (↑VRC)	gi|666862145	Glutamate dehydrogenase-like protein (hypothetical protein SAPIO_CDS10451)	*Scedosporium apiospermum*	23	39	1073
3	2.312 (↑VRC)	gi|666870829	Heat shock 70 kDa protein	*Scedosporium apiospermum*	16	19	836
4	0.350 (↓VRC)	gi|666862378	60S ribosomal protein-like protein (hypothetical protein SAPIO_CDS9923)	*Scedosporium apiospermum*	13	10	286
5	0.322 (↓VRC)	gi|310798588	RNA recognition domain-containing protein	*Colletotrichum graminicola M1.001*	12	22	358
		gi|576039574	malate dehydrogenase-like protein	*Chaetomium thermophilum var. thermophilum DSM 1495*	10	30	302
		gi|88766385	G-protein β subunit	*Metarhizium anisopliae*	11	19	300
6	0.285 (↓VRC)	gi|666862378	60S ribosomal protein-like protein (hypothetical protein SAPIO_CDS9923)	*Scedosporium apiospermum*	23	13	292
7	0.231 (↓VRC)	gi|380482569	Glyceraldehyde-3-phosphate dehydrogenase	*Pestalotiopsis fici* W106-1	12	21	411
		gi|666862386	D-arabinitol dehydrogenase-like protein (hypothetical protein SAPIO_CDS9932)	*Scedosporium apiospermum*	24	28	408
8	Unique in VRC	gi|302912421	Srp1 family-like protein (hypothetical protein NECHADRAFT_102628)	*Nectria haematococca* mpVI 77-13-4	47	42	572

3.4. Production of null mutants for relevant *L. prolificans* proteins

With the final objective of determining the role of VRC resistance-related proteins, we attempted to produce knock-out strains of some of the genes encoding for those proteins. Two proteins were selected to be deleted for their presumed relevance on VRC resistance: Hsp70, and Srp1 family-like protein. In this sense, successful amplification and deletion cassette construction were achieved for all the genes under study (Figure 5; Lanes A, B, and C), being these DNA constructs used to transform *L. prolificans* cells.

Surprisingly, among 40-50 antibiotic-resistant colonies of Srp1 and 3 of Hsp70 were capable of growing per transformation plate in the presence of hygromycin B. However, unfortunately, none of the *L. prolificans* strains validated in this study properly carried the deletion cassettes when compared to wild-type strain, being tested 50 strains in the case of Srp1 and the 3 of Hsp70. As it can be observed in Figure 5, which shows the validation process using a presumed Hsp70 mutant, no amplification was obtained for the *hyg* gene in the wildtype strain (Lane D), while there was positive amplification in the mutated strain (Lane E), evidencing the correct insertion of the deletion cassette. However, when using primers to amplify the Hsp70 gene region, the same band size was obtained with both strains (Lanes F and G), around 4,300 bp, while it was expected a size around 3,400 bp for a properly mutated Hsp70 gene. Therefore, we may conclude that, although the deletion cassettes were inserted, their location in *L. prolificans* genome was not correct.

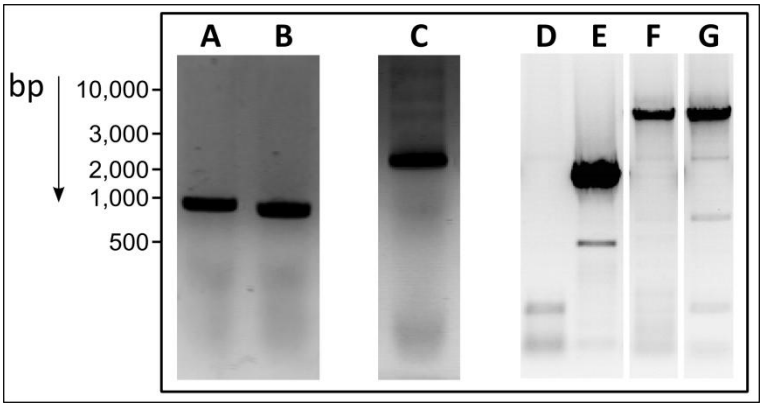


Figure 5. Representative gene deletion amplification results for heat shock protein (Hsp) 70 gene. Amplicons for upstream (A) and downstream (B) regions. Deletion cassette construction (C). Validation by PCR, using the amplification of *hyg* gene as a control of deletion cassette presence (D, wildtype; E, transformant), and of Hsp70 gene (F, wildtype; G, transformant).

4. Discussion

It is well known that the filamentous fungus *Lomentospora prolificans* causes serious, often fatal, infections above all among patients with some immunological disturbance. Remarkably, infections involving this fungal opportunist are on the rise, especially in countries as Spain or Australia (Alastruey-Izquierdo *et al.*, 2013; Slavin *et al.*, 2015). Unfortunately, the most challenging characteristic presented by the fungus is its inherent resistance to all systemically active antifungal drugs currently available (Lackner *et al.*, 2012), being infections caused by *L. prolificans* very difficult to manage and to eliminate. Consequently, morbidity and mortality rates associated with these mycoses are very high (Cortez *et al.*, 2008; Rodriguez-Tudela *et al.*, 2009).

Among all antifungal drugs, azoles have been proved to be the most effective against *L. prolificans* cells, the best results being obtained when using voriconazole (VRC) in both *in vitro* and clinical settings (Troke *et al.*, 2008). Interestingly, some combinatorial therapies have been applied, as VRC and terbinafine (Meletiadis *et al.*, 2003), suggesting that perturbing fungal cells in several ways may enhance antifungal drug activities (Cowen, 2008). However, up to now clinical results are not satisfactory and, in consequence, there is an urgent need for novel therapeutic approaches that help to eliminate the fungus. Therefore, since the molecular basis of this species multidrug resistance is completely unknown, the aim of this work was to analyze the changes on *L. prolificans* cells when exposed to the antifungal drug VRC.

Firstly, growth and morphological alterations induced in the presence of VRC were measured by microscopy. While fungal germination rate was not altered upon VRC exposure, hyphal morphology was dramatically modified, being cells significantly shorter and thicker when exposed to the antifungal drug. Moreover, calcofluor white staining showed that VRC-treated cells emitted fluorescence more intensely, which have been previously correlated to an increased chitin content (Costa-de-Oliveira *et al.*, 2013). These findings made us think that VRC might induce changes in the fungal CW that could be related to *L. prolificans* resistance.

Therefore, we delved into the alterations provoked by VRC exposure in the structure and composition of the CW. Interestingly, using electron microscopy we determined that, while the inner layer of the CW did not significantly change, *L. prolificans* doubled the thickness of the outer fibrillar layer upon VRC exposure. In these sense, similar observations have been reported in *A. fumigatus* in response to nikkomycin Z or micafungin, either alone or in combination (Chiou *et al.*, 2001). These cell wall layers, the compact inner and the fibrillar outer, have been previously reported in *C. albicans* to be composed by chitin plus β-glucans and mannans, respectively (Ene *et al.*, 2012).

In addition, compositional analysis by ion chromatography revealed that VRC-treated cells presented levels of rhamnose, glucosamine, glucose, and mannose significantly increased in the CW backbone; while in the cell surface those increased were galactose, glucose, and mannose. To our knowledge, these compositional changes have not been previously reported for any antifungal drug-induced effect, although inherent higher chitin content has been related to lower echinocandin susceptibility in *C. albicans* (Lee *et al.*, 2012). Taking together all our data, we can conclude that VRC induces enormous changes in both CW structure and composition, probably due to cell membrane instability promoted by ergosterol biosynthesis pathway impairment, as previously proposed for *C. albicans* (Sorgo *et al.*, 2011). Interestingly, since no loss of viability has been observed, these CW rearrangements may be related to a protective mechanism against VRC developed by the fungus.

To determine whether VRC-induced changes were occurring in whole proteomes or cell surface-associated proteins, a proteomics-based study of *L. prolificans* was performed. The results showed that, no significant differential protein expression was observed between VRC-treated and non-treated cell whole proteomes. This fact may be due to differences likely occurring in proteins with low abundance in the whole proteome, being difficult to detect them among the great amount of proteins present. On the contrary, a total of 72 protein spots were found either up- or downregulated when only cell surface subproteomes were scrutinized. In order to focus on the most relevant, the most over- and underexpressed protein spots upon VRC exposure were identified. Among them, Hsp70 (in two protein spots), glutamate dehydrogenase-like protein, and a Srp1 family-like protein

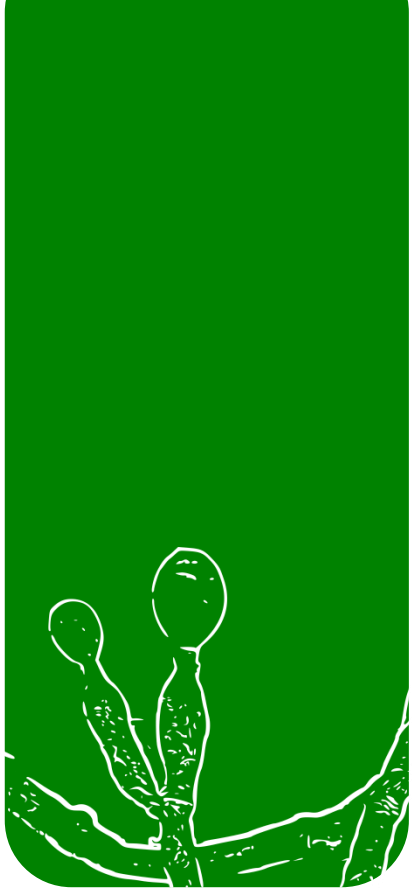
(unique in VRC-induced subproteome), were identified as upregulated; and, as downregulated, 60S ribosomal protein-like protein (in two protein spots), and two protein spots that, unfortunately, were identified as a mixture of proteins.

The interest in heat shock proteins (HSP), especially Hsp90, as antifungal targets has risen in the last years (Wirk, 2011). Beyond their role as chaperones to stabilize other protein structure in thermal shock conditions, HSPs have been related to orchestrate antifungal drug resistance or tolerance by regulation calcineurin, which have many roles in fungal cell growth, virulence, and stress resistance (Juvvadi *et al.*, 2014). Precisely, Hsp70, which is also involved in the abovementioned Hsp90-calcineurin pathway, has been recently related to amphotericin B and caspofungin resistance in *A. terreus* and *A. fumigatus*, respectively (Blatzer *et al.*, 2015; Lamoth *et al.*, 2015). Therefore, our results showing Hsp70 levels increased could be in accordance with these recent reports, suggesting that this protein could be relevant for *L. prolificans* VRC resistance.

In addition, a putative Srp1 protein was identified, this protein spot being only detected in VRC-treated cells. Although little is known about these proteins, they are putative suppressors of RNA polymerases (Yano *et al.*, 1992), being normally located in the nuclear membrane. However, they have been also detected in the fungal cell wall (De Groot *et al.*, 2008) and linked to cell thermal stress (Kowalski *et al.*, 1995). Regarding to 60S ribosomal proteins that were found downregulated upon VRC exposure, they are involved in protein translation, as structural parts forming ribosomes. Interestingly, they have been associated with antifungal stress by either being down- (Zhang *et al.*, 2009) or overexpressed (Chong *et al.*, 2012), which may be due to differences in mechanisms of action of the drugs used. It is worth highlighting that both Srp1 and 60S ribosomal proteins are related to protein synthesis, which might be related to the morphological changes occurring in *L. prolificans* cells in response to VRC. Finally, proteins related to amino acid, as glutamate dehydrogenase, and carbohydrate metabolism (as malate dehydrogenase, included in the protein spots having a mixture of proteins) have been found to be differentially expressed, showing that the fungal cell is regulating its metabolism.

In order to deeply comprehend the role of the most overexpressed proteins in VRC resistance or pathobiology, we wanted to construct knockout strains for Hsp70 and Srp1 genes. During this process, while all the steps concerning deletion cassette construction and *L.* prolificans wildtype strain transformation were successfully carried out, transformant DNA insertion in the correct *loci* was not achieved. Unfortunately, correct homologous recombination in filamentous fungi is a very unlikely event, being random integration of DNA fragments more usual due to nonhomologous end joining (NHEJ) pathway, in contrast with what happens in yeasts (Meyer, 2008). The NHEJ pathway mainly depends on the Ku heterodimer (Ku70/Ku80), being genetic defects on these proteins highly useful for improving transformant DNA insertion in correct *loci*, as reported for *A. fumigatus* (Krappmann *et al.*, 2006; Kress *et al.*, 2006) and other filamentous fungi (reviewed in Meyer, 2008). Consequently, our efforts in the future will be focused on obtaining null strains for any of these Ku subunits, in order to easily apply gene deletion protocols in the future and complete our study concerning VRC resistance-related proteins.

To conclude, in this work we have determined some mechanisms occurring in *L. prolificans* cells in response to VRC. These alterations include formation of shorter and wider hyphae, structural and compositional changes in the CW, and changes in the surfaceome, highlighting the overexpression of Hsp70 and Srp1-like protein. Although not yet proved, these cellular and molecular alterations described in this study could mediate *L. prolificans* resistance to VRC, since the antifungal drug is not able to kill fungal cells. Further research focused on these findings could provide evidence of their specific role in antifungal drug resistance and, in consequence, novel therapies could be designed to fight this therapy-refractory pathogen.



Chapter 4

Study of microglia immune response against the neurotropic fungus *Lomentospora prolificans*

1. Introduction

During the last decades, the number of fungal infections is on the rise worldwide and, since most of these mycoses are opportunistic and their severity closely related to host immune status, great efforts have been made to elucidate antifungal immune response. In this sense, *Candida albicans* and *Aspergillus fumigatus* have been the main targets of researchers, representing the models for studying immunity to yeasts and filamentous fungi, respectively (Romani, 2004; Romani, 2011). However, little is known about immune response to other fungi, such as the emerging pathogen *Lomentospora prolificans*, which represent new challenges as they may display differential pathobiology or virulence factors (Brown *et al.*, 2012).

Innate immune responses to filamentous fungi comprise both cellular and molecular events. After inhalation of conidia, epithelial and phagocytic cells are the first cellular barrier to confront the entering microorganisms. Among the latter, macrophages, neutrophils, and dendritic cells are the most important cell types during filamentous fungal infections, detecting, phagocytosing, and killing fungal cells (Brakhage *et al.*, 2010). On the other hand, molecular events, such as cytokine release, and opsonization of fungal cells by the complement system, may also participate by triggering immune responses against the invading agent. In this sense, a few studies concerning innate immunity management and recognition of *L. prolificans* infections have been released. There, both polymorphonuclear leukocytes (PMN) and monocyte-derived macrophages (MDM) from human origin have been proved to react to the fungus by inducing hyphal damage in an effector/target ratio-dependent manner. In addition, only the latter were observed to internalize *L. prolificans* conidia, successfully inhibit their germination, and develop an oxidative burst when exposed to human serum-opsonized conidia, all in a similar way to *A. fumigatus* (Gil-Lamaignere *et al.*, 2001). Moreover, the exposure of human monocytes to *L. prolificans* induced the release of greater amounts of Tumour necrosis factor-α (TNF-α) and Interleukin-6 (IL-6) than when exposed to *Aspergillus* spp. (Warris *et al.*, 2005).

