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# Immunoproteomics-based analysis of the immunocompetent serological response to *Lomentospora prolificans*

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DE), proteome, surfaceome, immunome, serum IgG.

**Abstract**

The filamentous fungus *Lomentospora prolificans* is an emerging pathogen causing severe infections mainly among the immunocompromised population. These diseases course with high mortality rates due to great virulence of the fungus, its inherent resistance to available antifungals, and absence of specific diagnostic tools. Despite being widespread in humanized environments, *L. prolificans* rarely causes infections in immunocompetent individuals likely due to their developed protective immune response. In this study, conidial and hyphal immunomes against healthy human serum IgG were analyzed, identifying immunodominant antigens and establishing their prevalence among the immunocompetent population. Thirteen protein spots from each morph were detected as reactive against at least 70% of serum samples, and identified by liquid chromatography tandem mass spectrometry (LC-MS/MS). Hence, the most seroprevalent antigens were WD40 repeat 2 protein, malate dehydrogenase, and DHN1, in conidia, and heat shock protein (Hsp) 70, Hsp90, ATP synthase  $\beta$  subunit, and glyceraldehyde-3-phosphate dehydrogenase, in hyphae. More interestingly, the presence of some of these seroprevalent antigens was determined on the cell surface, as Hsp70, enolase, or Hsp90. Thus, we have identified a diverse set of antigenic proteins, both in the entire proteome and cell surface subproteome, which may be used as targets to develop innovative therapeutic or diagnostic tools.

## Introduction

The filamentous fungus *Lomentospora prolificans* is an opportunistic pathogen closely related to the *Pseudallescheria/Scedosporium* species complex, being in fact, previously known as *Scedosporium prolificans*<sup>1</sup>. Among the members of the species complex, *L. prolificans* has been reported as one of the most virulent<sup>2</sup>, and the cause of infectious diseases which may course with a broad-spectrum of clinical manifestations, from cutaneous to disseminated infections<sup>3</sup>.

The importance of this pathogenic fungus lies with several factors. Clinical incidence of *L. prolificans* has been on the increase in recent years, with this fungus being considered an emerging pathogen<sup>4</sup>. In fact, *Scedosporium* spp./*L. prolificans* have been identified as the most common aetiological agents causing non-*Aspergillus* mould infections, in both Spain<sup>5</sup> and Australia<sup>6</sup>. Moreover, *L. prolificans* and *Scedosporium* spp. are especially relevant for cystic fibrosis patients, being present as colonizers of 6-17% of these patients<sup>7-9</sup>. Varying patient colonization patterns of these fungi may depend on geography, since, while *S. apiospermum* is distributed worldwide, *L. prolificans* clinical cases have been mainly reported in Spain, Australia, and the USA. Finally, it is worth highlighting the inherent resistance of *L. prolificans* to all the antifungals commonly used by clinicians. Currently, treatment with voriconazole alone or combined with other drugs such as terbinafine, is the most widely used strategy despite being well known that most of the strains are resistant<sup>3</sup>.

The absence of a truly effective therapy against *L. 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

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3 compounds. Until now, data from ecological studies have pointed out that distribution of *L.*  
4 *prolificans* is directly related to human activity, being widely distributed in human-impacted  
5 ecosystems, whereas it is absent in soils from natural environments<sup>10</sup>. Precisely for that  
6 reason, it is very remarkable that diseases caused by *L. prolificans* are almost exclusively  
7 developed in immunocompromised patients, despite humans being in contact with the fungus.  
8 Therefore, we can conclude that in the immunocompetent population our immune system  
9 provides successful protection against *L. prolificans*, in the same way as it does against most  
10 fungal pathogens.  
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23 The immune response against fungi comprises many mechanisms, both innate and adaptive<sup>11</sup>.  
24 Initial defences are mechanical and chemical barriers of the skin and mucosae, however, a  
25 response based on Pattern Recognition Receptors (PRR)-mediated inflammation and several  
26 cell types such as macrophages, neutrophils, or T lymphocytes may be activated.  
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32 Nevertheless, in this article attention is focused on the humoral response, as protective  
33 antibodies may also play an important role during *L. prolificans* infections.  
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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<sup>12,13</sup>. However, there is no  
evidence of protective antibodies against *L. prolificans* reported in literature at the present  
time. Interesting results have been obtained suggesting the use of specific antibodies, alone or



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3 in combination with conventional antifungal agents, may evolve into safe, broad-spectrum  
4 agents for prophylaxis in high-risk patients with impaired immune response. In addition to an  
5 inherent antifungal effect, antibody-based therapy may protect by promoting Th1 immunity,  
6 or altering fungal gene expression and intracellular trafficking. Moreover, the efficacy of  
7 certain vaccines indicates that antibody responses may provide a crucial contribution to  
8 antifungal immunity<sup>14</sup>.  
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18 Proteomics-based research offers the opportunity to identify and characterize the major  
19 antigens of microorganisms thereby providing the gateway to using them as therapeutic  
20 targets for new drugs, developing vaccines, or producing specific protective antibodies for  
21 passive immunotherapy. However, in the case of *L. prolificans*, proteomics studies have to  
22 tackle the problem of the absence of the completely annotated genome. In spite of this, gel-  
23 based proteomics have enabled the study of the proteome of some other fungi in the same  
24 status by comparison with orthologous proteins from other fungi whose genomes have been  
25 already annotated<sup>15,16</sup>. In fact, our research group recently used these techniques to identify  
26 the major antigens of *L. prolificans* recognized by immunoglobulins A from the saliva of  
27 immunocompetent people<sup>17</sup>.  
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43 In this work, we have analyzed the antigenicity of *L. prolificans* conidial and hyphal protein  
44 extracts against immunocompetent human sera using proteomics-based techniques. Thanks to  
45 which, the main antigens recognized by IgGs from sera in both morphs were identified, and  
46 their prevalence in the population studied.  
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## Materials & Methods

### Microorganism

The clinical isolate *Lomentospora prolificans* CECT 20842 was used in this study. Fungal spores and hyphae were obtained as previously described<sup>17</sup>. 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 on 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.

### 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, serum samples were coagulated and centrifuged in 5 ml glass tubes (Vacutainer; BD Biosciences, Madrid, Spain), being clean sera 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).

### Indirect immunofluorescence (IIF)

To analyze the localization of human IgG- or IgA-reactive antigens at cell level, IIF was

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3 performed. Fungal cell suspensions were obtained as previously mentioned, air-dried on  
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5 slides overnight, and fixed with acetone for 20 min. After which, slides were air-dried and  
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7 blocked with phosphate buffered saline (PBS), 5% (w/v) bovine serum albumin (BSA) at  
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9 room temperature for 30 min. Pooled human sera or saliva was then diluted 1:50 in PBS, 5%  
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11 (w/v) BSA, and slides incubated with it at 4°C overnight. Next, slides were washed three  
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13 times with PBS, 10 min each, and incubated at room temperature for 30 min with FITC-  
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15 labelled anti-human IgG or anti-human IgA antibodies (Sigma-Aldrich, St Louis, MO, USA)  
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17 diluted 1:100 in PBS, 0.025% (w/v) Evans Blue, 0.05% (v/v) Tween 20. After incubation,  
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19 slides were washed as mentioned above and, then, coverslips were placed using  
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21 Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich). A negative control was also  
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23 performed without incubation with human samples. Cells were visualized under a confocal  
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25 microscope Olympus FluoView FV500 (Tokyo, Japan).  
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### 32 **Protein extraction**

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36 After obtaining fungal cells, these were pelleted by centrifugation and re-suspended in PBS  
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38 containing 1% (v/v) 2-mercaptoethanol and 1% (v/v) ampholytes (GE Healthcare, Freiburg,  
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40 Germany) to facilitate cell disruption. Total protein extracts were obtained according to the  
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42 previously standardized workflow<sup>17</sup>. Briefly, glass beads were added and cells disrupted  
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44 using the MillMix 20 bead beater (Tehtnica, Slovenia) for 20 min at 30 Hz. Cell debris was  
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46 removed by centrifugation and supernatant was stored at -20°C until use.  
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52 To analyze cell surface-associated proteins (CSP), conidiospores and hyphae were boiled in  
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54 extraction buffer (50 mM Tris-HCl pH 8.0, 0.1 M EDTA, 2% [w/v] SDS, 10 mM DTT) for  
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56 10 min, as previously reported<sup>18</sup>. Next, suspensions were centrifuged, and the supernatant  
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3 stored at -20°C until used. To verify there was no cytoplasmic protein leakage and cell  
4 integrity was maintained, cells were pelleted after CSP extraction, washed three times with  
5 PBS, then stained with propidium iodide (1 mM) and calcofluor white (40 ng/ml) for 30 min  
6 at room temperature and protected from light. Autoclaved cell suspensions (15 min, 121°C)  
7 were used as positive control for cell breakage.  
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### 14 **Two-dimensional electrophoresis (2-DE) and image acquisition**