One of the first steps of cell-mediated immunity is related to the recognition of pathogen-associated molecular patterns (PAMP) by pattern recognition receptors (PRR) present in

immune cells. Among PRRs, Toll-like receptors (TLR) and C-type lectin receptors (CLR), such as mannose receptor (MR) or dectins, have been shown to be very important for sensing fungal cells (Netea *et al.*, 2008). Concerning the recognition of *L. prolificans*, indirect evidences of the important role of dectins and TLR have been reported, since after exposure of human PMN to *L. prolificans* hyphae, RNA expression of dectin-1 was 1.5-fold, and 1-fold increased in the case of TLR2 and TLR4 (Lamaris *et al.*, 2008). Moreover, *Drosophila melanogaster* null mutants for TLRs were more susceptible to infections with this species (Lamaris *et al.*, 2007). Interestingly, *L. prolificans* PAMPs, such as *O*-linked oligosaccharides in cell wall proteins, have been shown to promote pro-inflammatory responses and to likely play roles during phagocytosis (Xisto *et al.*, 2015), but no specific receptor was reported to be related with this molecules.

In spite of having all this antifungal immune mechanisms, when the host immune system is deeply compromised, fungal cells are able to trespass the epithelial barrier and disseminate through the bloodstream, infecting other organs and areas of the host. In the case of *L. prolificans*, it is well-known that the fungus tends to invade the Central Nervous System (CNS), causing serious neurological disorders, usually leading patient death (Cortez *et al.*, 2008; Rodriguez-Tudela *et al.*, 2009). Microglia represents the resident immune cells in the CNS, being the main cell type that confronts invading microorganisms at their arrival to the brain. These phagocytic cells usually remain at a resting state to maintain homeostasis in the brain (e.g. as scavenger cells removing dead cells or protein aggregates). However, microbial PAMPs are able to activate them, dramatically changing their morphology and physiology and increasing their expression of PRRs or other molecules, as integrins or cytokines (Rock *et al.*, 2004). These immune responses are highly regulated during infection and they are terminated when the microbial stimuli are stopped, in order to avoid damage in nervous tissue by an excessive inflammation (Aloisi, 2001).

In this sense, although microglial function against other fungi more related to fungal CNS infections, as *Cryptococcus neoformans* (Blasi *et al.*, 1995), have been previously explored, little is known about their role during filamentous fungal infections, especially in the case of *L. prolificans*. Therefore, in this study we evaluated *in vitro* interactions between microglia and *L. prolificans*, showing their ability to phagocytize fungal cells, but determining that

microglia is inefficient against the fungus. Taking together the data reported here, it has been established the basis to delve into the understanding of the antifungal immunity to this species in the CNS and the pathobiology of *L. prolificans* neurotropism.

2. Materials & Methods

2.1. *Lomentospora prolificans* strain, growth, and maintenance

For this study, it was used the *L. prolificans* strain CECT 20842, previously obtained from a patient with disseminated infection in the Hospital Marqués de Valdecilla (Santander, Spain). Fungal spores were cryopreserved at -80°C and the strain was reconstituted twice a month on Potato dextrose agar (PDA) (Pronadisa, Madrid, Spain). To prepare conidia, 7-day old fungal cultures on PDA plates were washed twice with sterile saline (0.9% [w/v] NaCl). Cell suspensions were filtered through gauze to remove hyphae and other debris, and cell concentration calculated using a Bürker counting chamber.

2.2. Microglia and macrophage cell lines

Two murine cell lines were used to evaluate their interactions with *L. prolificans*. On the one hand, the microglial cell line BV-2, previously immortalized with a retrovirus carrying the *v-raf*/*v-myc* oncogene by Blasi and co-workers (Blasi *et al.*, 1990); on the other hand, the peritoneal macrophage-like cell line J774A.1, which was derived from the ascitic fluid of Balb/C mice (Ralph *et al.*, 1975), both retaining most of the morphological, phenotypical, and functional properties described for freshly isolated cells. Cell lines were maintained and propagated in a humidified atmosphere (95% relative humidity) and 5% CO_2, at 37°C in DMEM supplemented with 200 mM L-Glutamine and 10% heat-inactivated FBS (Complete Medium; CM), and a mixture of antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin) and antimycotics (0.25 µg/ml amphotericin B). All culture media components were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Primary cultures of microglia

Primary microglia cultures were kindly provided by Dr. Carlos Matute, leader of the Laboratory of Neurobiology, University of the Basque Country (UPV/EHU), Spain. Primary microglia were derived from mixed glial cultures obtained from the cerebral cortex of neonate Sprague-Dawley rats (P0-P2) as previously described (McCarthy and de Vellis,

1980), with modifications. First, extracted brain cortices were placed in 10 ml of HBSS without Ca^{2+} and Mg^{2+}, supplemented with 400 µl of 0.05% trypsin (Gibco, Madrid, Spain) and 40 µl of 0.4% DNAse (Sigma-Aldrich), and incubated 15 min at 37°C to enzymatically dissociate the tissue. To stop the reaction, IMDM supplemented with 10% of FBS was added, and tissue centrifuged 5 min at 300 g. Supernatants were discarded, and pellets were resuspended in the abovementioned medium to mechanically dissociate them by using needles of decreasing diameter (21G and 23G). Cell suspension was centrifuged for 5 min at 400 g, resuspended in medium, and seeded in poly-D-lysine- (Sigma-Aldrich) pre-treated culture flasks.

After two weeks in culture, flasks were shaken for 1 h at 300-400 rpm and 37°C, being supernatants containing microglia placed on Petri dishes, and left 48 h at 37°C to let it adhere. Then, supernatant was eliminated, removing non-adherent cells and obtaining 95-99% microglia pure cultures. Primary microglia cultures were maintained in CM with antibiotics and antimycotics.

All procedures and experiments involving animals, including their housing and care, were carried out according to the guidelines of the European Union Council (Directive 2010/63/EU) and Spanish Government regulations (RD 53/2013), and with the approval of the ethics committee of the University of the Basque Country (UPV/EHU).

2.4. Fungal-immune cell co-cultures, phagocytosis assay, and germination dynamic

To evaluate the interactions between immune cells and *L. prolificans*, BV-2 and J774A.1 cells were scrapped from flasks, counted under the microscope using a Bürker counting chamber, and stained with Trypan blue to determine their viability. Cell passages with less than 80% of viability were discarded. Then, cells were placed on 24-well plates at a density of $5x10^4$ cells per well containing 12 mm-diameter cover slips and 400 µl of CM only supplemented with antibiotics. After an overnight incubation, immune cells were co-cultured with *L. prolificans* conidia at a Multiplicity of Infection (MOI) of 1 (one conidium per mammalian cell). At the end of each incubation time, cover slips were extracted from wells, and placed in another plate containing cold PBS in order to stop phagocytic activity. By using an

inverted light microscope, at least 300 immune cells were counted per well determining the percentage of them that were engulfing conidia. In addition, rates of germination and branching of *L. prolificans* were determined, being the latter defined as the proportion of fungal cells with more than one hyphal tip. Control wells in absence of immune cells were also counted to measure these fungal growth parameters.

In the case of primary microglia cultures, dishes containing cells were washed with HBSS without Ca^{2+} and Mg^{2+}, and treated with trypsin for 5 min at 37°C. Once the cells were detached, cold CM was added to stop the reaction, and cell suspension was centrifuged. Harvested cells were resuspended in CM only with antibiotics, and cell number and viability were determined as abovementioned. To minimize the number of sacrificed mice, experiments with primary microglia were performed plating 10^4 cells per well in 200 µl of CM only with antibiotics using 96-well plates. In the case of experiments performed using primary microglia, counts to determine phagocytosis, germination, and branching rates were performed directly on the 96-well plates.

2.5. Receptor blocking assay

To determine the role of PRRs on microglial phagocytosis of *L. prolificans,* mannan from *Saccharomyces cerevisiae* (Sigma-Aldrich) and laminarin from *Laminaria digitata* (Sigma-Aldrich) were used to block MR and dectin-1, respectively. Prior to the addition of *L. prolificans* conidia, immune cells were treated with the blocking reagents (15 min with mannan, or 10 min with laminarin), as previously described (Slesiona *et al.*, 2012). After this incubation time, media containing the inhibitors were removed, and cells washed once with CM only with antibiotics, being, then, conidia were added.

2.6. Visualization of fungus-immune cell interactions by immunofluorescence

To better visualize the interactions between fungal and immune cells, immunofluorescence staining was performed. After each co-culture time, cover slips were washed three times with PBS, and cells fixed with 3% paraformaldehyde for 20 min. Then, cells were washed three times with PBS, and cell membranes permeabilized with 0.1% Triton X-100 in PBS for 4

min. Excess of Triton X-100 was washed with PBS three times and, then, Phalloidin-Atto 488 1/200 (Sigma-Aldrich) and calcofluor white 40 µg/ml in PBS was added, to stain actin filaments and fungal chitin, respectively. Finally, after 35 min kept in the dark, unlabelled compounds were washed as mentioned above and, then, cover slips were placed using Fluoromount Aqueous Mounting Medium (Sigma-Aldrich). Fungus-immune cell interactions were visualized under a microscope Nikon Eclipse Ni (Nikon, Tokyo, Japan). All incubations were performed at room temperature.

2.7. Measurement of immune cell survival

Cellular damage induced by *L. prolificans* to immune cells was measured by Calcein-AM (Life Technologies, CA, USA), which is a non-fluorescent compound that easily penetrates cell membrane. Intracellular hydrolysis of this probe by cellular esterases of living cells produces Calcein, a fluorescent compound that is retained inside the cytoplasm. Therefore, after each experimental time, cells were washed three times with sterile pre-warmed PBS, and Calcein-AM added at a final concentration of 1 µM in fresh CM. Next, cells were incubated for 30 min at 37°C, and washed three times with PBS, being emitted fluorescence measured by a spectrophotometer Synergy HT (Biotek, MA, USA) using excitation wavelength at 485 nm and emission at 527 nm. Arbitrary fluorescence units were used to calculate the percentage of cell survival in comparison with non-infected cells at each time point.

2.8. Quantification of ROS, RNS, and pro-inflammatory cytokines

To determine the response of immune cells to *L. prolificans* infection, the production of ROS, RNS, and two pro-inflammatory cytokines, TNF-α and IL-6, was evaluated. Intracellular ROS were measured using a fluorescent probe, CM-H_2DCFDA (Life Technologies), as a general oxidative stress indicator. After each incubation time, wells were washed three times with pre-warmed PBS, and the probe was added diluted in PBS at a final concentration of 5 µM for 20 min at 37°C. Then, the probe was removed and pre-warmed PBS was added to each well for another 20 min at 37°C. Finally, fluorescence was detected spectrophotometrically using excitation wavelength at 492 nm and emission at 517 nm. As abovementioned, arbitrary fluorescence units were compared to those obtained in control

wells, being the results expressed as percentage of control.

Supernatants of cells cultured alone or with *L. prolificans* were used to measure RNS and pro-inflammatory cytokines. On the one hand, RNS were measured by the Griess method. Briefly, 150 µl of culture supernatant of each time point and condition were placed in 96-well plates, and mixed with 20 µl of Griess reagent: equal volumes of 1% (w/v) Sulfanilamide in 5% (v/v) H_3PO_4, and 0.1% (w/v) N-(1-Naphthyl)ethylenediamine dihydrochloride. Then, samples were homogenized by adding 130 µl of distilled H_2O, and plates incubated for 30 min. Samples absorbance were measured at 548 nm, and nitrite concentration values inferred from a standard curve using sodium nitrite. On the other hand, TNF-α and IL-6 levels in supernatants were determined by sandwich ELISA following manufacturer's instructions (Life Technologies; Carlsbad, CA, USA).

2.9. Effect of pH stress on *Lomentospora prolificans* survival

Conidiospores of *L. prolificans* were incubated for three or seven days at 37°C and 200 rpm in PBS with adjusted pH ranging from 3 to 8. At the end of each experimental incubation time, serial dilutions were plated in triplicate on PDA plates to test fungal viability. After 24 h of incubation at 37°C, the number of colony forming units (CFU) was recorded. Survival at pH 7 was set as 100%, and the relative survival in the rest of the pH range compared to pH 7 was determined.

2.10. Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5 software (GraphPad Software, CA, USA). At least three biological replicates were performed to measure each parameter. Differences of the mean were analyzed by using the Student's paired or unpaired *t*-test.

3. Results

3.1. Phagocytosis and fungal growth dynamic during *Lomentospora prolificans*-immune cell co-cultures

Lomentospora prolificans has previously shown a marked tendency to develop infections in the CNS after its dissemination through the bloodstream. As abovementioned, microglia is the resident and most relevant immune cells in this area, being crucial for infective and non-infective processes. Therefore, its efficiency in the response against *L. prolificans* infections might be contributing to the neurotropism showed by the fungus. Consequently, in this work the ability of these phagocytic cells to recognize, phagocytize, and produce reactive and signalling molecules in response to the fungus was evaluated. Concretely, BV-2 and J774A.1 cell lines were used to analyze their interactions with *L. prolificans*, being compared with primary microglial cells.

BV-2 cells were able to successfully phagocytize fungal cells at all times assessed, showing a phagocytosis peak of 19.65±3.26% after 4 h of co-culture. Compared to microglial cells, the macrophage-like cell line J774A.1 showed a very significant increased ability to internalize *L. prolificans* conidia in all experimental times, with a phagocytosis minimum of 19.79±2.51% at 2 h, and a maximum of 48.87±7.40% at 8 h post infection. Due to the great differences found between the two cell types, we wanted to validate these results using primary microglia cultures. These cells showed even lower values compared to BV-2 cells, corroborating the different phagocytic capacity observed between microglia and monocytes. Remarkably, none of the cellular models used were able to completely phagocytize all *L. prolificans* conidia at any time assessed (Figure 1 A).

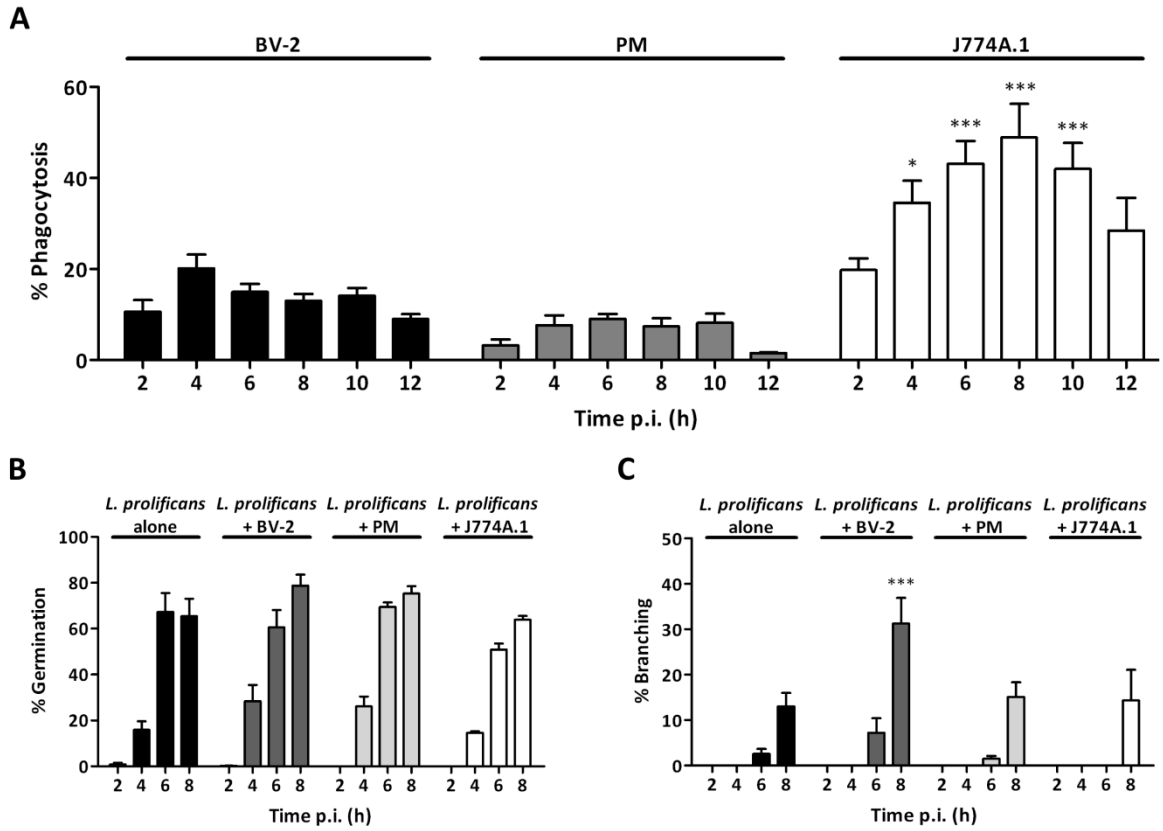


Figure 1. Analysis of phagocytosis dynamic and *Lomentospora prolificans* growth during co-culture experiments. A) Percentage of cells engulfing at several times post infection (p.i.) during co-cultures of *L. prolificans* with BV-2, primary microglia (PM), and J774A.1. Results are shown as mean ± SEM, n≥5. *p<0.05, ***p<0.0001 in comparison with BV-2 cells. B, C) *L. prolificans* growth dynamics were also monitored by analyzing germination (B) and hyphal branching (C). Results are shown as mean ± SEM, n≥3, ***p<0.0001 in comparison with *L. prolificans* alone.

Fungal cell germination rates were also analyzed during co-cultures with immune cells and, while *L. prolificans* did not change its germination rate in any co-culture condition in comparison with when cultured alone (Figure 1 B), a significant increase on hyphal branching percentage was observed when the fungus was in contact with BV-2 cells (Figure 1 C). In addition, J774A.1 cells were able to slightly delay hyphal branching, the fungus starting to develop this feature from 8 h of co-culture. However, branching rate data obtained with the microglial cell line could not be confirmed using the primary microglial cultures.

3.2. Immune cell survival is dramatically affected by *Lomentospora prolificans*

Phagocytosis is one of the main stages in pathogen clearance by immune cells. However, some microorganisms, including fungal pathogens, are able to escape from the processes occurring after phagocytosis, such as microbial killing. In the co-culture models used in this study, it was determine by using the fluorescent vital staining Calcein-AM that *L. prolificans* was able to produce cell death in both microglial models, being significantly higher in comparison with non-infected cultures from 6 h of co-culture in BV-2 cell and from 2 h in primary microglia. Conversely, cell survival of peritoneal macrophages was maintained until 10 h post infection (Figure 2).

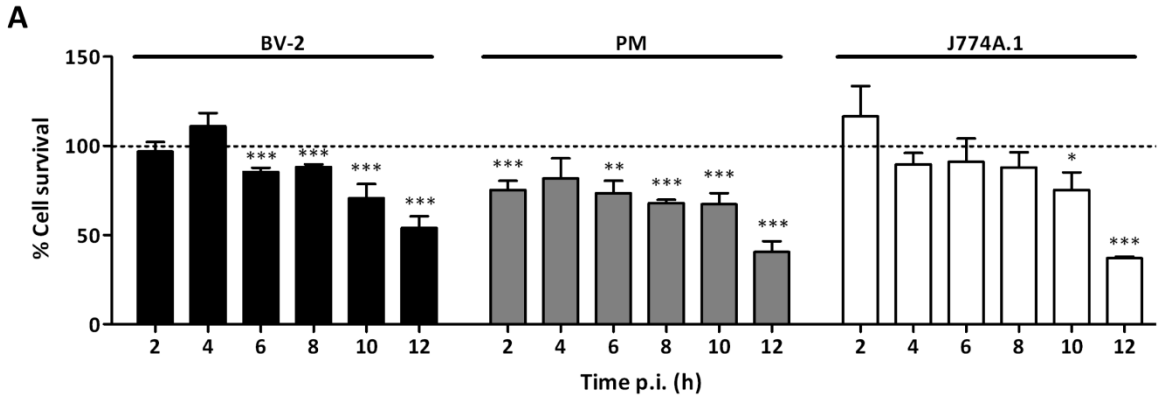


Figure 2. Cell damage induced by *Lomentospora prolificans* cells to phagocytic cells. Microglial cells, both BV-2 cell line and primary microglia (PM), and J774A.1 macrophage-like cells survival was measured in the presence of fungal cells at different times post infection (p.i.). Results are shown as mean ± SEM, n≥5, of the percentage of survival in comparison with non-infected cells, representing 100% of survival (spotted line). *p<0.05, **p<0.01, ***p<0.0001 in comparison with non-infected cultures.

To clearly observe fungus-immune cell interactions, we performed immunofluorescence staining of fungal chitin with calcofluor white, and immune cell actin filaments with Phalloidin-FITC (Figure 3). Interestingly, we observed how germination rates and hyphal length were increasing along time, while cells were phagocytosing fungal cells. Moreover, already engulfed conidia were able to germinate inside phagocytes and to pierce cell membranes. This fact may be contributing to the observed cell death induced by fungal cells.

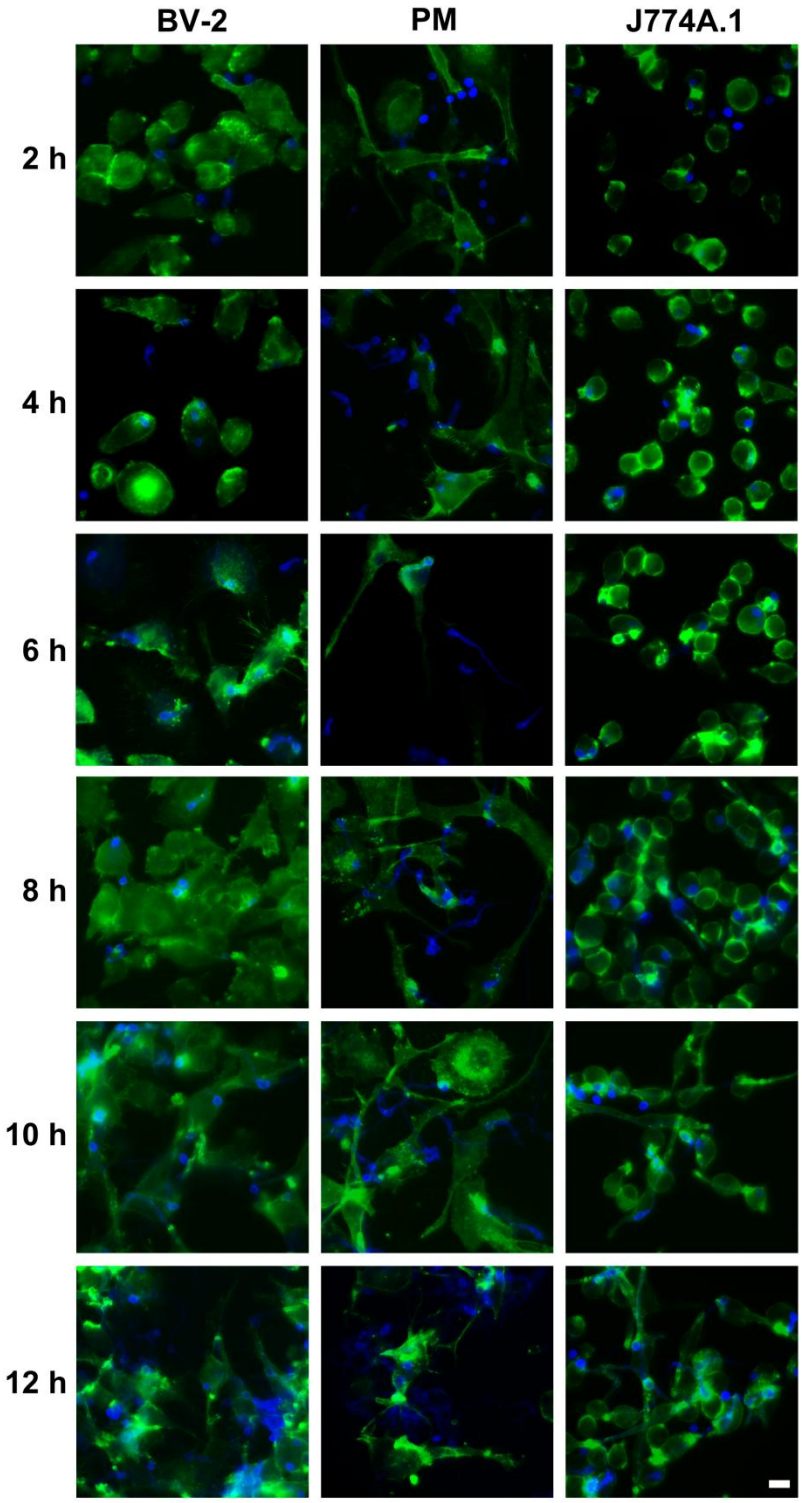


Figure 3. Immunofluorescence micrographs of *Lomentospora prolificans*-immune cell interactions. Co-cultures of fungal cells and BV-2, primary microglia (PM), and J774A.1 were visualized using immunofluorescence staining at different time points.

3.3. *Lomentospora prolificans* survival upon pH stress

After *L. prolificans* conidia being phagocytized, extreme acidic environments may be found inside phagolysosomes. Therefore, since the absence of nutrients in the media and the acid pH may resemble the environment that the fungus face inside phagolysosomes (Slesiona *et al.*, 2012), we determined the viability of fungal spores after 3 or 7 days of culture in PBS at several pH values.

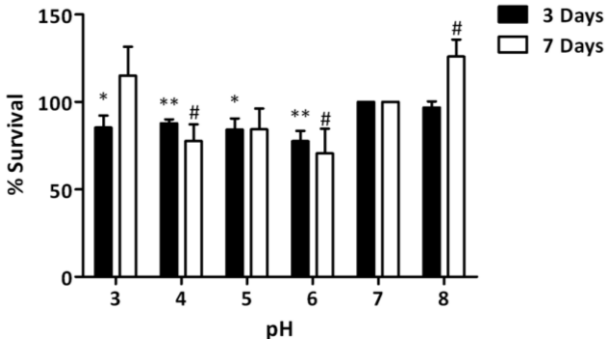


Figure 4. Survival of *Lomentospora prolificans* conidia at different pH. Fungal survival at different pH in PBS was determined at 3 and 7 days of culture. Results are shown as mean ± SEM, n=3, of the percentage of conidial survival compared to pH 7 (set as 100% of survival). *p<0.05, **p<0.01, compared to pH 7 for 3 days. #<0.05, compared to pH 7 for 7 days.

Interestingly, *L. prolificans* cells were able to maintain their viability in all the pH range tested. Despite losing viability up to 22.45% at 3 days and up to 29.38% at 7 days in neutral pH (both being at pH 6), most of the *L. prolificans* cells were able to survive pH stress, maintaining high levels of viability both in basic and acidic conditions.

3.4. Oxidative and nitrosative bursts developed against *Lomentospora prolificans*

Reactive compounds, as oxygen- or nitrogen-containing molecules, are key mediators of microbial killing after their engulfment. These molecules promote molecular stress in proteins or DNA, leading them to malfunction or degradation. Therefore, we evaluated the production of ROS and RNS by immune cells in response to *L. prolificans* infection. Interestingly, ROS production levels by BV-2 cells were significantly lower in comparison with those produced by J774A.1, only being slightly higher at 2 h post infection. ROS

production by the latter followed a similar dynamic as their phagocytic rate, matching the phagocytic peak with the highest ROS production (Figure 5 A). Concerning to RNS release, both cell lines responded to fungal exposure by producing RNS without relevant differences between them (Figure 5 B and C). Remarkably, no detectable levels of either ROS or RNS were detected in primary cultures of microglia.

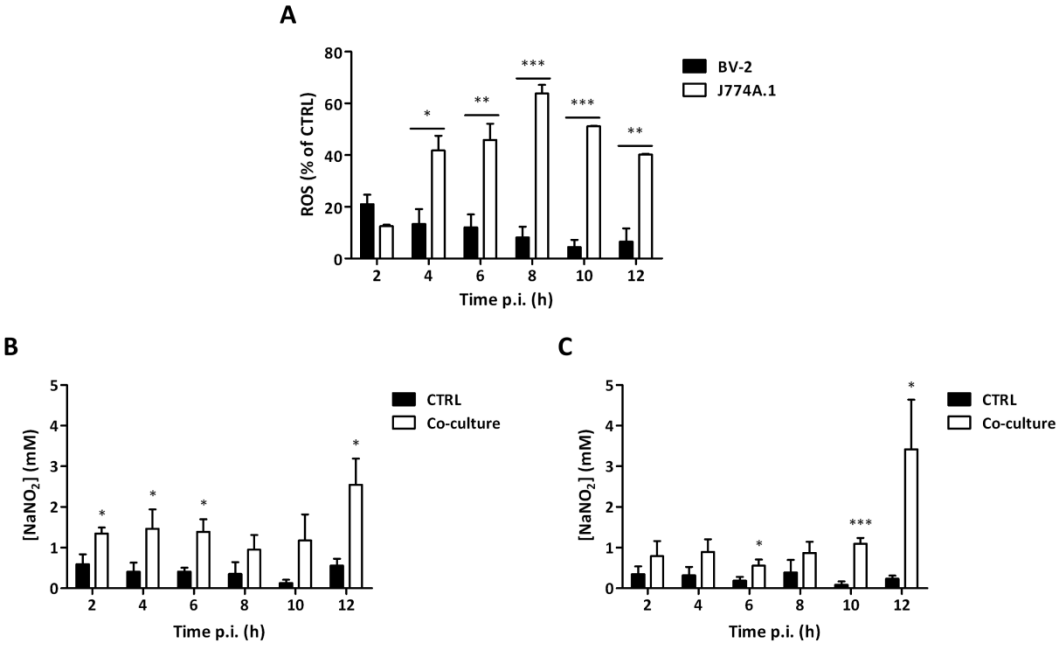


Figure 5. Release of ROS and RNS by phagocytic cells. Intracellular ROS production was monitored upon *Lomentospora prolificans* exposure at different times post infection (p.i.). Results are shown as mean ± SEM of percentage respect to non-infected cells, n=4. *p<0.05, **p<0.01, ***p<0.0001. RNS release was measured from non-infected (black bars) or fungus-infected (white bars) BV-2 (B) and J774A.1 (C) cells. Results are shown as mean ± SEM, n=4. *p<0.05, ***p<0.0001 compared to non-infected cultures.