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20 Fungal proteins were precipitated in four volumes of acetone, 10% (w/v) trichloroacetic acid,  
21 0.07% (v/v)  $\beta$ -mercaptoethanol, at -20°C for 1 h, and pelleted by centrifugation for 15 min at  
22 14,100 g. Cleaned protein extracts were re-suspended in rehydration buffer (7 M urea, 2 M  
23 thiourea, 4% [w/v] CHAPS, 2% [v/v] ampholytes, 2% [v/v] 2-mercaptoethanol) and  
24 incubated at room temperature for 1 h for correct protein dissolution. Protein concentration  
25 was measured using the Pierce 660 nm Protein Assay Reagent (Thermo Scientific, Rockford,  
26 IL, USA).  
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38 Four hundred  $\mu$ g of protein were loaded onto 18-cm-long Immobiline DryStrip pH 3-10 (GE  
39 Healthcare) for isoelectric focusing (IEF), after adding 1% (v/v) 2-mercaptoethanol, 1% (v/v)  
40 ampholytes, and 0.002% (w/v) bromophenol blue to the samples. Strips were left to rehydrate  
41 overnight, then followed by IEF at 500 V for 2,000 Vhr, 1,000 V for 9,000 Vhr, 8,000 V for  
42 20,000 Vhr, and 8,000 V for 100,000 Vhr; 50  $\mu$ A per strip. Next, proteins were reduced and  
43 alkylated for 15 min each in equilibration buffer (6 M urea, 75 mM Tris-HCl pH 6.8, 25.5%  
44 [v/v] glycerol, 2% [w/v] SDS, 0.002% [w/v] bromophenol blue) with 1% (w/v) DTT and  
45 2.5% (w/v) iodoacetamide, respectively. The SDS-PAGE was performed in the PROTEAN II  
46 xl Cell (Bio-Rad, Hercules, CA, USA) at 20 mA per gel, under refrigerated conditions. Broad  
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3 range Precision Plus Protein™ Standards (Bio-Rad) were used as molecular weight markers.  
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5 Gels were stained with Coomassie brilliant blue (CBB) as previously described<sup>19</sup>, and then  
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7 digitalized using ImageScanner III (GE Healthcare).  
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### 10 11 12 **Immunoblot using human sera**

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16 Proteins were electrotransferred following 2-DE to Hybond-P PVDF membranes (GE  
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18 Healthcare) at 150 mA for 20 h. Correct transference was assessed by Ponceau red stain  
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20 (0.2% [w/v] Ponceau red, 1% [v/v] acetic acid). To detect antigenic proteins, immunoblot  
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22 experiments were performed using human sera, both pooled and individually. All incubations  
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24 were carried out at room temperature, except when noted. First, membranes were blocked for  
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26 2 h in TBS (50 mM Tris-HCl pH 7.5, 150 mM NaCl) containing 5% (w/v) low-fat dried milk  
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28 and 0.1% (v/v) Tween 20 (TBSM). Next, membranes were incubated with human sera  
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30 diluted 1:100 in TBSM at 4°C overnight. Afterwards, antibody excess was removed by  
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32 washing 3 times with TBS, 5 min each, and an HRP-labeled anti-human IgG diluted  
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34 1/100,000 in TBSM added as a secondary antibody. Immunogenic proteins were detected  
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36 using the enhanced chemiluminescence kit ECL Prime system (GE Healthcare) following  
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38 manufacturer's instructions in a G:BOX Chemi system (Syngene, Cambridge, United  
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40 Kingdom).  
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### 47 48 **Bioinformatic image analysis and heat map creation**

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51 Gel and Western blot images were analyzed using ImageMaster™ 2D Platinum Software  
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53 (GE Healthcare) to detect protein and antigenic spots, respectively. To do that, automatic spot  
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55 detection was used, applying the following parameters: smooth, 4; min. area, 5; and saliency,  
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3 3. Then, the results were manually reviewed and corrected, modifying them as little as  
4 possible so as to ensure reproducibility. The Western blots of the pooled sera were carried out  
5 in triplicate and analyzed to obtain a reference pattern. Then, the prevalence and relative  
6 volume of the antigens of each individual serum was calculated by comparison with the  
7 pattern of the pool.  
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16 After obtaining relative volume data, heat maps for both conidial and hyphal antigenic  
17 proteins were created using TM4 free available software<sup>20</sup>.  
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### 20 21 22 23 **Identification of seroprevalent antigens by LC-MS/MS**

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27 For protein identification, antigenic spots recognized on Western blots by over 70% of the  
28 sera tested were excised manually from fresh CBB-stained gels, and subjected to in-gel  
29 digestion with trypsin. Gel pieces were swollen in digestion buffer containing 50 mM  
30  $\text{NH}_4\text{HCO}_3$  and 12.5 ng/ $\mu\text{l}$  proteomics grade trypsin (Roche, Basel, Switzerland), and the  
31 digestion processed at 37°C overnight. The supernatant was recovered and peptides were  
32 extracted twice: first, with 25 mM  $\text{NH}_4\text{HCO}_3$  and acetonitrile (ACN), and then with 0.1%  
33 (v/v) trifluoroacetic acid and ACN. The recovered supernatants and extracted peptides were  
34 pooled, dried in a SpeedVac (Thermo Electron, Waltham, MA, USA) dissolved in 10  $\mu\text{l}$  of  
35 0.1% (v/v) formic acid (FA) and sonicated for 5 min.  
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49 LC-MS/MS spectra were obtained using a SYNAPT HDMS mass spectrometer (Waters,  
50 Milford, MA, USA) interfaced with a nanoAcquity UPLC System (Waters). An aliquot (8  $\mu\text{l}$ )  
51 of each sample was loaded onto a Symmetry 300 C18, 180  $\mu\text{m}$  x 20 mm precolumn (Waters)  
52 and washed with 0.1% (v/v) FA for 3 min at a flow rate of 5  $\mu\text{l}/\text{min}$ . The precolumn was  
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3 connected to a BEH130 C18, 75  $\mu\text{m}$  x 200 mm, 1.7  $\mu\text{m}$  (Waters), equilibrated in 3% (v/v)  
4 ACN and 0.1% (v/v) FA. Peptides were eluted with a 30 min linear gradient of 3–60% (v/v)  
5 ACN directly onto a NanoEase Emitter (Waters). Capillary voltage was set to 3,500 V and  
6 data-dependent MS/MS acquisitions performed on precursors with charge states of 2, 3, or 4  
7 over a survey m/z range of 350–1990.  
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16 Obtained spectra were processed using VEMS<sup>21</sup>, and searched for against the NCBI non-  
17 redundant (nr) database restricted to Fungi (version 20150309) using the online MASCOT  
18 server (Matrix Science Ltd., London; <http://www.matrixscience.com>). Protein identification  
19 was carried out by adopting the carbamidomethylation of cysteines as a fixed modification  
20 and, oxidation of methionines as a variable modification. Up to one missed cleavage site was  
21 allowed, and values of 50 ppm and 0.1 Da were set for peptide and fragment mass tolerances,  
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34 Due to the *L. prolificans* genome sequence being incompletely annotated as yet, antigenic  
35 protein identification was performed by comparison with orthologous proteins from other  
36 fungi whose genomes were already available in the NCBI nr database.  
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### 42 ***In silico* immunogenic protein analyses**

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47 Several online available bioinformatic tools were used in this study to characterize the  
48 antigens identified. First, we analyzed protein functionalities inferred from homology using  
49 the PEDANT web server (<http://pedant.gsf.de>)<sup>22</sup>, which predicts cellular roles and functions,  
50 and categorizes proteins according to the Functional Catalogue (FunCat)<sup>23</sup>. Protein  
51 localization was also predicted by TargetP (<http://www.cbs.dtu.dk/services/TargetP/>) which  
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3 detects the presence of signal peptides for several destinations, including secretion and  
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5 mitochondria. As this tool is based on canonical signal peptides, we applied another available  
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7 online application, SecretomeP 2.0 (<http://www.cbs.dtu.dk/services/SecretomeP/>), which  
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9 predicts protein secretion via non-conventional pathways. In both predictive methods, a value  
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11 is assigned per protein ranging from 0 to 1, with a score of  $\geq 0.5$  considered positive for a  
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13 given situation. Finally, we analyzed the possible adhesin-like properties of each antigen  
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15 identified using the Faapred web server<sup>24</sup>. A score threshold of -0.8 was established.  
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## 20 21 **Results**

### 22 23 24 25 **Recognition of conidia and hyphae given by saliva and serum samples from** 26 27 **immunocompetent individuals at cell level** 28 29

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32 Since conidia is the morph which mainly penetrates via airways and hyphae the morph used  
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34 to invade tissues, it is likely that the recognition patterns given by saliva and sera will be  
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36 different on both conidia and hyphae morphs.  
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40 Therefore, to determine whether sera and saliva of human immunocompetent population  
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42 display distinct humoral recognition of conidia and hyphae of *L. prolificans*, we performed  
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44 IIF using pooled sera samples and compared the results obtained with pooled saliva samples.  
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46 Interestingly, whereas IgAs from saliva were able to almost exclusively detect the conidial  
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48 morph, the IgG from sera recognize both conidia and hyphae (Figure 1).  
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### 52 53 54 **Humoral response of immunocompetent individuals to *L. prolificans* proteomes** 55 56 57 58 59 60