3.5. Release of pro-inflammatory cytokines by microglia and macrophages

Release of pro-inflammatory signalling molecules, such as cytokines, is a key process during microorganism invasion in order to recruit other immune cells to the site of infection. Among all the molecules involved in the inflammatory response, in this study we measured TNF-α and IL-6 to evaluate the pro-inflammatory effect of *L. prolificans* cells on BV-2 and J774A.1 cells (Figure 6).

Both cell types were observed to produce basal levels of TNF-α and IL-6 in the absence of any stimulation (Figure 6, black bars). However, when cells were exposed to *L. prolificans*, time-dependent increasing concentrations of both cytokines were released to the culture medium. In the case of BV-2 cells, significantly higher levels of TNF-α and IL-6 were produced from 8 h and 10 h, respectively, after conidia inoculation in comparison with control (Figure 6 A and C). Regarding to J774A.1 macrophage-like cells, TNF-α followed a similar pattern as in the case of BV-2 cells (Figure 6 B). However, IL-6 release was very fast, starting to be significantly higher from the 4 h post infection (Figure 6 D). More importantly, significantly higher concentrations of released cytokines in the extracellular medium were achieved by J774A.1 cells for both TNF-α and IL-6 than BV-2 microglial cells.

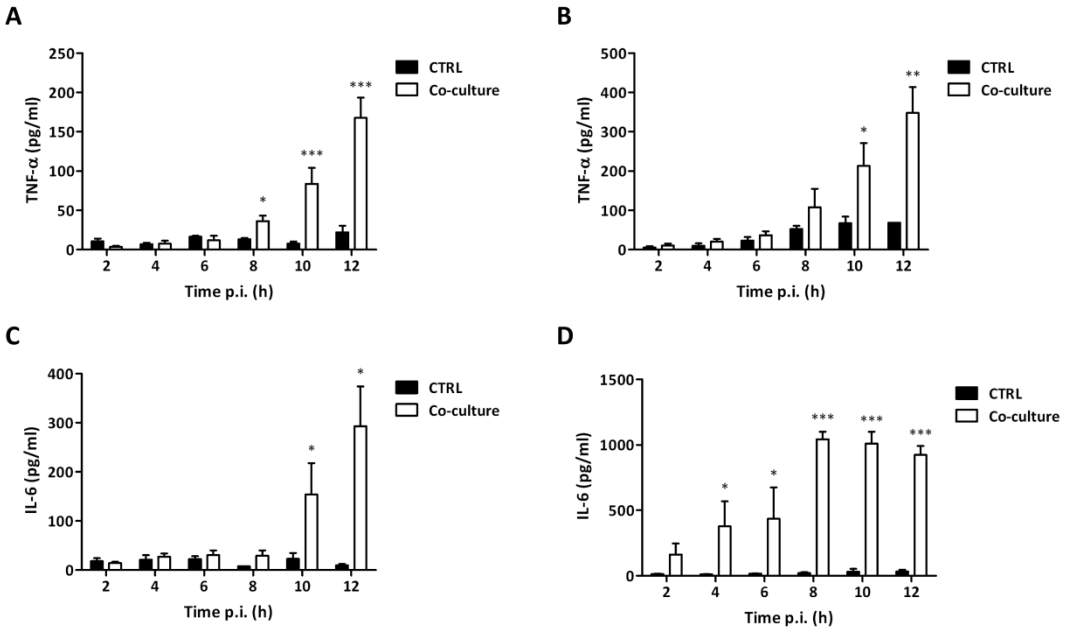


Figure 6. Pro-inflammatory cytokines production by immune cells after stimulation with *Lomentospora prolificans*. Tumour necrosis factor-α and Interleukin-6 release by BV-2 (A, C) and J774A.1 (B, D) cells were measured when co-cultured with the fungus at different times post infection (p.i.). Results are shown as mean ± SEM, n=4. *p<0.05, ***p<0.0001 compared to non-infected cultures.

3.6. Role of mannose receptor and dectin-1 on microglial phagocytosis of *Lomentospora prolificans*

Since PRR are essential for recognition of pathogens, we wanted to evaluate the role of two CLR on the phagocytic process of *L. prolificans* by microglia, MR and dectin-1. In order to investigate this, phagocytic capacity of these cells was evaluated in the presence of specific blocking agents, mannan and laminarin (Figure 7).

First, using 2 mg/ml of mannan to block MR, phagocytosis of *L. prolificans* significantly decreased in short incubation times (2 and 4 h of co-culture), being inhibited up to 71.31%. This tendency was also observed, but to a lesser extent, at 6 h after inoculation of conidia (Figure 7 A). In addition, to evaluate whether inhibitor concentration was relevant in the process, decimal dilutions were tested (from 0.1 to 1000 µg/ml) at 4 h of co-incubation (in which phagocytosis peak was previously shown to be achieved by BV-2 cells). Interestingly, mannan blocked microglial MR in a concentration-dependent manner (Figure 7 B) at concentrations lower than 100 µg/ml (79.51% of phagocytosis inhibition).

Additionally, laminarin was used to block the participation of dectin-1 receptors in the studied process. In this case, using 2 mg/ml produced a higher inhibition that mannan at all times assessed, being the highest inhibition 2 h post infection (84.76%) (Figure 7 C). Moreover, when varying laminarin concentrations were applied to co-cultures, a dose-dependent kinetic was observed at all concentrations used (Figure 7 D). More interestingly, when both blocking agents were used simultaneously on microglial cells, no synergistic effect was observed at 4 h of co-culture, being the inhibition values achieved not significantly different from those obtained using laminarin only (Figure 7 E).

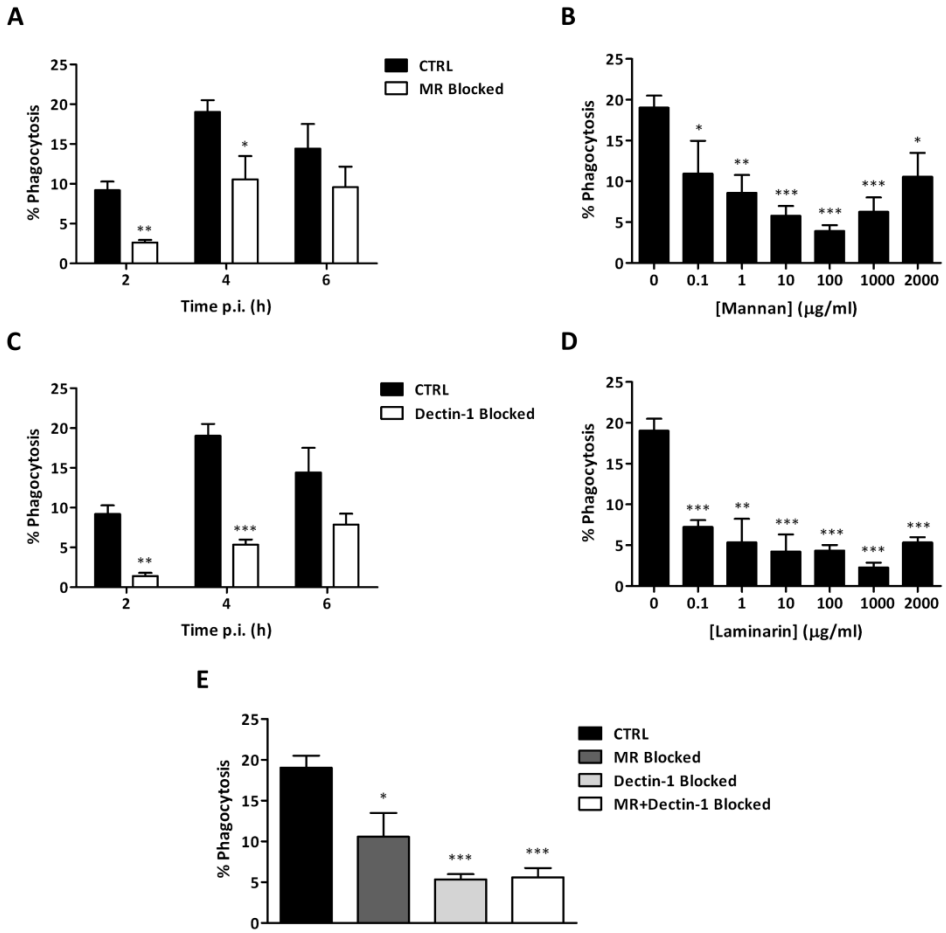


Figure 7. Role of mannose receptor (MR) and dectin-1 during *Lomentospora prolificans* recognition by microglia. Mannan was used as an inhibitor of MR, being phagocytosis studied at several times post infection (p.i.). (A) and different blocking agent concentrations (B). Similarly, laminarin was used for blocking dectin-1 (C, D). Simultaneous blocking of MR and dectin-1 was also carried out (E). Results are shown as mean ± SEM, n≥3. *p<0.05, **p<0.01, ***p<0.0001 compared to co-cultures without blocking agent.

4. Discussion

Fungal infections, especially those with invasive profiles, are increasingly becoming a great cause of morbidity and mortality among certain population groups at risk, such as immunocompromised patients. In spite of the existence of antifungal drugs and diagnostic methods, the treatment and management of these infections are usually difficult and, in some cases, patients die due to lack of efficacy of the clinical tools existing nowadays (Brown *et al.*, 2012). In this sense, both the immune status of the patient and the biology of the infectious agent are essential for disease progression. While immune antifungal responses to relevant fungi, such as *Aspergillus* or *Candida*, have been extensively studied, little is known about the immunity to most of the other fungi (Romani, 2004; Romani, 2011). Hence, host immune responses against etiologic agents of endemic mycoses or emerging pathogens are poorly understood. In consequence, since there are important differences among fungal pathogens related to biology, physiology, pathology, or virulence, which may trigger distinct immune mechanisms, they should be well studied in order to completely understand them and, ultimately, to develop efficient treatment strategies or diagnostic techniques.

The filamentous fungus *Lomentospora prolificans* is one of these emerging pathogens (Bouchara *et al.*, 2009), showing high virulence and resistance to a wide range of antifungal compounds. Moreover, this fungus is mainly related to disseminated infections in patients with underlying diseases (Rodriguez-Tudela *et al.*, 2009) and, in consequence, from the airways, which are the main portal of entrance, it is able to colonize other organs or areas. In this way, *L. prolificans* has shown a tendency to develop infections in the CNS, producing fungal abscesses (Cortez *et al.*, 2008) and, more infrequently, meningitis (Uno *et al.*, 2014), being consequently termed as a neurotropic fungus. During these infections, microglia, the resident immune cells in the CNS, has important roles related to microbial clearance and other immune cell attraction (Mariani and Kielian, 2009). Remarkably, while some studies have focused on the role of monocytes/macrophages during these infections, the role of microglia in *L. prolificans* infections is completely unknown. Therefore, since we hypothesized that an immune inefficiency may be behind *L. prolificans* neurotropism, in this work we performed a study of the interactions between microglia and fungal cells. To that

end, we carried out co-cultures using microglial cells, both BV-2 cell line and primary cell cultures, and *L. prolificans*.

First, we analyzed the dynamic of the *L. prolificans* conidia phagocytic process over time. Microglial BV-2 cells were able to uptake fungal spores, being at 4 h post infection the highest percentage of phagocytosis recorded. Surprisingly, these results obtained were very low in comparison to those reported with the same cell line but with other fungi, such as *Candida* spp. (Neglia *et al.*, 2006; Orsi *et al.*, 2010) or *Cryptococcus neoformans* (Blasi *et al.*, 1992; Blasi *et al.*, 1995). In spite of the fact that experimental differences among reports should be taken into account (the use of cell lines or primary cultures, experimental times, MOI used, etc.), phagocytic processes may differ depending on several biological and physical features. In fact, it is well described that phagocytosis is highly dependent on the species (Luther *et al.*, 2006) or the genetic background of the microorganism (Neglia *et al.*, 2006). In this sense, the composition and structure of the fungal cell wall in the different species or, even, morphotypes are important, since PAMPs may be more or less exposed to be recognized by phagocytes (Gersuk *et al.*, 2006).

Taking into account our results, we proposed that this cell type may be inefficient for *L. prolificans* clearance from the brain. Hence, we compared these results with other phagocytes, the macrophage-like cell line J774A.1. In addition, we utilized primary cultures of microglia to validate whether the BV-2 cell line was acting unsuccessfully against the fungus. Interestingly, J774A.1 cells were able to phagocytize *L. prolificans* conidia at significantly higher rates, achieving phagocytic rates almost 2.5 times higher than BV-2 cells. These last data are more similar to previously published results concerning human MDM that reached 64.5±4.8% of cells phagocytosing (Gil-Lamaignere *et al.*, 2001). In accordance with the inefficient phagocytic capacity shown by BV-2 cells, primary microglial cultures showed even lower phagocytosis rates, which supported our hypothesis.

In addition, *L. prolificans* growth was also examined by measuring its germination rate and the proportion of branched hyphae produced. This fact was relevant for the study since yeast-to-hypha transition in yeast, and germination of conidia in filamentous fungi, have been described as highly important virulence factors during infective processes (Brand,

2012; Mayer *et al.*, 2013). In this sense, none of the three cellular models were able to stop or delay fungal germination, being *L. prolificans* able to produce hyphae, both inside and outside phagocytes**.** Moreover, fungal germination inside immune cells produced their death by piercing immune cell membranes. In fact, germination became so excessive that no reliable data could be obtained after 8 h of co-culture. As a consequence, *L. prolificans* induced microglial cell death at very short experimental times, especially in primary cultures, while J774A.1 cells were able to maintain their viability up to the tenth hour post infection. These findings contrast with previously results reported by Gil-Lamaignere and co-workers who observed around 80% inhibition of germination after 7 h of co-culture with MDM (Gil-Lamaignere *et al.*, 2001).