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3 Proteomes of *L. prolificans*, of both conidia and hyphae, were resolved by 2-DE and proteins  
4 electrotransferred to PVDF membranes to identify IgG-reactive antigens. Firstly, pooled sera  
5 collected from ten immunocompetent volunteers were used to analyze the overall  
6 immunomes of both morphs. Developed chemoluminescent images were acquired and, later,  
7 analyzed using the ImageMaster™ 2D Platinum Software. Estimations of isoelectric point  
8 (*pI*) and relative molecular mass (*Mr*) of each immunogenic spot were carried out. In  
9 addition, relative volume values were calculated as a measurement of the protein spot  
10 antigenic capacity.  
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23 A large number of protein spots were found to react to serum IgG from healthy donors, with  
24 some being intensely recognized. Important differences were found between the recognition  
25 given by each volunteer, making it even more important to detect the most prevalent antigens  
26 among the healthy population (Supplementary figures 1 and 2). To be precise, up to 169  
27 antigenic spots were detected scattered on the conidial immunome (Figure 2), with *Mr*  
28 ranging from 18.3 to 196.9 kDa, and *pI* from 3.8 to 9.4. However, 102 hyphal protein spots  
29 were observed to be IgG-reactive (Figure 3). Although hyphal antigens were found scattering  
30 all the *Mr* (from 17.8 to 190.4 kDa) and *pI* (from 4.1 to 7.9) spectra, the majority and most  
31 intense were present on the acidic side of the immunome.  
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#### 45 **Antigen seroprevalence of *L. prolificans* in immunocompetent population**

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49 To identify the major antigens recognized by serum IgG in immunocompetent individuals,  
50 frequency and relative volumes of each antigen were analyzed by 2-D Western Blot using the  
51 sera from ten donors separately and compared to immunomes obtained using pooled samples.  
52 Thus, seroprevalence values were obtained, and identification of the conidial and hyphal  
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3 proteins forming those antigenic spots present in at least the 70% of the human serum  
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5 samples was performed by LC-MS/MS. A total of 26 protein spots were excised from fresh  
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7 CBB-stained gels, 13 belonging to conidia and the other 13 to hyphae, then subjected to mass  
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9 spectrometric analyses. All immunoreactive spots were correctly identified corresponding to  
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11 different 12 and 9 conidial (Table 1) and hyphal (Table 2) proteins, respectively.  
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16 Regarding the identified conidial antigens, G-protein  $\beta$  subunit-like protein, malate  
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18 dehydrogenase (Mdh) (present in two antigenic spots), and DHN1 were found to be the most  
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20 prevalent immunogenic proteins, the first being recognized by 100% of the individuals tested,  
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22 and the latter by 90%. Concerning hyphal immunodominant antigens, Heat shock protein  
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24 (Hsp) 70-like protein was found to be the most seroprevalent antigen, recognized by 100% of  
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26 the healthy human sera samples. Other immunodominant hyphal antigens recognized by 90%  
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28 of the individuals were: another Hsp70, Hsp90, putative ATP synthase  $\beta$  subunit protein, and  
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30 glyceraldehyde-3-phosphate dehydrogenase (Gapdh). In both morphs, immunodominant  
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32 conidial and hyphal antigens also presented the highest relative volume values in comparison  
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34 to all the spots analyzed as pictured in the heat maps (Figure 4 A and C). Moreover, a relation  
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36 between relative volume and prevalence in the most seroprevalent antigens was observed in  
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38 both morphs (Figure 4 B and D). Interestingly, Hsp70-like proteins, Mdh, and G-protein  $\beta$   
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40 subunit-like proteins were detected in both morphs among the immunodominant antigens  
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42 identified in this study but with varying seroprevalence values. Unfortunately, two protein  
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44 spots, one per morph, turned out to be composed by a mixture of proteins (C9 and H7).  
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52 In addition, the presence of several protein species of some of the identified antigens has  
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54 been confirmed, presenting different Mr and/or *pI*. In the case of conidia, Mdh was present in  
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56 C2 and C3 spots, both being recognized by 90% of the tested sera. Nevertheless, hyphal  
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3 Hsp70 identified in both H1 and H2 spots, was found to react against 100 and 90% of the  
4 sera. In addition, spots: H4, H6 and H10 were formed by ATP synthase  $\beta$  subunit, and  
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7 recognized by 90, 80 and 70% of the tested sera, respectively.  
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### 10 11 **Study of the functionality and cellular location of the major antigens of *L. prolificans*** 12 13

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16 Proteins identified by LC-MS/MS were subjected to bioinformatic analyses to study their role  
17 and localization in fungal cells. To do so, we explored the orthologous protein profiles using  
18 several software and databases. First, we studied the functionality of each antigen by  
19 extracting data from FunCat database. Most of the proteins presented several functions in the  
20 cell, all of which were taken into account and displayed in Figure 5A. Several differences  
21 were found among the *L. prolificans* morphs. The major conidial immunogenic proteins were  
22 found to be mainly related to primary metabolism and energy production, and less  
23 importantly to protein fate, and cell rescue, defence and virulence. However, energy  
24 production and cellular transport-related antigens were the most abundant in the hyphal  
25 immunome.  
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40 In addition, prediction of the identified antigens localization was performed using the TargetP  
41 web application (Figure 5B). On the one hand, we observed that most antigens of both  
42 morphs were non-typically secreted or targeted to mitochondria. Among others, Mdh (present  
43 in C2 and C3 protein spots) and enoyl-CoA hydratase (spot C13) from conidia; and ATPase,  
44 both  $\alpha$  and  $\beta$  subunits (H4, H6, H10 and H11 spots) from hyphae, were predicted to have  
45 mitochondrial signal peptides. On the other hand, among all the proteins identified, only one  
46 conidial antigen present in C9 spot was predicted to have secretion signal peptide. However,  
47 as already mentioned, this antigenic spot was found to be formed by a mixture of three  
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3 proteins. Despite this, we analyzed all antigen sequences using the web application  
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5 SecretomeP, which not only takes into account the presence of consensus signal peptides, but  
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7 also the markers for non-conventional secretion pathways. Hence, we were able to determine  
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9 that conidial C3, C5, C9, C12 and C13; and hyphal H4, H5, H6, H7, H9, H10 and H11  
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11 antigenic spots contained proteins predicted as likely to be secreted.  
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16 Finally, we performed a bioinformatic prediction of adhesin-like proteins using the Faapred  
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18 web server. Several major antigens were found to have adhesin-like properties, representing  
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20 38.46% and 23.07% of the identified conidial and hyphal antigenic spots, respectively. These  
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22 included the conidial G-protein  $\beta$  subunit-like protein, Mdh, DHN1, and fructose-  
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24 bisphosphate aldolase (Fba)-like protein, likewise the hyphal Gapdh, Mdh, and G-protein  $\beta$   
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26 subunit-like protein.  
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### 32 **Detection of immunodominant antigens in *L. prolificans* cell surface**

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36 To determine whether antigenic proteins were present on the surface of *L. prolificans* cells,  
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38 CSP antigenicity analysis was performed. We detected IgG-reactive proteins spots with 2-D  
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40 immunoblot using pooled serum samples in both conidial and hyphal surfaceomes.  
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42 Interestingly, in both cases most of the antigenic spots were found on the acidic side of the  
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44 immunome. To focus on the most relevant proteins, we compared whole immunomes with  
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46 cell surface immunomes to find the antigenic spots with the higher percentages of prevalence  
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48 in the population studied. So, we selected four and three protein spots, in conidia and hyphae  
49  
50 respectively, which had similar *pI* and *Mr* to those in whole immunomes. Interestingly,  
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52 Hsp70 and Eno, in conidia (Figure 6A), and Hsp70, Hsp90, and ATP synthase subunit  $\beta$ , in  
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54 hyphae (Figure 6B), were detected in the surfaceome of *L. prolificans*. The other two protein  
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3 spots form conidial cells, CCS3 and CCS4, returned different protein identification results  
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5 from those expected, moreover, worse identification parameters were obtained due to small  
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7 spot size.  
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## 10 11 **Discussion**

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16 *Lomentospora prolificans* infections are dangerous, often lethal, mycoses mainly affecting  
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18 immunocompromised patients. Although clinical cases concerning this fungus are not very  
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20 common worldwide, the relevance of this pathogen and the related genus *Scedosporium* in  
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22 countries like Spain or Australia is increasing. Furthermore, they are the cause of over 30%  
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24 non-*Aspergillus* mould infections<sup>5,6</sup>. In addition, the absence of fast diagnostic tools and  
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26 effective treatments to fight these infections results in high mortality rates. As a result, in  
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28 recent years research has focused on the characterization of new biomarkers that may be used  
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30 as therapeutic or diagnostic targets<sup>25</sup>.  
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36 Thus, proteomics-based techniques have been widely used to study host-pathogen  
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38 interaction-related proteins, such as antigens or adhesins<sup>26-29</sup>. Using these techniques,  
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40 combined with immunoblot, our research group recently described a wide panel of novel *L.*  
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42 *prolificans* antigens recognized by human salivary IgA<sup>17</sup>. To understand the humoral  
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44 response to this fungus in-depth, we performed here an analysis of serum IgG-mediated  
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46 recognition of *L. prolificans* proteomes in immunocompetent individuals, who seem to be  
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48 significantly less susceptible to these infections. The use of sera from healthy young people is  
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50 of special relevance because, even though they are probably in contact with the fungus daily,  
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52 they do not suffer from *L. prolificans* infection. This is likely due to the immune response  
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54 they have developed since, although innate immunity is of major relevance during fungal  
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3 infections, acquired immunity also play important roles in clearance of these pathogens<sup>12</sup>.  
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5 Therefore, the results obtained may give us significant information about the antigens  
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7 targeted. The most prevalent of these molecules could be studied as therapeutic targets or to  
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9 develop protective vaccines, and antibodies against them may be useful in passive  
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11 immunotherapy.  
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16 Firstly, it was observed that immunocompetent human sera IgG were reactive to both conidia  
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18 and hyphae using IIF, in contrast to the results obtained when analyzing IgA-mediated  
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20 recognition in saliva samples, which mainly focused on conidia. Hence, it was determined the  
21  
22 differential humoral recognition that distinct immunoglobulin isotypes display against *L.*  
23  
24 *prolificans*. This fact is consistent with the biological scenario where conidia represent the  
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26 morph used by the fungus for dispersion and enter hosts primarily through airways, while  
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28 hyphae are commonly considered the infective morph, their formation being a virulent  
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30 mechanism shared with many other pathogenic fungi.  
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36 Interestingly, a large number of protein spots were found to be reactive with healthy human  
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38 serum IgG, many of which were intensely recognized. In fact, up to 169 and 102 antigenic  
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40 spots were detected scattered over the entire conidial and hyphal immunomes, respectively.  
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42 Thus, our data suggest there is a strong complex humoral response against *L. prolificans*  
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44 among the immunocompetent individuals analyzed. This fact contrasts with studies  
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46 concerning other pathogenic fungi against which very little<sup>30</sup> or, even, no detectable antibody  
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48 recognition in non-infected patient sera has been found<sup>15,31</sup>. However, this may be the result  
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50 of different experimental procedures. Nevertheless, the results presented in our study are  
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52 consistent with previously reported data concerning IgA-mediated humoral response in the  
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54 mucosae of the airways<sup>17</sup>, and other studies regarding *L. prolificans* and *Scedosporium* spp.  
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3 environmental distribution, where the trend of these fungi to occupy human-impacted  
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5 ecosystems has been highlighted<sup>10,32</sup>. In this sense, the unique biogeography of *L. prolificans*  
6  
7 is also remarkable with regard to its relation with human hosts, since all serum donors were  
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9 resident in the Basque Country, in Northern Spain, where this fungus is becoming an  
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11 increasingly relevant pathogen<sup>33</sup>. This fact contrasts with the situation in other European  
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13 countries, like France<sup>32</sup> or Austria<sup>34</sup>, where little or no trace of the fungus has been found. It  
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15 is also worth highlighting the cross-reactivity of some or all of the antigens identified may be  
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17 occurring with proteins from other fungi or microorganisms. Therefore, more research may  
18  
19 be necessary to identify common and specific epitopes.  
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25 However, among all the detected antigens, a total of 26 seroprevalent immunogenic spots, 13  
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27 from conidia (Table 1) and 13 from hyphae (Table 2), were identified by LC-MS/MS.  
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29 Despite the *L. prolificans* genome being sequenced albeit incompletely annotated and not  
30  
31 publicly available<sup>35</sup>, all antigens were successfully identified by comparison to other fungi as  
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33 orthologous proteins, the majority of which belong to the closely related fungus *S.*  
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35 *apiospermum*, whose genome has recently been released<sup>36</sup>.  
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40 With regard to conidial antigens, G-protein  $\beta$  subunit-like protein, Mdh (present in two  
41  
42 different protein spots), and DHN1 were the most seroprevalent antigens since they were  
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44 detected by at least 90% of the human sera used, with the first protein spot recognized by  
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46 100%. Among the other proteins found to be antigenic were Fba-like protein, Hsp70-like  
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48 protein, and Eno, which have been widely described as antigens in many microorganisms.  
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50 The fact that in some of the conidial identifications, to be specific, DHN1, Fructose-  
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52 bisphosphate aldolase, Polyketide synthase-like protein, and Enoyl-CoA hydratase, only one  
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54 single peptide matched and the MASCOT score, despite being statistically significant, was  
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3 relatively low must be recognized. In this sense, these identifications can be considered as not  
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5 totally definitive.  
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10 Regarding hyphal antigens, Hsp70 was detected in H1 and H2 spots, and recognized by 100  
11 and 90% of the tested sera. In addition, Hsp90, putative ATP synthase  $\beta$  subunit, and Gapdh  
12 were also targeted by 90% of the human sera. Interestingly, ATP synthase  $\beta$  subunit was  
13 detected in other two antigenic spots (H6 and H10), with variable humoral recognition. The  
14 presence of several protein species was evidenced in this study and, surprisingly, some of  
15 these proteins species exhibited different seroprevalence. Little variations among protein  
16 species, which may affect epitopes, might explain the fact that these antigens present distinct  
17 relevance for humoral response. Additionally, both conidial and hyphal immunodominant  
18 antigens presented variable relative volume values, as measured by image analysis and  
19 depicted in Figure 4. Remarkably, a comparative analysis revealed that the most  
20 seroprevalent antigens (C1 to C4, and H1 to H4) also showed the highest relative volume  
21 values. This may indicate that, in addition to present high seroprevalence, these antigens are  
22 recognized by large amounts of serum IgG.  
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40 Interestingly, some of the immunogenic proteins detected in this study, namely ATP synthase  
41  $\alpha$  and  $\beta$  subunits, Fba, Mdh, and Hsp70, were previously identified by our research group as  
42 reactive to human salivary IgA<sup>17</sup>. This finding makes them especially relevant for the  
43 development of prophylactic therapies such as vaccines, since with the use of these antigens,  
44 patients may develop antifungal antibodies to protect them from *L. prolificans* at different  
45 infection levels.  
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3 Metabolism-related proteins represent one of the most important functional group in our  
4 study, gathering immunodominant antigens like: Mdh, Gapdh, Fba-like protein, or Eno,  
5 among others. Curiously, these proteins have been widely described as antigenic in many  
6 other microorganisms. In addition, G-proteins are enzymes with GTPase activity involved in  
7 many cell signalling processes since they are usually connected to a wide variety of receptors  
8 (called G protein-coupled receptors). In pathogenic fungi, these proteins have been related to  
9 important processes such as: cell growth, asexual and sexual development, or virulence<sup>37,38</sup>.  
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11 Despite detecting G-protein  $\beta$  subunit-like proteins as antigens in both morphs (C1 and H13  
12 spots), the immunogenicity of these proteins has been poorly described, only being detected  
13 in *A. fumigatus* immunoproteome<sup>39</sup>, in addition to being linked to its cell wall<sup>40</sup>. Finally,  
14 several Hsp have been identified, in both conidial and hyphal immunomes. These proteins  
15 were first described during thermal shock experiments, where they were upregulated as their  
16 main function is to maintain other protein structures as chaperones. However, they are known  
17 to play more roles in fungal cells, being related for example to antifungal resistance or fungal  
18 biofilm dispersion<sup>41,42</sup>.  
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38 Cellular localization of immunogenic proteins is crucial to develop novel diagnostic or  
39 therapeutic strategies<sup>43-45</sup>. As already mentioned, most of the antigens identified participate in  
40 pivotal processes in the fungal cell. The immunodominant nature of these normally internal  
41 proteins may be given by their release from fungal cells via secretion or cell lysis, or their  
42 presence in the cell wall. In this sense, cell wall-linked proteins are very interesting since they  
43 represent available targets for diagnosis, or new therapeutic treatments, such as protective  
44 antifungal antibodies. To analyze this, we used two experimental approaches: firstly, *in silico*  
45 analyses of the identified antigens, and, secondly, by analyzing the cell surface subproteome.  
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3 Bioinformatic analyses predicted that conidial antigens C3, C5, C9, C12, and C13; and  
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5 hyphal antigens H4, H5, H6, H7, H9, H10, and H11 may be secreted or anchored to the  
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7 fungal cell wall. However, this was not an unprecedented finding since many of these  
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9 proteins were found to be secreted and/or attached to the fungal cell wall in other important  
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11 pathogenic fungi<sup>44,46,47</sup>. Additionally, many of the proteins, such as Gapdh or Mdh (and  
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13 others identified in the immunomes, but with no positive results when using the predictive  
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15 bioinformatic tools), have been widely related to unusual functions, such as: invasion of host  
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17 tissues, biofilm formation, and adaptation to stress, known as moonlighting proteins<sup>48</sup>.  
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19 Moreover, antigens C1, C2, C3, C4, C6, H5, H7, and H13 were predicted to have adhesin-  
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21 like properties, which might indicate these proteins are important for *L. prolificans* virulence.  
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27 To detect empirically human serum IgG-reactive proteins in the cell wall of *L. prolificans*, we  
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29 performed experiments to analyze cell surface subproteomes. When using this strategy, the  
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31 presence of several fundamental proteins in *L. prolificans* cell wall, such as Eno, Gapdh,  
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33 Hsp70, or Hsp90 were determined for the first time. Therefore, more research should be  
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35 focused on these proteins since they may well represent promising novel targets for therapies  
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37 or detection methods. In fact, Eno or Hsp90 are well-described useful antigens as vaccines<sup>45</sup>  
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39 or as part of detection methods<sup>30,49</sup> applied to *C. albicans* invasive infections. More  
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41 interestingly, recent studies targeting the Hsp90-calcineurin axis have proven the efficacy of  
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43 specific inhibitors against *L. prolificans*<sup>50</sup>, and azole- and echinocandin-resistant *A. fumigatus*  
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45 strains<sup>51</sup>. In addition, profiling the serological response to *L. prolificans* proteomes in patients  
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47 suffering from these fungal infections is a critical field of research for the future, as these  
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49 data, combined with the study presented here, might lead to improving the knowledge on  
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51 these proteins likewise determining which one may represent valuable molecular targets. It is  
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53 worth highlighting the fact that many of the proteins identified in this study, among them  
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3 Eno, Gapdh, Hsp70, and Hsp90, found on cell walls, have been also described as antigens in  
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5 other fungi<sup>52,53</sup>. Therefore, this suggests that the cross-reactivity between species may play a  
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7 significant role in the detection of fungi, whose clarification could be the aim of future  
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9 research.  
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14 In conclusion, we have analyzed the immunomic profile of *L. prolificans* conidia and hyphae  
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16 describing a panel of novel immunodominant antigenic proteins recognized by serum IgG of  
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18 immunocompetent individuals using a proteomics-based approach. Moreover, the presence of  
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20 some relevant antigens such as Eno, Hsp70, or Hsp90 on the surface of fungal cell walls has  
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22 been described. In this sense, due to the limited number of antifungal compounds and  
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24 inherent multidrug resistance of *L. prolificans*, these antigens could be an interesting subject  
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26 for research, in order to develop novel diagnostic tools or therapies, such as compounds to  
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28 inhibit their pathways, specific antifungal monoclonal antibodies, or protective vaccines.  
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## Figure Legends