Interestingly, hyphal branching rates were found to be increased in the presence of BV-2 cells, which may be considered as a more virulent phenotype. Conversely, J774A.1 cells were able to induce a slight delay on hyphal branching to 8 h. In this sense, physical properties, such as size and shape of the particle to be engulfed, are relevant for the phagocytic process (Champion and Mitragotri, 2006; Paul *et al.*, 2013), being larger and non-spherical elements more difficult to phagocytize. Therefore, it is logical that phagocytosis rates decrease proportionally with increasing germination or branching rates, being hyphae formation an important virulence factor in filamentous fungi (Brand, 2012). Unfortunately, branching rate data obtained from BV-2 cell line were not validated in primary cultures, indicating that this phenotype may not be expressed by *L. prolificans* during CNS infections.

After phagocytosis, immune cells may contribute to fungal clearance by both killing invading cells and/or promoting pro-inflammatory signals to attract other immune cells (Brakhage *et al.*, 2010). Certain mechanisms concerning killing fungal pathogens are known, but the whole process is not completely understood yet. In this sense, phagolysosome acidification, or production of ROS and RNS are important mechanisms during fungal killing by macrophages. Thus, firstly we measured *L. prolificans* survival at different pH values, from 3 to 8, during 3 and 7 days. Interestingly, fungal cells were able to survive these stressful environmental conditions, maintaining high cell viability (more than 70%) in all the pH range, including very acidic conditions as pH 3. This capacity would permit fungal cells to persist inside phagolysosomes until they start to germinate and escape from phagocytes by

piercing cell membranes. These two strategies, persistence and escape, have already been described in different *Aspergillus* species (Slesiona *et al.*, 2012), but not used combined by the same microorganism.

Moreover, we quantified the production of both ROS and RNS by immune cells when exposed to fungal cells. While RNS release was not very high in comparison with non-infected cells, and not significantly different between microglia and macrophage cellular models, ROS production was highly induced in the latter, being consistent with previously reported data using human PMNs and MDMs (Gil-Lamaignere *et al.*, 2001). On the contrary, primary microglial cells did not developed either oxidative or nitrosative bursts, corroborating the inefficiency showed by the microglial cell line. More interestingly, ROS production and phagocytosis dynamics match with each other in J774A.1 cells, suggesting that they are coupled in order to eliminate the fungal threat. In addition, the release of TNF-α and IL-6 was determined, showing that, although both cell types produced these pro-inflammatory molecules, macrophage-like cells released higher cytokine quantities and, in the case of IL-6, in shorter experimental times. Although microglial pro-inflammatory responses are highly regulated in order to avoid neuronal tissue damage (Aloisi, 2001), microglial decreased responses might also be related to inefficiency since high cytokine production has been reported when these cells are challenged with other microbes (Frande-Cabanes *et al.*, 2014).

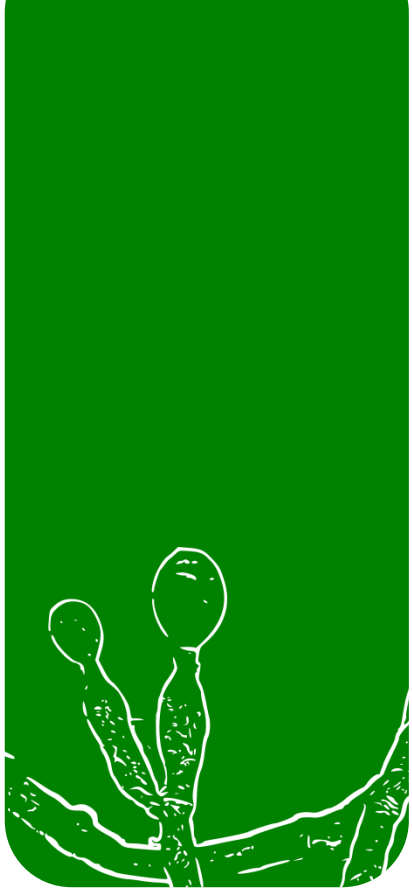
Finally, we evaluated the role of two PRR during the phagocytic process of *L. prolificans* by microglia, MR and dectin-1. These receptors have been previously described to be very important during recognition of fungal PAMPs, being dectin-1, for example, involved in distinguishing *C. albicans* (Gantner *et al.*, 2005) or *A. fumigatus* (Gersuk *et al.*, 2006) morphotypes. MR and dectin-1 are involved in the recognition of *N*-linked mannans and β-glucans, respectively, in the fungal cell wall (Netea *et al.*, 2008; Gresnigt *et al.*, 2012), being both related to phagocytosis and pro inflammatory responses, among others.

Interestingly, we showed that both PRR were mediating *L. prolificans* conidia uptake by microglial BV-2 cells. First, we observed that individual blockade of both MR and dectin-1 successfully inhibited phagocytosis of *L. prolificans* in short incubation times (2 and 4 h of

co-culture), but in a less extent at 6 h after inoculation of conidia. Since inhibitors were removed before inoculation of *L. prolificans*, the cells may be adapting to the infective process along time. Speculatively, microglia may be, for example, expressing more molecules of the receptor blocked or of others in order to improve its phagocytic rates. However, this hypothesis needs further research to be proved. Moreover, we showed that phagocytosis inhibition was inhibitor concentration-dependent, except for high levels of mannan which resulted in less inhibition capacity. It is also remarkable that the double inhibition of MR and dectin-1 did not result in a synergistic response, achieving similar phagocytosis levels as when only dectin-1 was inhibited. This finding of the role of dectin-1 is very important since, although dectin-1 has been indirectly related to the molecular recognition of *Scedosporium* spp. (Roilides *et al.*, 2009), no direct evidence of this has been reported until now.

To conclude, in this work we have described the interactions between *L. prolificans* and microglia or peritoneal macrophages. On the one hand, we have observed that the fungus is able to maintain its germination and hyphal branching rates, and to survive in a wide range of environmental pH, especially in highly acidic conditions. On the other hand, we have characterized some features related to microglia that may be contributing to this pathogenic species tendency to invade the CNS, such as impaired phagocytic capacity, decreased pro-inflammatory cytokine release and ROS production, or inability to avoid or delay hyphal infective growth. Moreover, we have proved that both MR and dectin-1 play key roles during microglial phagocytosis, being the latter receptor of remarkable importance in this process.

General Discussion



The filamentous ascomycete *Lomentospora prolificans* is a pathogen, whose prevalence in clinical settings has increased in the last decades, being termed as an emerging pathogen (Salkin *et al.*, 1988; Cortez *et al.*, 2008). In this sense, it is worth highlighting that it currently represents the most relevant cause of non-*Aspergillus* filamentous fungal infections, together with the related genus *Scedosporium,* in countries such as Spain or Australia (Alastruey-Izquierdo *et al.*, 2013; Slavin *et al.*, 2015). As an opportunist, *L. prolificans* mainly infects patients suffering from underlying conditions, most of them related to immunosuppressed states (transplant recipients, AIDS patients, etc.). Moreover, this species is prone to get disseminated through the bloodstream, being the most usual and mortality-carrying pattern of infection (Rodriguez-Tudela *et al.*, 2009). Remarkably, among all the organs that is able to invade, *L. prolificans* tends to enter the central nervous system (CNS), being reported as a neurotropic pathogen (Marco de Lucas *et al.*, 2006; Kantarcioglu *et al.*, 2008). There, it is able to establish fungal abscesses or, less frequently, meningitis, leading to neurological symptoms and, often, to the death of the patient.

In order to deeply understand *L. prolificans* pathogenesis, we proposed for this study a multifaceted analysis of fungal virulence factors and host immune response to this species by applying molecular and cellular approaches, with the final goal of finding novel candidate for therapeutic or diagnostic targets.

In the first part of this work, it was studied the adaptive host immune response to *L. prolificans* mediated by salivary IgA and serum IgG among immunocompetent population, by using immunoproteomics-based techniques. These approaches have been shown to be helpful to study fungal pathobiology (Thomas *et al.*, 2006), and the discovery of novel biomarkers (Kniemeyer *et al.*, 2011) or targets for antifungal compounds (Parsons *et al.*, 2003). However, to our knowledge, large-scale analyses of *L. prolificans* proteins have never been performed, currently being a completely unexplored field. Consequently, we developed a quick and reproducible workflow for working on proteomics with this species, establishing proper fungal culture conditions, and disruption methods.

Applying the newly described workflow, we analyzed the antibody-mediated immune response to *L. prolificans* among healthy individuals. The study of immunocompetent

immune response is interesting since, although this fungus is mainly distributed in human-impacted ecosystems, as parks or playgrounds, agricultural or industrial lands, etc. (Kaltseis *et al.*, 2009; Harun *et al.*, 2010; Rougeron *et al.*, 2014), the number of clinical cases reported in this group of population is remarkably low (Rodriguez-Tudela *et al.*, 2009). Therefore, our hypothesis was that an effective protective immune response should be present in immunocompetent individuals to avoid these mycoses and, in consequence, immunodominant antigens might be candidates to be studied as therapeutic targets.

To prove this, we used two types of biological human samples obtained from healthy volunteers, saliva and serum, to study the recognition mediated by IgA and IgG, respectively. At cell level, it was observed that while salivary IgA recognized almost solely conidia, serum IgG displayed a complete recognition of both morphotypes. This is consistent with the biological scenario in which conidia, the dispersive morph, enter the host body through the airways, being in this area present predominantly IgA. On the other side, serum IgG may be acting against both conidia and hyphae in order to stop any invasion.

Then, immunoreactive protein spots were detected and identified by applying immunomics, being antigens selected by intensity or by prevalence in the case of salivary IgA or serum IgG, respectively. Hence, a large panel of proteins was identified proving their antigenicity against IgA and/or IgG isotypes. Interestingly, conclusions can be drawn when comparing datasets concerning IgA- and IgG-reactive proteins (Figure 1). While some of the antigens share their recognition in the two morphotypes or by both Ig isotypes, only Hsp70 was detected in all of them. This fact proves the Hsp70 high immunogenic properties, making this antigen very interesting for the development of therapeutic strategies, such as vaccines, by which the host may develop protection at different levels (airways, blood, etc.).

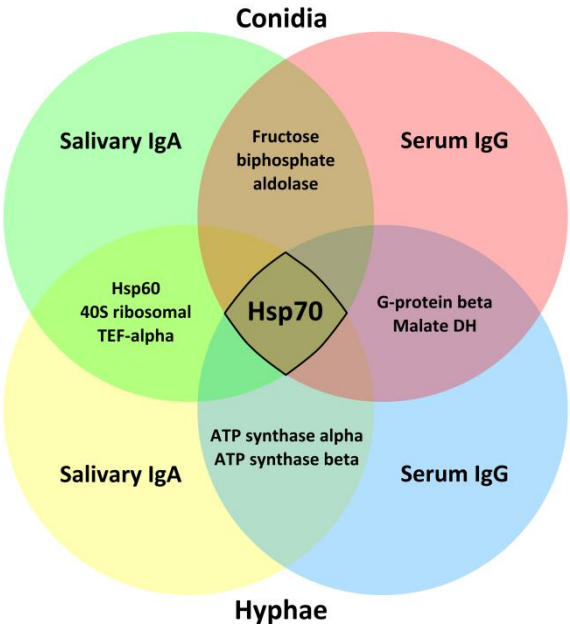


Figure 1. Summarized results of immunoreactive proteins shared by both morphotypes and immunoglobulin isotypes. Hsp, heat shock protein; TEF, translation elongation facto; DH, dehydrogenase.

In addition, the development of protective antibodies against the identified antigens could be a key factor to avoid fungal infections. In this sense, monoclonal antibody production technology has been proved to be very useful to develop specific therapeutic approaches based on antigenic or virulence-related proteins. Although no research efforts have been focused yet on this topic concerning *L. prolificans*, very promising results have been obtained with other pathogenic fungi such as *Candida albicans*, *Aspergillus fumigatus*, or *Histoplasma capsulatum* (Casadevall and Pirofski, 2012). Moreover, identified antigenic proteins could be useful as diagnostic markers for *L. prolificans* infections, such as catalase for *Scedosporium boydii* (Mina *et al.*, 2015) or Hsp90 for *C. albicans* (Pitarch *et al.*, 2014).

It is also worth highlighting that the antigenicity of some of the proteins identified in this study has been previously described in other pathogenic fungi, as *Candida* or *Aspergillus*, as pointed out throughout this thesis. Therefore, the wide recognition of *L. prolificans* proteins described in this study may not be specific to this species, but may be related to a panfungal or panmicrobial immune response targeting highly conserved proteins, such as of glycolytic enzymes. In this sense, we have not delved into cross-reactivity among other fungal species, and this fact should be taken into account when analyzing a certain antigenic protein.

Therefore, more in-depth studies should be performed in order to determine the suitability of these proteins as therapeutic or diagnostic targets.

After the immunomics-based work, we designed a study to analyze the molecular basis of *L. prolificans* resistance to the most effective antifungal drug, voriconazole (VRC), since its inherent resistance is one of the major concerns related to this fungus. The emergence of this and other species intrinsically resistant to available antifungals, and the increasing number of strains with acquired-resistance belonging to genera such as *Aspergillus* (Vermeulen *et al.*, 2013), is becoming a serious problem. This represents a great challenge when facing the fungus in clinical settings, increasing patient mortality rates up to 95-100%, length of stay, and hospital costs (Heng *et al.*, 2012). Despite there exist novel compounds with very promising activity against *L. prolificans*, such as *N*-chlorotaurine (Lackner *et al.*, 2015), increased experience using them is needed, including clinical trials. Therefore, the absence of a truly effective therapy to eliminate *L. prolificans* infections is a hotspot in which more research should be focused on.

Therefore, we applied a wide range of molecular techniques to determine the changes on the cell wall architecture and composition, and at proteomic level in response to VRC exposure. Interestingly, although VRC did not delay or stop germination in fungal cells, dramatic morphological changes were induced, meaning that *L. prolificans* cells are stressed but somehow are able to overcome the situation (Figure 2). In this sense, since we observed that VRC-treated cells were more intensely stained with calcofluor white, and taking into account that this dye binds to chitin polymers present in fungal cells, it was proposed that *L. prolificans* was modifying its cell wall as a consequence of the antifungal drug-induced stress. Indeed, structural and compositional alterations was promoted by VRC, the fungus highly thickening the outer fibrillar layer, associated mostly with mannans (Ene *et al.*, 2012), and increasing the content of mainly glucose and mannose in both the whole cell wall and cell surface. In this sense, our data are consistent with some studies pointing out that the presence of higher chitin content in *C. albicans* cell wall is associated with less sensibility to echinocandins (Lee *et al.*, 2012). These findings are notable as only a few studies describe modifications in the cell wall as a mechanism of antifungal drug resistance.