**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  $\mu\text{m}$ .

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

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

**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).

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5 **Figure 5.** Classification of serum IgG-reactive immunodominant *Lomentospora prolificans*  
6 antigens by function and cellular localization. Functional Catalogue (FunCat) was searched to  
7 determine the functions carried out by the identified antigens in fungal cells, both conidia and  
8 hyphae (A). Similarly, bioinformatic tools were applied to obtain information about the  
9 cellular localization and adhesin-like properties of these proteins (B).  
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18 **Figure 6.** Representative 2D proteomes and immunoblots of *Lomentospora prolificans* cell  
19 surface proteins (CSP). Conidial (A) and hyphal (C) CSP extracts were obtained by  
20 chemically shaving entire cells and separated by 2D SDS-PAGE. Serum IgG-reactive  
21 proteins of conidia (B) and hyphae (D) were detected using Western blot and interesting  
22 spots were identified by LC-MS/MS (refer to Table 3).  
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FIGURE 1

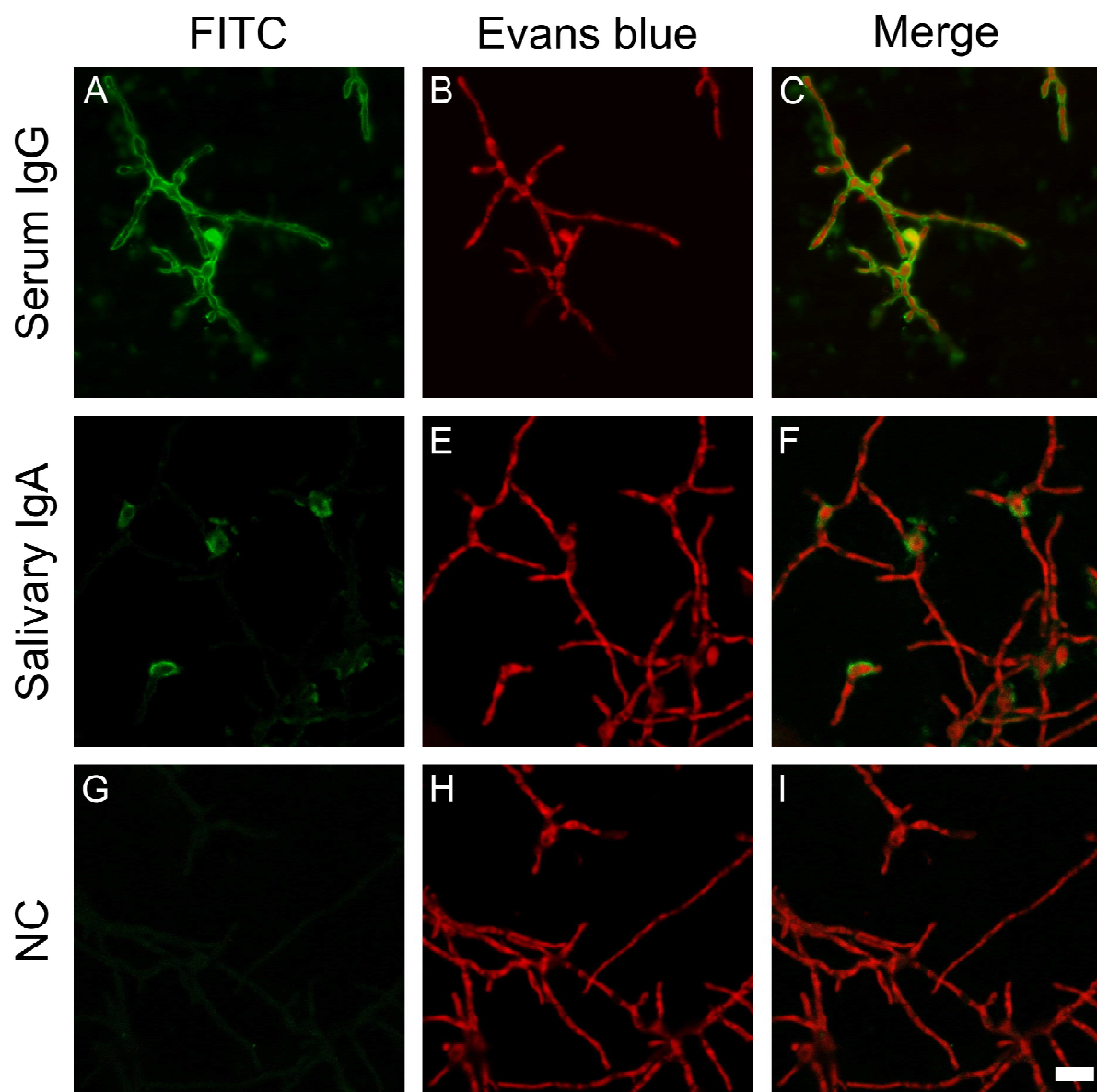


FIGURE 2

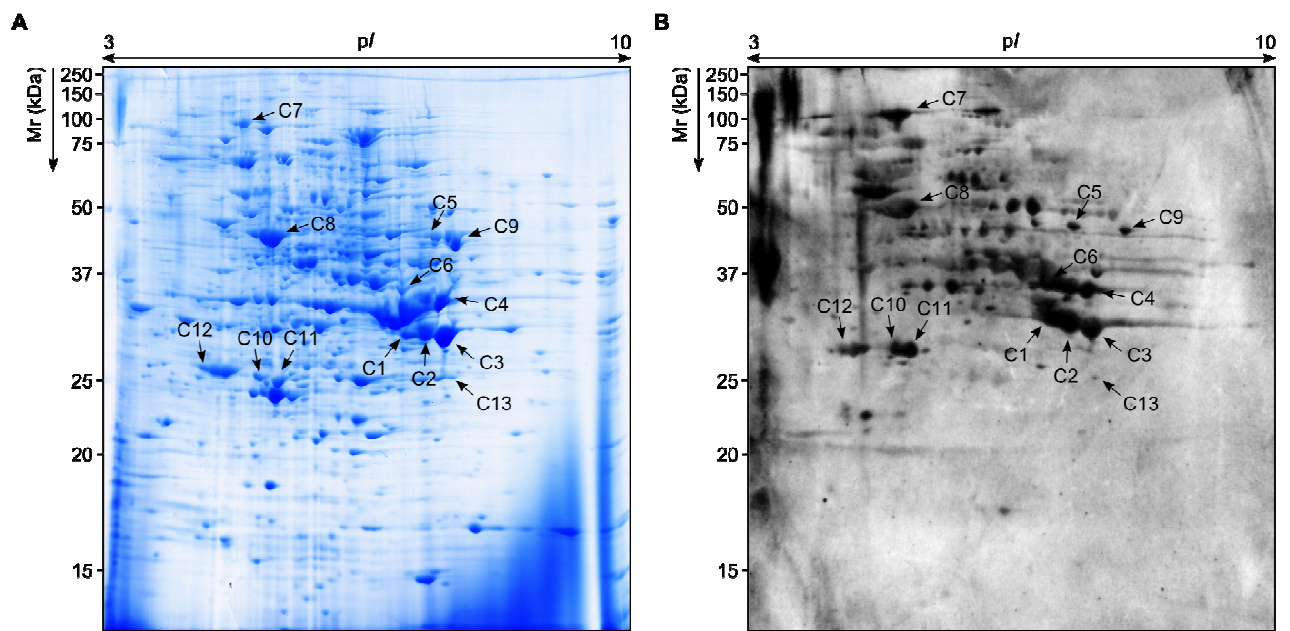


FIGURE 3

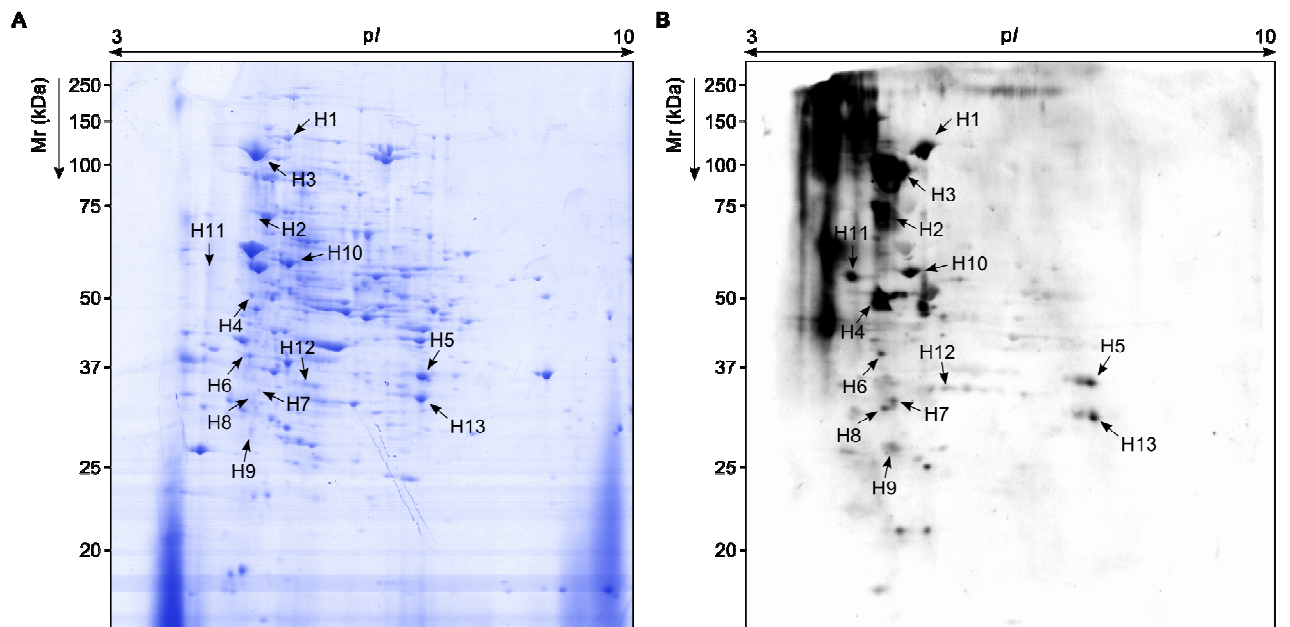




FIGURE 4

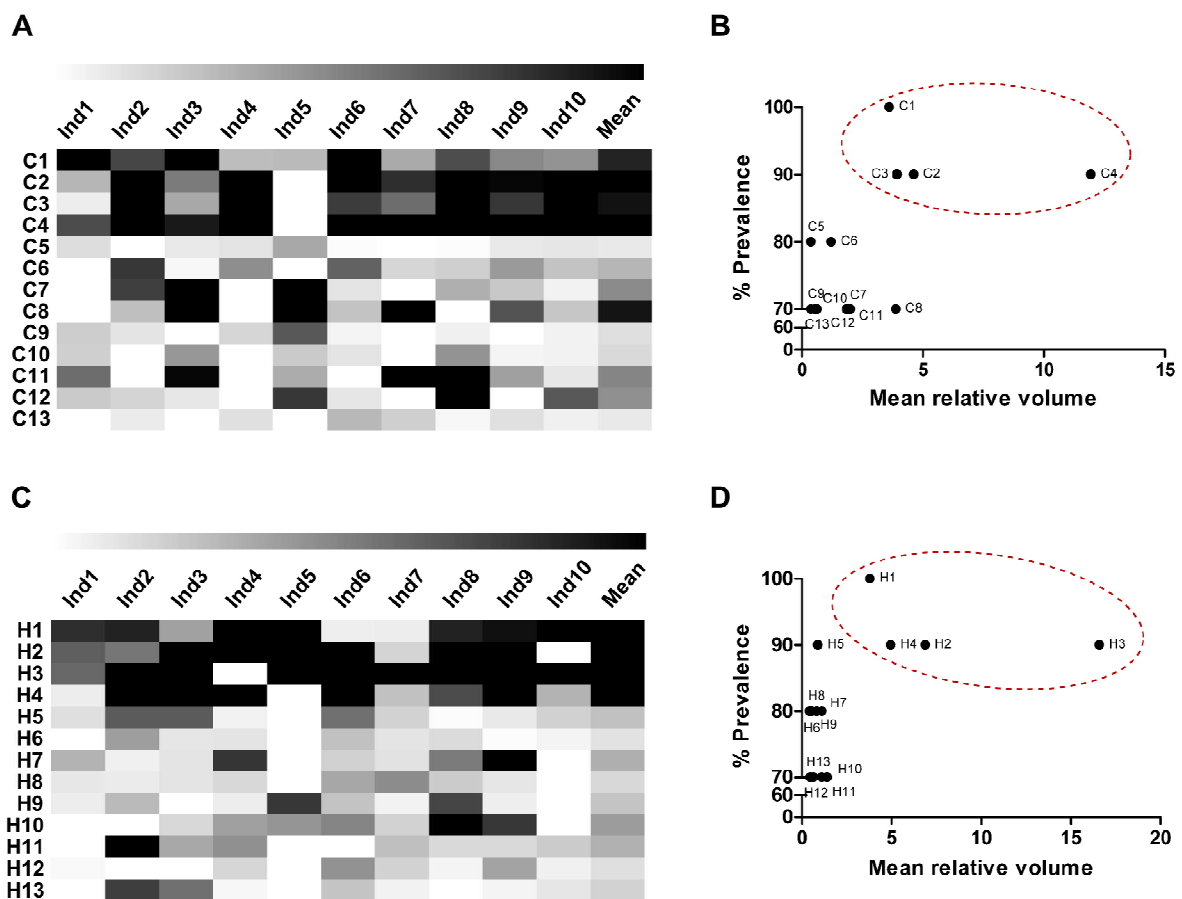


FIGURE 5

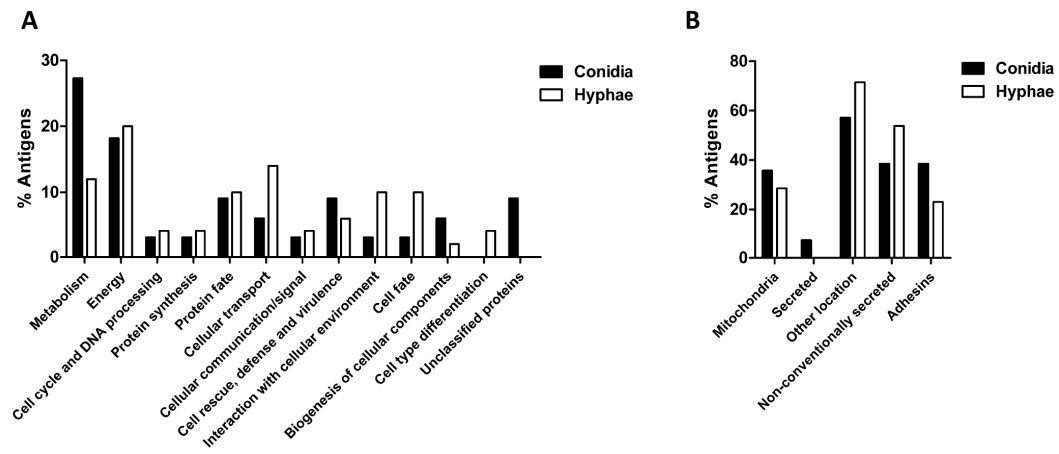


FIGURE 6

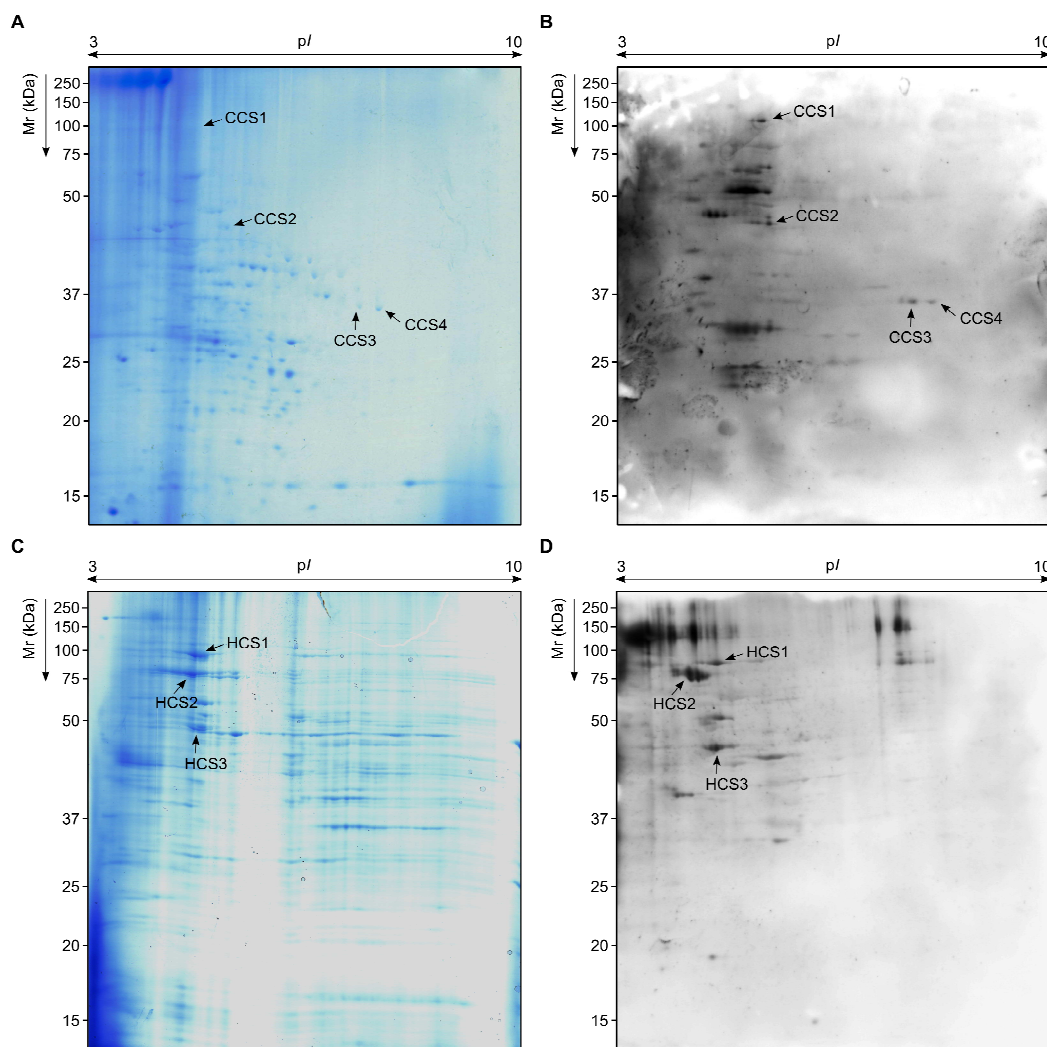


TABLE 1

**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 (%)	MASC OT score	Theor. pI/Mr (kDa)	Exper. pI/Mr (kDa)
C1	100	<b>gi 629729816</b>	<b>WD40 repeat 2 (G-protein <math>\beta</math> subunit-like protein)</b>	<i>Metarhizium robertsii</i> ARSEF 23	14	27	615	6.75/35.42	7.01/33.74
C2	90	<b>gi 666869544</b>	<b>Malate dehydrogenase, mitochondrial<sup>#</sup></b>	<i>Scedosporium apiospermum</i> <i>Chaetomium thermophilum</i> var. <i>thermophilum</i> DSM 1495	12	29	510	7.67/35.08	7.28/32.75
C3	90	<b>gi 576039574</b>	<b>Malate dehydrogenase-like protein<sup>#</sup></b>	<i>thermophilum</i> DSM 1495	5	18	320	8.57/35.60	7.54/32.33
C4	90	gi 83638421	DHN1	<i>Tuber borchii</i>	1	2	63	6.64/3476	7.48/37.01
C5	80	gi 666862968	Cysteine transaminase	<i>Scedosporium apiospermum</i>	5	11	324	6.22/45.78	7.27/48.75
C6	80	gi 666864016	Fructose-bisphosphate aldolase-like protein (hypothetical protein SAPIO_CDS8665) <sup>#</sup>	<i>Scedosporium apiospermum</i>	1	4	110	5.52/39.68	6.95/37.95
C7	70	<b>gi 666864352</b>	<b>Heat shock protein70-like protein (hypothetical protein SAPIO_CDS6996)<sup>#</sup></b>	<i>Scedosporium apiospermum</i>	10	10	471	5.10/79.45	5.06/116.75
C8	70	gi 666868085	Enolase	<i>Scedosporium apiospermum</i>	42	28	572	5.19/47.50	5.16/52.17
C9	70	gi 666863970	Poly(A)+ RNA export protein	<i>Scedosporium apiospermum</i>	3	8	186	7.12/39.94	7.92/48.13
		gi 666865792	Putative fumarate reductase protein	<i>Scedosporium apiospermum</i>	3	5	158	6.17/66.85	
		gi 380482569	Acetyl-CoA acetyltransferase	<i>Colletotrichum higginsianum</i>	2	5	126	6.35/41.77	
C10	70	gi 530461331	ABC-type transporter-like protein (hypothetical protein CGLO_16789)	<i>Colletotrichum gloeosporioides</i> 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)	<i>Fusarium verticillioides</i> 7600	1	4	52	5.26/25.38	5.32/29.44
C12	70	gi 666863172	Had superfamily-like protein (hypothetical protein SAPIO_CDS8905)	<i>Scedosporium apiospermum</i>	3	8	106	4.87/26.50	4.49/29.29
C13	70	gi 302417738	Enoyl-CoA hydratase	<i>Verticillium alfalfae</i> 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.

<sup>#</sup>Antigens that have been also described to be detected by human IgA<sup>17</sup>.

TABLE 2

**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 (%)	MASC OT score	Theor. pI/Mr (kDa)	Exper. pI/Mr (kDa)
H1	100	<b>gi 666864352</b>	<b>Heat shock protein 70-like protein (hypothetical protein SAPIO_CDS6996)<sup>#</sup></b>	<i>Scedosporium apiospermum</i>	15	14	634	5.10/79.45	5.40/121.61
H2	90	<b>gi 666870829</b>	<b>Heat shock 70 kDa protein<sup>#</sup></b>	<i>Scedosporium apiospermum</i>	19	31	887	5.10/71.25	4.76/70.52
H3	90	gi 666870612	Heat shock protein 90-like protein	<i>Scedosporium apiospermum</i>	25	28	1157	4.92/80.16	4.89/97.72
H4	90	gi 631232067	Putative ATP synthase subunit $\beta$ protein <sup>#</sup>	<i>Togninia minima</i> UCRPA7	22	42	1084	5.36/55.76	4.76/46.59