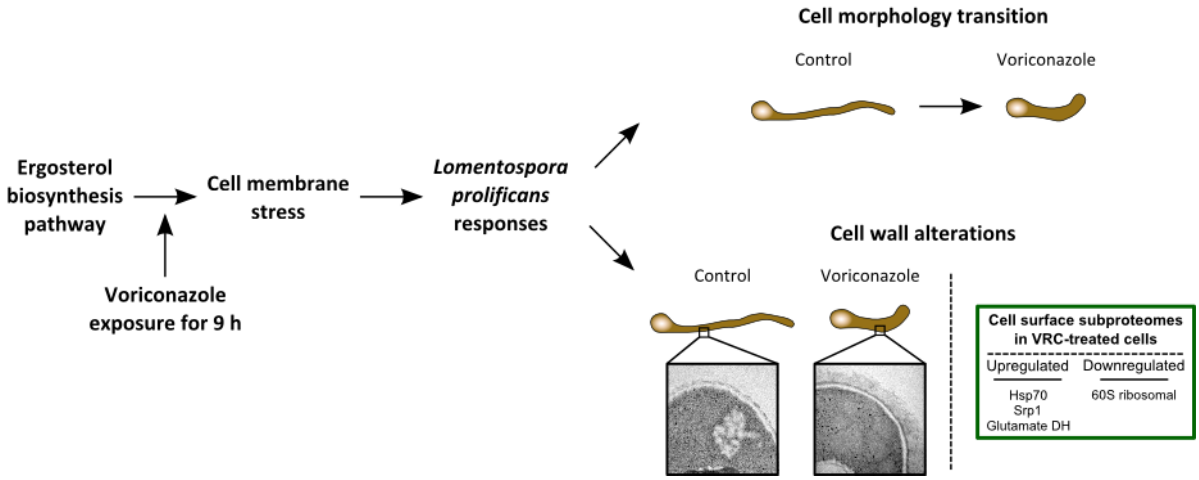


Figure 2. Schematic summary describing cellular and molecular alterations promoted by *Lomentospora prolificans* cells in response to voriconazole. Hsp, heat shock protein; Srp1, Serine-rich RNA polymerase I suppressor protein; DH, dehydrogenase.

It is also interesting that the analysis of cell surface-associated proteins showed the presence of some upregulated protein spots in the presence of the antifungal drug, including heat shock protein (Hsp) 70. The identification of this protein as relevant for VRC resistance is very remarkable, and should be taken into account for further research, also regarding to its relevance as antigenic protein in both conidia and hyphae. Supporting the data we have obtained in this work, this protein, included in the signalling pathways in which Hsp90 and calcineurin are also involved, has been proved to be essential for *A. fumigatus* and *A. terreus* tolerance to caspofungin and amphotericin B, respectively (Blatzer *et al.*, 2015; Lamoth, Juvvadi, *et al.*, 2015). Although pifithrin-μ, a specific Hsp70 inhibitor, has been tested against *L. prolificans* showing very poor activity (Lamoth, Alexander, *et al.*, 2015), combination therapy using this compound with VRC has not been tested at the moment, which could be interesting for future research. In addition, studying the role of Hsp70 in *L. prolificans* pathobiology using null mutants is essential to deeply understand the molecular basis of its virulence and antifungal resistance, experiments that will surely be carried out in the future in our laboratory.

Finally, we focused on studying the *in vitro* interactions between *L. prolificans* and microglial cells. As already mentioned, *L. prolificans* is prone to develop disseminated infections and to invade different host areas, especially in highly immunodepressed individuals. Remarkably, the fungus tends to trespass the blood-brain barrier, forming fungal abscesses or meningitis

in CNS. In these cases, although patient immune status is compromised, microglial cells might play key roles in fungal clearance (Mariani and Kielian, 2009). Therefore, in this work we analyzed the immune response mediated by microglia against *L. prolificans* to elucidate whether inefficiency of these phagocytic cells could be one of the factors contributing to this fungus neurotropism.

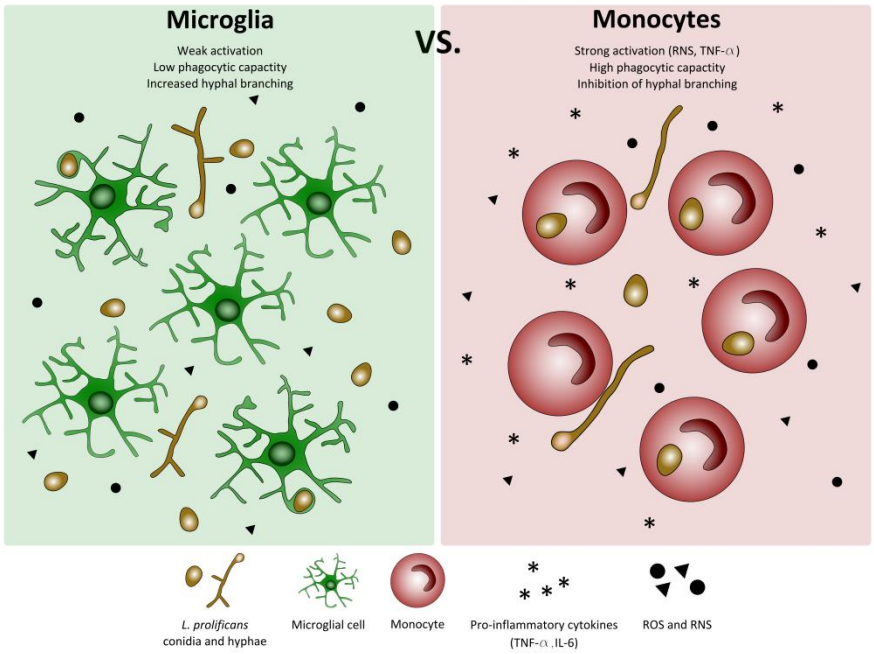


Figure 3. Schematic summary of the results showing microglia inefficiency against *Lomentospora prolificans* cells.

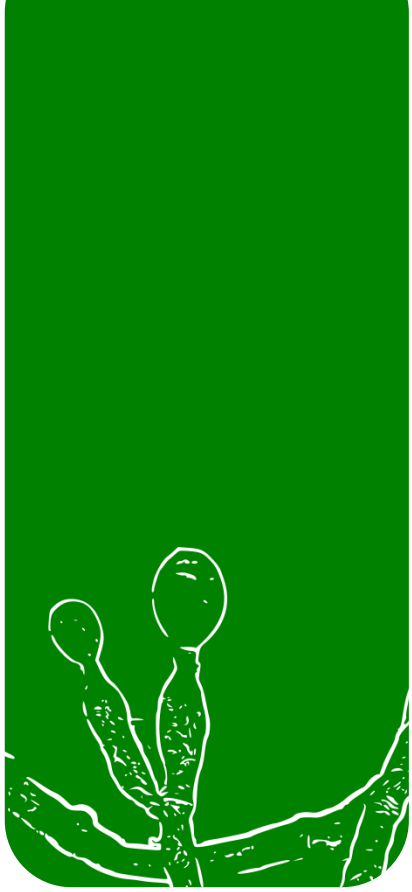
In this way, we characterized microglial responses to the fungus, and compared them with monocytes/macrophages and the available data in the literature (Figure 3). Precisely, it was observed that, although microglia was able to phagocytize fungal cells in a mannose receptor- and dectin-1-dependent manner, its efficiency in this process was very low in comparison with the other phagocytes or when challenged with other fungal species, as *Candida* (Neglia *et al.*, 2006; Orsi *et al.*, 2010) or *Cryptococcus* (Blasi *et al.*, 1992; Blasi *et al.*, 1995). Moreover, other responses concerning fungal killing (as the production of ROS and RNS) or pro-inflammatory signalling were also less efficient in microglial cells, strengthening our hypothesis.

To conclude, in this thesis we have settled the basis for working on proteomics with *L. prolificans*, and have deeply described the antibody-mediated humoral response to this

fungus in immunocompetent population by studying conidial and hyphal immunomes against salivary IgA and serum IgG. Moreover, we have determined several cellular and molecular events occurring in *L. prolificans* cells when exposed to VRC, which, together with other mechanisms yet to be described, could be related to this species antifungal resistance. In this sense, we have identified a wide panel of novel *L. prolificans* proteins, both antigens and related to antifungal resistance. Among them, Hsp70 has been shown as one of the most interesting proteins since in this work has been identified as antigen in both morphs, being recognized by salivary IgA and serum IgG, and overexpressed in the presence of VRC, proving its relevance during drug resistance. Additionally, we have characterized some aspects of microglia-fungus interactions that may be related with the prominent neurotropism observed in disseminated infections, including decreased phagocytic capacity.

Therefore, the results obtained in this study can be very useful to deepen into the finding of new molecular targets which may contribute to the development of novel therapeutic and diagnostic systems. Furthermore, it has shed light on the better understanding of different pathobiological factors, such as VRC resistance and neurotropism.

Conclusions



1. At cellular level, salivary IgAs and serum IgGs from immunocompetent humans display differential recognition of *Lomentospora prolificans* morphotypes, the first recognizing mainly conidia and the latter both conidia and hyphae.

2. Human salivary IgAs from immunocompetent individuals target a great amount of *Lomentospora prolificans* proteins, being identified 22 and 23 immunodominant in conidia and hyphae, respectively. Among them, conidial catalase and hyphal heat shock protein 60 were found to be the most immunoreactive.

3. Antigenicity of *Lomentospora prolificans* immunomes against serum IgGs from immunocompetent population was analyzed, identifying 12 conidial and 9 hyphal antigenic proteins as the most seroprevalent. Precisely, G-protein β subunit and heat shock protein 70 have been described as the most seroprevalent antigens in conidia and hyphae, respectively, being the latter protein also identified in the cell surface.

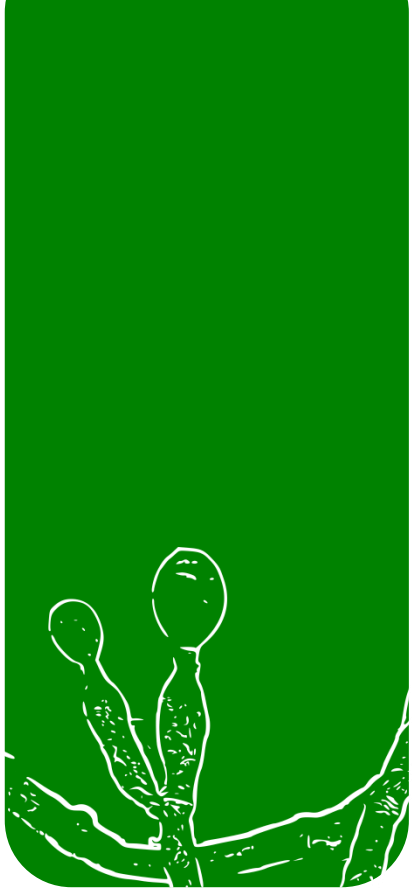
4. *Lomentospora prolificans* cells promote sustained alterations in cell morphology, cell wall composition and structure, and cell surface subproteome in response to the antifungal drug voriconazole.

5. Among the wide set of novel proteins of *Lomentospora prolificans* identified in this study, heat shock protein 70 stands out as one of the most relevant, being recognized by salivary IgA and serum IgG in both morphotypes, and overexpressed upon voriconazole exposure.

6. Microglial cells are inefficient to manage *Lomentospora prolificans* infections *in vitro*, showing deficient phagocytic rates, loss of viability, and decreased production of ROS and pro-inflammatory cytokines TNF-α and IL-6.

7. Phagocytosis of *Lomentospora prolificans* cells by microglia is mediated by two C-type lectin receptors, mannose receptor and dectin-1, having the latter a more important involvement in this process.

Resumen y Conclusiones



Resumen

El hongo filamentoso *Lomentospora prolificans* es un patógeno cuya prevalencia en el ambiente hospitalario se ha visto incrementada en gran medida en las últimas décadas, siendo conocido como un patógeno emergente. En este sentido, es importante resaltar que esta especie, junto con el género afín *Scedosporium*, se encuentran en primer lugar como agentes etiológicos de micosis causadas por especies de hongos filamentosos no pertenecientes al género *Aspergillus* en países como España o Australia.

Las infecciones causadas por esta especie tienen una amplia gama de manifestaciones clínicas, desde micosis cutáneas o subcutáneas, a diseminadas. Al igual que muchos otros hongos, como patógeno oportunista *L. prolificans* causa infección principalmente en individuos con patologías subyacentes, generalmente relacionadas con la inmunosupresión. Así, pacientes oncológicos, pacientes trasplantados, o individuos infectados con el virus de inmunodeficiencia adquirida son proclives a sufrir una infección por este hongo. Además, en estos tipos de pacientes, *L. prolificans* tiende a producir infecciones diseminadas, utilizando el torrente sanguíneo para invadir otros órganos del hospedador distintos al lugar de entrada. Entre ellos, el sistema nervioso central se ha mostrado como uno de las áreas por la cual el hongo tiene mayor tendencia, conociéndose por tanto como un patógeno neurotrópico.

Por todo ello, nos planteamos un **objetivo principal** que consistía en un estudio polifacético de la patobiología de *L. prolificans*, centrándonos en los factores de virulencia del hongo y la inmunidad antifúngica mediada por el hospedador, para profundizar en el conocimiento de estas micosis y contribuir al desarrollo de nuevas terapias.

Asimismo, propusimos una serie de **objetivos específicos** en este estudio para poder llevar a cabo el trabajo, siendo:

1. Caracterizar los inmunomas de los conidios e hifas de *L. prolificans* reconocidos frente a IgAs de la saliva de individuos inmunocompetentes.
2. Analizar la antigenicidad de las proteínas de conidios e hifas de *L. prolificans* frente a

IgGs séricas, determinando la seroprevalencia de antígenos inmunodominantes entre la población inmunocompetente.

3. Estudiar las modificaciones celulares y moleculares que le ocurren a las células de *L. prolificans* en respuestas al compuesto antifúngico voriconazol.
4. Caracterizar las interacciones *in vitro* entre la microglía y *L. prolificans* para dilucidar los factores de virulencia del hongo y la respuesta inmune de los fagocitos.

En la primera parte de este trabajo, por tanto, estudiamos la respuesta inmune mediada por IgAs de saliva e IgGs de suero en individuos inmunocompetentes mediante el uso de técnicas inmunoproteómicas. En este sentido, debido a que no se conocían estudios previos utilizando estas técnicas con *L. prolificans*, diseñamos un esquema de trabajo en proteómica para poder aplicarlo en esta especie. De esta manera, diseñamos una metodología completa que incluía condiciones de cultivo, ruptura celular, y condiciones para la electroforesis bidimensional.

Aplicando la metodología descrita, nos dispusimos a analizar los inmunomas de *L. prolificans*, de ambos morfotipos, conidio e hifa. Para ello, utilizamos dos tipos de muestras biológicas obtenidas de individuos inmunocompetentes, saliva y suero, determinando aquellos antígenos que eran reconocidos por las IgAs e IgGs presentes en estos fluidos, respectivamente. Debido a que la distribución ambiental del hongo es amplia en ambientes en los que el ser humano tiene mucha presencia, y a que los individuos sin la respuesta inmune afectada no suelen verse afectados por estas micosis, nuestra hipótesis consistió en que esta población debía poseer una respuesta inmune protectora efectiva evitando el desarrollo de infecciones. De esta manera, los anticuerpos, tanto los específicos para una especie, como los que están dirigidos para grupos de microorganismos, ayudan en la protección frente a infecciones, siendo microbicidas directamente o ayudando a desencadenar otros tipos de respuestas, como por ejemplo la fagocitosis.

En primer lugar, en este estudio se ha demostrado que los dos isotipos de inmunoglobulinas analizados, IgA salivar e IgG sérica, muestran diferentes patrones de reconocimiento del hongo a nivel celular. Así, las IgAs reconocen principalmente el morfotipo conidial, mientras que las IgGs muestran un reconocimiento más amplio, tanto en conidios como en hifas. Esto

puede relacionarse con el escenario biológico en el que, mientras que los conidios, o forma dispersiva, es mayoritariamente inhalada, penetrando por las vías respiratorias, las hifas son el morfotipo relacionado con la invasión, por lo que una respuesta a nivel de tejido interno debe ser más necesaria.

Por otro lado, en consonancia con la hipótesis planteada, se encontró una amplia respuesta mediada por anticuerpos, tanto IgAs como IgGs, en estos individuos, identificándose aquellas proteínas más inmunoreactivas, en el caso del reconocimiento mediado por IgAs, y más seroprevalentes, en el caso de los anticuerpos séricos. Un total de 66 proteínas diferentes con características de antígeno han sido identificadas en este trabajo. Curiosamente, entre todas estas nuevas proteínas, destaca la Hsp70, la cual ha sido descrita como antígeno en ambos morfotipos, conidios e hifa, además de ser reconocida tanto por IgAs como por IgGs. Este hecho evidencia sus grandes propiedades inmunogénicas, siendo esta proteína muy interesante para el desarrollo de nuevas terapias, como vacunas, mediante las cuales el hospedador adquiriría protección a diferentes niveles (vías respiratorias, sangre, etc.).

Por otro lado, *L. prolificans* ha sido ampliamente descrito como un hongo refractario a las terapias antifúngicas que existen en la actualidad, presentando alta resistencia y tolerancia a los compuestos antifúngicos. En este sentido, el agente antifúngico voriconazol (VRC) es el que mejores resultados ha mostrado, siendo utilizado en clínica como primera opción terapéutica. Sin embargo, su eficacia es aún baja y dependiente del aislado que se encuentra en cada paciente. Por ello, a pesar de que en estudios recientes se han presentado nuevas sustancias con resultados prometedores frente a *L. prolificans*, se necesita más investigación y experiencia clínica con los mismos (como por ejemplo ensayos clínicos) para ser aplicados a pacientes. Por tanto, existe una gran necesidad de desarrollar nuevos compuestos o estrategias que permitan reducir las altas tasas de morbi-mortalidad asociadas a estas infecciones fúngicas.

Con el objetivo de comprender los mecanismos moleculares subyacentes a esta resistencia, en este trabajo de tesis doctoral se han estudiado los cambios que se producen en el hongo a nivel celular y molecular cuando se le expone a VRC, aplicando también las técnicas

proteómicas establecidas anteriormente. Así, se ha observado que, aunque el compuesto antifúngico no modifica la tasa de germinación del hongo, la morfología de las hifas cambia drásticamente, volviéndose más cortas y gruesas respecto al control. Además, se pudo determinar que las células tratadas con VRC se teñían más intensamente con blanco de calcofluor, un colorante fluorescente que permite observar la quitina presente en la pared de los hongos, indicando cambios en este orgánulo celular. En este sentido, se observó que, tanto la estructura, como la composición de la pared celular fúngica sufrían grandes modificaciones tras la exposición al VRC, sugiriendo que el estrés producido por el este compuesto en la membrana plasmática, puede ser compensado de alguna manera mediante estas modificaciones en la pared.

Por otra parte, el estudio proteómico se realizó en dos fases: proteoma de célula completa, y subproteoma de superficie celular. A pesar de que no se encontraron cambios significativos a nivel de proteoma completo, se observaron variaciones interesantes en el subproteoma de superficie celular, encontrándose varias proteínas sobre- e infraexpresadas en presencia del compuesto antifúngico. Entre ellas, las proteínas Hsp70 y Srp1 se identificaron como las más sobreexpresadas, mientras que dos *spots* formados por la proteína 60S ribosomal se observaron como los más infraexpresados. De nuevo, la Hsp70 destacó entre todas las proteínas identificadas, ya que su papel en la tolerancia o resistencia a agentes antifúngicos, junto con otras proteínas con actividad de chaperona, ya ha sido previamente descrita para otros hongos filamentosos, como *Aspergillus fumigatus* of *A. terreus*, proponiéndose en este estudio como una diana interesante con la que continuar la investigación. Lamentablemente, no fue posible la producción de cepas con estos genes inactivados debido a la capacidad de los hongos filamentosos de integrar secuencias nucleotídicas sin necesidad del complementariedad de bases. Por tanto, su relevancia en la resistencia a antifúngicos o, incluso, en la virulencia del hongo deberán ser más ampliamente analizados en el futuro.

Finalmente, la última parte de este estudio se centró en el análisis de las interacciones *in vitro* entre las células de la microglía y *L. prolificans*. Como se ha mencionado anteriormente, el hongo se ha descrito como neurotrópico ya que, al diseminarse por el torrente sanguíneo, tiene tendencia a infectar el sistema nervioso central, especialmente en

individuos profundamente inmunodeprimidos. Así, propusimos este estudio para determinar si la actividad de estas células fagocíticas podrían tener un papel en esta tendencia de *L. prolificans*.

En este sentido, se observó que la microglía presentaba ciertas características disminuidas en comparación con monocitos/macrófagos. Más concretamente, a pesar de que la microglía fue capaz de fagocitar las células fúngicas, y que este proceso esta mediado parcialmente por el receptor de manos y la dectina-1, la eficiencia de este tipo celular para deshacerse de la infección producida por *L. prolificans* fue muy baja. Así, los índices de fagocitosis, producción de ROS, o la activación de cascadas pro-inflamatorias se encontraban disminuidas en la microglía en comparación con el otro tipo de fagocito analizado.

En definitiva, este proyecto de tesis doctoral ha establecido las bases en varios campos que permitirán profundizar en el estudio de la patobiología de las micosis causadas por *L. prolificans*. En primer lugar, se ha descrito un flujo de trabajo en proteómica mediante el cual se ha descrito la amplia respuesta mediada por anticuerpos que los individuos inmunocompetentes han desarrollado frente a esta especie. Esta respuesta mediada por anticuerpos ha sido detallada en conidios e hifas del hongo tanto a nivel celular como a nivel proteómico, permitiendo la identificación de posibles dianas frente a las que desarrollar nuevas estrategias terapéuticas. Asimismo, se han descrito una serie de eventos celulares y moleculares que ocurren en las células fúngicas en respuesta al compuesto antifúngico VRC, los cuales, junto a otros mecanismos aún por detallar, pueden ser la base de la resistencia a fármacos presentada por *L. prolificans*. Tanto el estudio inmunómico, como el proteómico en presencia de VRC han servido para identificar por primera vez un gran número de proteínas pertenecientes a esta especie. Curiosamente, entre todas estas nuevas proteínas, destaca la Hsp70, la cual ha sido descrita como antígeno en ambos morfotipos, siendo reconocida tanto por IgAs como por IgGs, y como proteína sobreexpresada en respuesta al estrés creado por el VRC, probando su relevancia durante el proceso de tolerancia al fármaco. Además de todo ello, se han caracterizado las interacciones entre las células de microglía y *L. prolificans*, observándose que estas células fagocíticas son ineficientes frente al hongo, mostrando bajos índices de fagocitosis, y unas respuestas oxidativa y pro-

inflamatoria disminuidas.

Por todo ello, los resultados presentados y discutidos en esta tesis podrían ser muy útiles en la búsqueda de nuevas diana moleculares con las que se contribuyera al desarrollo de nuevas estrategias, tanto diagnósticas como terapéuticas. Además, se ha profundizado en la descripción de importantes factores patobiológicos del hongo, como son la resistencia al VRC y el neurotropismo.

Conclusiones

1. Las IgAs de la saliva e IgGs del suero de individuos inmunocompetentes muestran patrones de reconocimiento diferentes de los morfotipos de *Lomentospora prolificans* a nivel celular, las primeras reconociendo principalmente conidios, mientras que las segundas tanto conidios como hifas.

2. Las IgAs de la saliva de individuos inmunocompetentes tienen como diana una gran cantidad de proteínas de *Lomentospora prolificans*, habiendo sido identificadas 22 y 23 antígenos inmunodominantes en conidios e hifas, respectivamente. Entre ellos, la catalasa en conidios, y la *heat shock protein* 60 en hifas, fueron las que mayor inmunoreactividad presentaron.

3. La antigenicidad de los inmunomas de *Lomentospora prolificans* frente a IgGs séricas en la población inmunocompetente fue analizada. Entre el amplio número de proteínas reactivas, se identificaron 12 y 9 antígenos en conidios e hifas, respectivamente, siendo los más seroprevalentes. Concretamente, la subunidad β de la proteína G, en conidios, y la *heat shock protein* 70, en hifas, fueron los antígenos más seroprevalentes, siendo esta última también detectada en la superficie celular.

4. Las células de *Lomentospora prolificans* promueven grandes alteraciones en la morfología celular, la composición y estructura de la pared celular, y el subproteoma superficial en respuesta al compuesto antifúngico voriconazol.

5. Entre el amplio grupo de proteínas identificadas por primera vez en *Lomentospora prolificans* en este estudio, la *heat shock protein* 70 destaca como una de las más relevantes, habiendo sido identificada como antígeno en conidios e hifas, reconocida tanto por IgAs como por IgGs, además de encontrarse sobreexpresada en presencia de voriconazol.

6. Las células de la microglía son ineficientes durante las interacciones con *Lomentospora prolificans in vitro*, mostrando bajos índices de fagocitosis, pérdida de

viabilidad, y una producción disminuida de las ROS y de las citocinas pro-inflamatorias TNF-α and IL-6.

7. La fagocitosis de *Lomentospora prolificans* por la microglía está mediada por dos receptores lectina tipo-C, el receptor de manosa y la dectina-1, teniendo esta última un papel más importante en el proceso.

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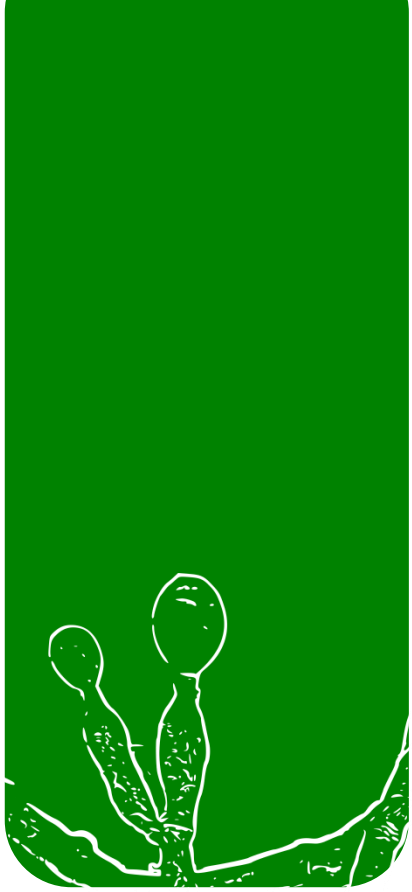

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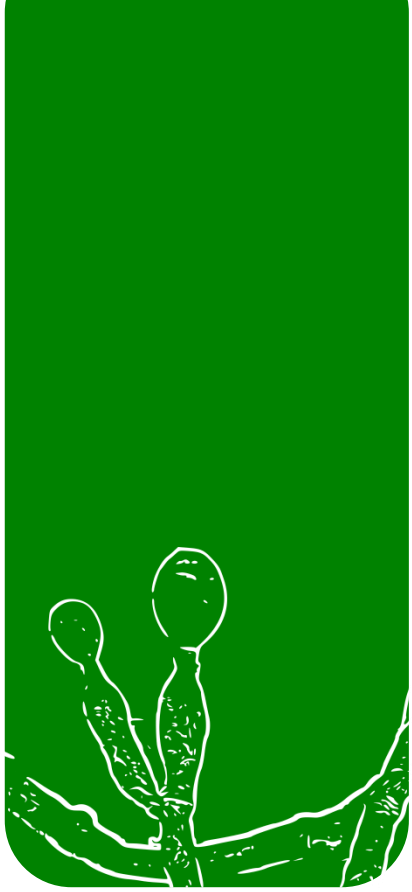
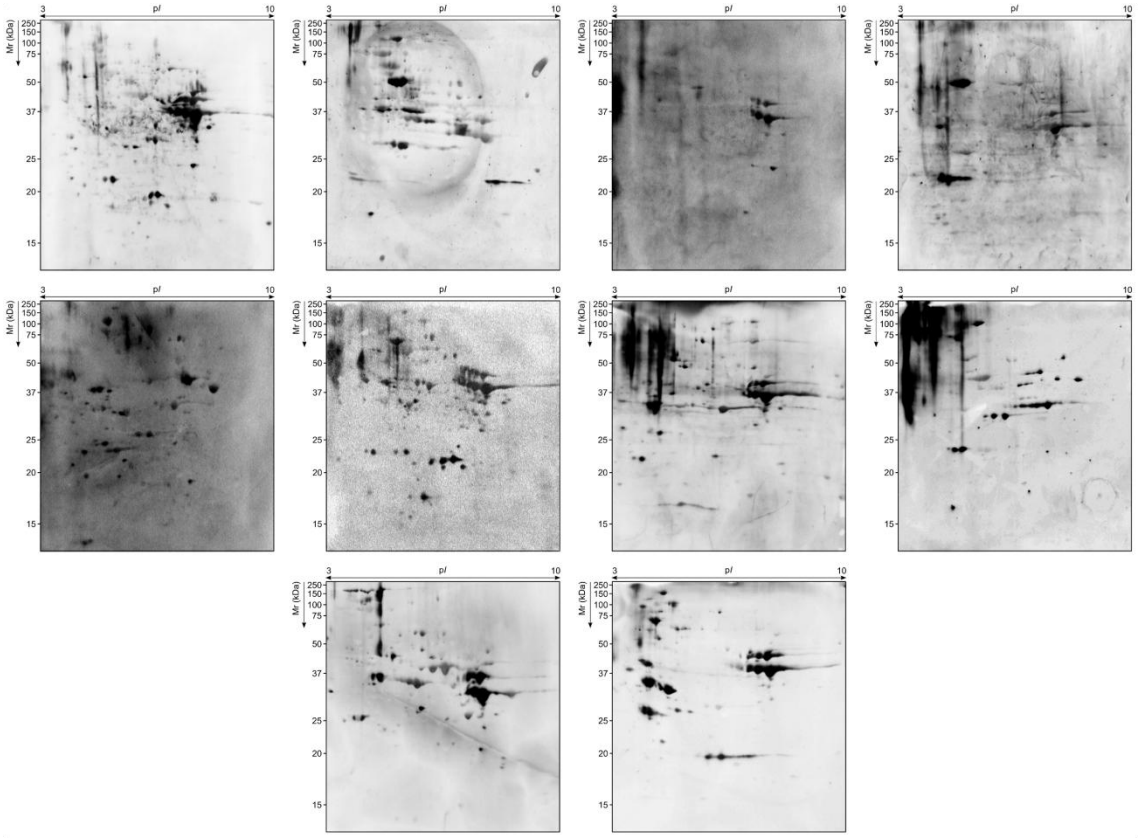
Supplementary Material