H5	90	gi 630042184	Glyceraldehyde-3-phosphate dehydrogenase	<i>Pestalotiopsis fici</i> W106-1	23	21	612	7.01/36.30	7.93/33.41
H6	80	gi 116204743	ATP synthase $\beta$ chain, mitochondrial precursor <sup>#</sup>	<i>Chaetomium globosum</i> CBS 148.51	7	19	328	5.10/55.68	4.75/33.35
H7	80	<b>gi 685397755</b>	<b>Malate dehydrogenase<sup>#</sup></b>	<i>Gaeumannomyces graminis</i> var. <b><i>tritici</i> R3-111a-1</b>	7	26	392	5.87/34.67	4.93/31.25
		gi 477529454	Pyridoxine biosynthesis protein	<i>Colletotrichum orbiculare</i> MAFF 240422	12	23	367	5.74/32.77	
H8	80	gi 666868259	14-3-3-like protein (hypothetical protein SAPIO_CDS3671)	<i>Scedosporium apiospermum</i>	17	40	617	4.92/30.90	4.80/30.29
H9	80	gi 666863507	40S ribosomal S1-like protein (hypothetical protein SAPIO_CDS8739)	<i>Scedosporium apiospermum</i>	5	14	163	10.10/29.46	4.93/26.01
H10	70	gi 34447120	Mitochondrial ATPase $\beta$ -subunit <sup>#</sup>	<i>Zygosaccharomyces rouxii</i>	6	15	353	5.06/54.08	5.21/52.63
H11	70	gi 666868184	ATP synthase subunit $\alpha$ -like protein (hypothetical protein SAPIO_CDS3582) <sup>#</sup>	<i>Scedosporium apiospermum</i>	11	23	636	9.18/59.65	4.29/51.56
H12	70	gi 666864705	Transaldolase-like protein (hypothetical protein SAPIO_CDS7435)	<i>Scedosporium apiospermum</i>	19	29	611	5.78/35.14	5.72/32.65
H13	70	<b>gi 666863913</b>	<b>G-protein <math>\beta</math> subunit-like protein (hypothetical protein SAPIO_CDS8541)</b>	<i>Scedosporium apiospermum</i>	36	46	808	6.55/35.37	7.96/29.29

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

<sup>#</sup>Antigens that have been also described to be detected by human IgA<sup>17</sup>.

TABLE 3

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

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

<sup>#</sup>Spots present in the whole immunome with similar pI and Mr.

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**Author Contributions**

AP, IB, and AA performed the experiments. ARG, AR, and FLH conceived the experiments and supervised the work. All authors have actively contributed in writing the manuscript and gave approval to the final version of it.

**Notes**

The authors declare no competing financial interest.