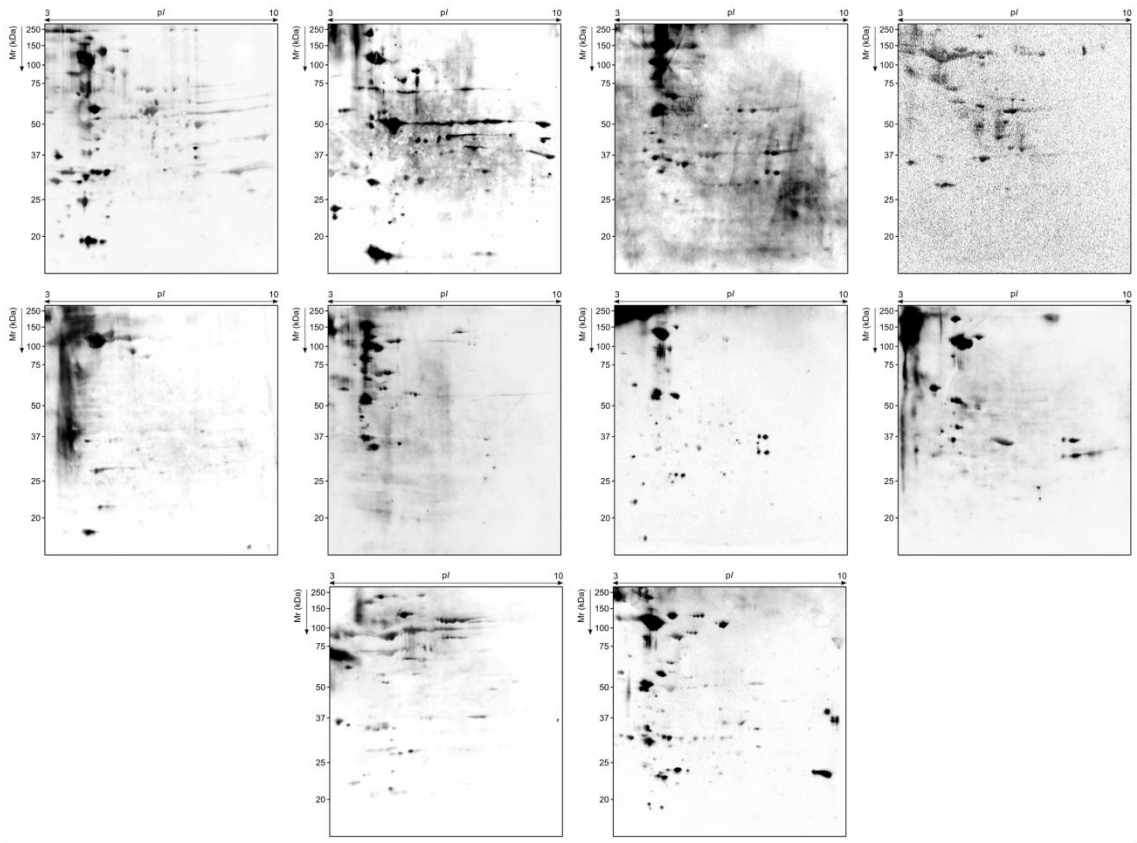
Supplementary material 1

Two-dimensional immunoblots using the ten serum samples individually against conidial proteome.



Supplementary material 2

Two-dimensional immunoblots using the ten serum samples individually against hyphal proteome.



Supplementary material 3

Sequences of genes which deletion was attempted. Putative coding sequences are shown in bold letter plus 1 kb upstream and downstream.

Heat shock 70 kDa protein

TTTGAAGATCAGAGAGCCAGCGAGGGGGCCTTACACCGCATTTGCTGTTTTGCCATTGAG
CGGAAAGTGAGCAGGCGACGGAGGTTGGTGGGTACTGAGCCCTAGTTTCTAGCCCGTTGT
ATCGGCTACGCACGAGGACTCTGCGTTACTATGGGAAAGCCGTGGCCTGGGCCCGCCGAC
GTGGGGTACAGGTACCTCTAAGGAACCCCACTATGAACATCTGAGTATGACATCAATCCA
TGGTGAGAAAATTAATTCTATCCACGATGAATGACGGTGCGCTGGGAGCTCCAACTCTAA
ACACTGGTGAAAGCATTTAGAGAAAACTCGAAGACGCTAGCAGAAAATAGAAGCGCTGAG
CATGATCTGGATTGTTCAACTTGCACGTGGGATAAGGAATTGAGAAGCCTGGCTGCCATT
GTAGGAATGTTCCAGCTTCGTCCAGGACTCAAGCAGAAACAGGAAGTGCACCGATCGGGA
AGCTAGCAAAGATCCAGAGCTTTAACTAGAAAAACCCAGAAAACAAAGGAAGACCAGGGG
TTTCCTGTTTGGGGGGGCAATTGGGGGCAGCCTGATGGGCGGAGCGTGGAAAACTCTGGT
GGTTGCCCCAGCATTCTGGAGCTGGCGCTGGAAGAGAAAAGAACCCCGTAGAAGTGGCGG
GGTACCACTCGTACCTTCCCGCCCCGCAACGCTCTGAGTATTAAGCTGCCATCTTCGACA
TCGAGGTCAGAGTTTGGAACTTTTTTTTCTCTTTCCTCTACACAAATCACAATCCAAACA
CCTCTCTCTGAGTTTTTAGACCTATAAAAGAGGAGCCATCCTGATCTCTGCTCTCTTTTT
CTTCCCTCCCACAAATTCCTGCCTGTTGAACCCAAACCAATTCAACACACCCCTGTTTCC
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CCTATCATCTTTTTCTTAATTCCACACATCACACATCCATC**ATG**GCCCCCGCGATTGGTA
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TTGCCAACGACCAGGGTAACCGTACCACCCCCTCCTATGGTAAGTAAACGAGAAATATTC
ACAAAGGGCGGATGGCCTGTTACAGCCACCCCTCCCCTCTTTCGCAACCCCCCGGTTCTT
GCACGCCCTCCCAGCGCGGGAGGGCTTGGGAGAATTTGTGGCAGCGTGCTTCTCTATGTC
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ACGCGGCCAAGAACCAGGTCGCTATGAACCCCAAGAACACAGTCTTCGATGCCAAGCGTC
TTATTGGACGCAAGTTCAACGACGCCGAGGTCCAGGCTGACATGAAGCACTTCTCCTTCA
CTGTCAAGGACGACAAGGGTGGCAAGCCTGTCGTCGAGGTCGAGTTCAAGGGCGAGACCA
AGCAGTTCACTCCCGAGGAGATTTCCGCTATGATCCTCACCAAGATGCGAGAGACTGCCG
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ACTCTCAGCGTCAGGCTACCAAGGATGCCGGTCTCATTGCCGGTCTCAATGTCCTCCGTA
TCATTAACGAGCCCACCGCGGCCGCCATTGCCTACGGTCTCGACAAGAAGCACGAGGGTG
AGCGCAACGTCCTCATCTTCGATCTTGGTGGTGGTACTTTCGATGTCTCCCNNNNNNNNN
NGAGGACGGTATCTTCGAGGTCAAGGCTACCGCTGGTGACACTCATCTTGGTGGTGAAGA
TATGGACAACCGCCTGGTGCAGCACTTCATCAACGAGTTCAAGCGCAAGCACAAGAAGGA
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CTTCTACACCTCCATCACCCGTGCCCGTTTCGAGGAGCTCTGCCAGGACCTCTTCCGATC
CACTCTCCAGCCCGTCGACCGCGTCCTCTCCGATGCCAAGATCGACAAGTCCAACGTCCA
CGACATCGTCCTCGTCGGTGGTTCCACCCGTATCCCCCGTGTCCAGAAACTCATCTCGGA
CTACTTCAACGGCAAGGAGCCCAACAAGTCCATCAACCCCGATGAGGCTGTTGCCTACGG
TGCTGCCGTCCAGGCTGCCATCCTTTCCGGTGACACCTCCAACAAGACCACCAACGAGAT
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CAAGCTCATTCCCCGCAACACCACCATCCCTACCAAGAAGTCCGAGATCTTCTCTACCTT
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TCCCCAGATTGAGGTCACCTTCGACGTTGATGCTTCCGGTATCATGAACGTCTCCGCTGT
CGAGAAGGGCACTGGCAAGTCGAACAAGATTGTCATCACCAACGACAAGGGCCGTCTCTC
CAAGGAGGAAATCGAGCGTATGCTTTCGGAGGCCGAGAAGTACAAGGAGGAGGATGAGGC
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CACCCTCTCTGACGAGAAGGTTGACGAGAAGCTCGACGCCTCCGACAAGGAGAAGCTCAA
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GTACGAGGAGCGCCAGAAGGAGCTCGAAGGTGTCGCCAACCCCATCATGATGAAGTTCTA

CGGTGCCGGTGGCGCTGGTGGCATGCCTGGTGGTATGCCCGGTGGTCCTGGCGGTCCCGG
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TGAGGAGGTCGACTAAATGCACCAACTTCATGATGATACCCGCACGTCCTTTTCCTAAAT
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CTTTTCCTTCGGACGTTTTTTAAGTCGTTTCGGCATCTTAACGAATAAAGGAGAGATGGA
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GATCAAATGAAAACAGTTCAACAAACTTGGCACTTTCTCTTCCGTAAATGTCTTTCTTCT
TCTGTGATCTCTAGCGTCCCGTAGCGATATGCCAGTCCTCTGTCTTGGTGCCCGTCGTTT
CGAACATCACAAGCAAAGTGTGTGATTGATGGCATGCAAAATTTAGAAAGAGTTGAGTTA
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TGAATCTCCATCAACTGCCACTCTTCATCGCTCATGTCGTCCGGTACGAACGGCTCCTTG
AACGCCGTCCCCGGCGGTACCTTCTCATCCCTACTCACCCATACGTCTTCGCTATTCGGC
TTGCCGCCGTACTTCTCCAGCTCCTTCCTTTTATAGTGTTCCTTGACCATCTCGGCTTTC
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CACGAGCGCACGGAGCCGTCGCGGTAGATGGCGTTCCACTGGCCGCCGATGCCCTGGCAC
GCGTACGCCTGGTCGAAAGCCTGGCGGCAGGACATGTCGGTGGGCCGGAGAGAGTGGGCG
AGGGCTTCGGAGCCCGGGGTGGGGTTGCGGGAGGTGGAGGTTGCGAGGGGCGCGGGCTTC
TCGGGGGCGGAGCGGGAGGTTAAGAAAGGGAGATAGGTGGATATGGCGGAGGAGATGGCT

Srp1 family-like protein (hypothetical protein SAPIO_CDS8550)

ACATGCATACTACTGCGTCAACAAGAACTCGGTAGTGCTACGTTAGAGCCGTTGAACAGG
CGGGGTGGCATCCCGATTATTATATCCTGGGTCTGTTTCCGAGGCTTCCTTTGATGATAT
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TGGAGCCGTTTAACTTAGCCCGTTCCTGAAATCCCGGTCATTGGTGGCTTGCGTGACGGT
CATACTTGCCACTTGCTGTTGATAGAGAAGAGAATGGAGTGCCCGGCGATAATATGCCCA
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CGCCTACGAGTTCGAACGGTATGTTATCCGCGCGTCGACACCTCCATCGTGACGGCGAAC
CATTATACTTTCCTCCCGATGTGTCGAGTTTCCATTCCTCCCCCACAAAAAGCTTCTTTC
GCCCTTCGTACTCCCGCCGCGTGCTCGCGCACGTCAACGCCCATCAACATCTTCGCCTCC
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CGTCTTCGCCCTCTCGTCCTCTCCTCGTCCTCGTCGTCGTCGTAGTCCGTCGGTCCATCG
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GCGACTACTCTCCCCGCAAGGATGATCGTCGTGACCGCGACCGAGATTATGACCGCGATC
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TGATAGGGAGACCAAGGATGATAGGGACGACCGTGACCGCCGTGAGAACGGTGCCAATGG
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TAACTCGGCCCGTTCCTATAGCTCTTGACAGTCCCCCTCCTCATGATGATCTCGATGTTG
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TGCCGAGTTTCTTCCCTTTACTCACTCGGGTCATTTTCGGGATGACCATTCACAGTACCT
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CCTAAGTGTAGGAATGTGATGCTCGCGCCTCCCCTAGTCCCTTATCTCTATTGGCGTGTT
TCGGATTCTCGACCCTGGCTGGCTGACATGGTCCTGCCACTGGCGCTGTGCTGCTGGTTT

Supplementary material 4

Primers used in this study.

Primer name	Primer sequence	Primer application
HSP70 up F	TAGCTACATCTCTCGGTGCG	Hsp70 upstream region Forward
HSP70 up R	gtcgtgactgggaaaaccctggcgAGCAGAGATCAGGATGGCTC	Hsp70 upstream region Reverse
HSP70 down F	tcctgtgtgaaattgttatccgctTTATCGAGCCGGGTTCTTTG	Hsp70 downstream region Forward
HSP70 down R	AGCAGACCTCTCCAAAACCT	Hsp70 downstream region Reverse
HSP70 dis F	ACCCCACTATGAACATCTGAGT	Hsp70 cassette amplification Forward
HSP70 dis R	TCCGCCATATCCACCTATCTC	Hsp70 cassette amplification Reverse
SRP1 up F	ACTGCGTCAACAAGAACTCG	SRP1 upstream region Forward
SRP1 up R	gtcgtgactgggaaaaccctggcgGGATGAGGTGACGGATGACA	SRP1 upstream region Reverse
SRP1 down F	tcctgtgtgaaattgttatccgctTTCCCCTCCGCTAAGACATG	SRP1 downstream region Forward
SRP1 down R	CCCTCAGTTGAAGGCTCGAT	SRP1 downstream region Reverse
SRP1 dis F	ATCCTGGGTCTGTTTCCGAG	SRP1 cassette amplification Forward
SRP1 dis R	CGCGAGCATCACATTCCTAC	SRP1 cassette amplification Reverse
M13F	CGCCAGGGTTTTCCCAGTCACGAC	*hyg* gene Forward
M13R	AGCGGATAACAATTTCACACAGGA	*hyg* gene Reverse