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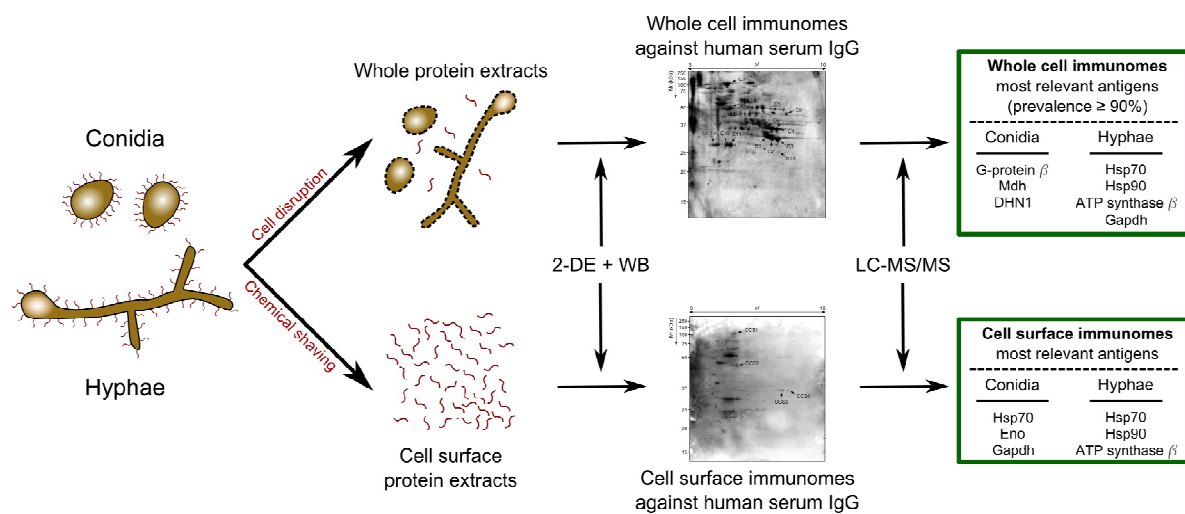
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## Abstract graphic



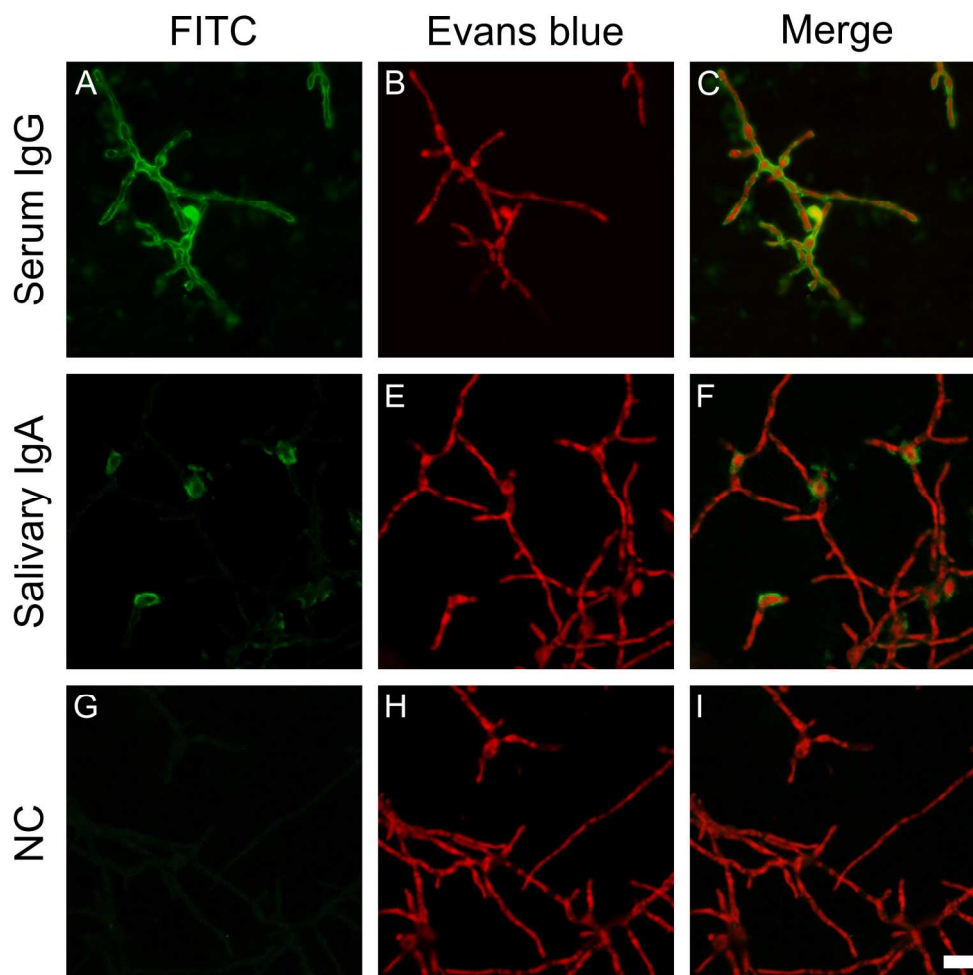


Figure 1. Indirect immunofluorescence staining to detect IgG- and IgA-reactive *Lomentospora prolificans* cells. Fungal cells were incubated with human serum (A-C), saliva (D-F), or without any sample (Negative Control, NC, G-I). Antigens were detected using FITC-labelled secondary antibodies (first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

171x168mm (300 x 300 DPI)

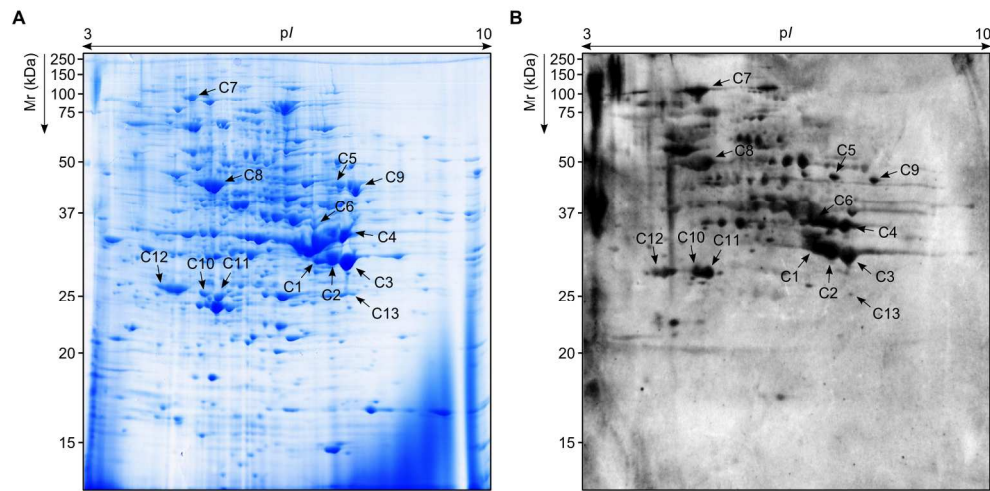


Figure 2. Representative 2-DE proteome and immunoblot of conidia from *Lomentospora prolificans*. Conidial total protein extracts resolved by 2D SDS-PAGE and CBB-stained (A), or electrotransferred to PVDF membranes to detect serum IgG-reactive proteins using Western blot (B). Numbers shown correspond to the most prevalent antigens identified by LC-MS/MS (refer to Table 1).  
182x90mm (300 x 300 DPI)



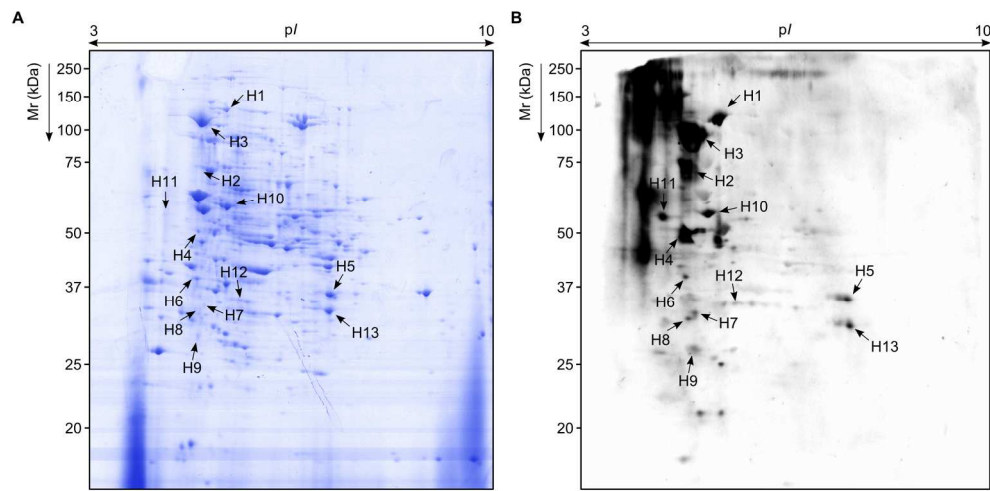


Figure 3. Representative 2-DE proteome and immunoblot of hyphae from *Lomentospora prolificans*. Hyphal total protein extracts resolved by 2D SDS-PAGE and CBB-stained (A), or electrotransferred to PVDF membranes to detect serum IgG-reactive proteins using Western blot (B). Numbers shown correspond to the most prevalent antigens identified by LC-MS/MS (refer to Table 2).  
182x90mm (300 x 300 DPI)

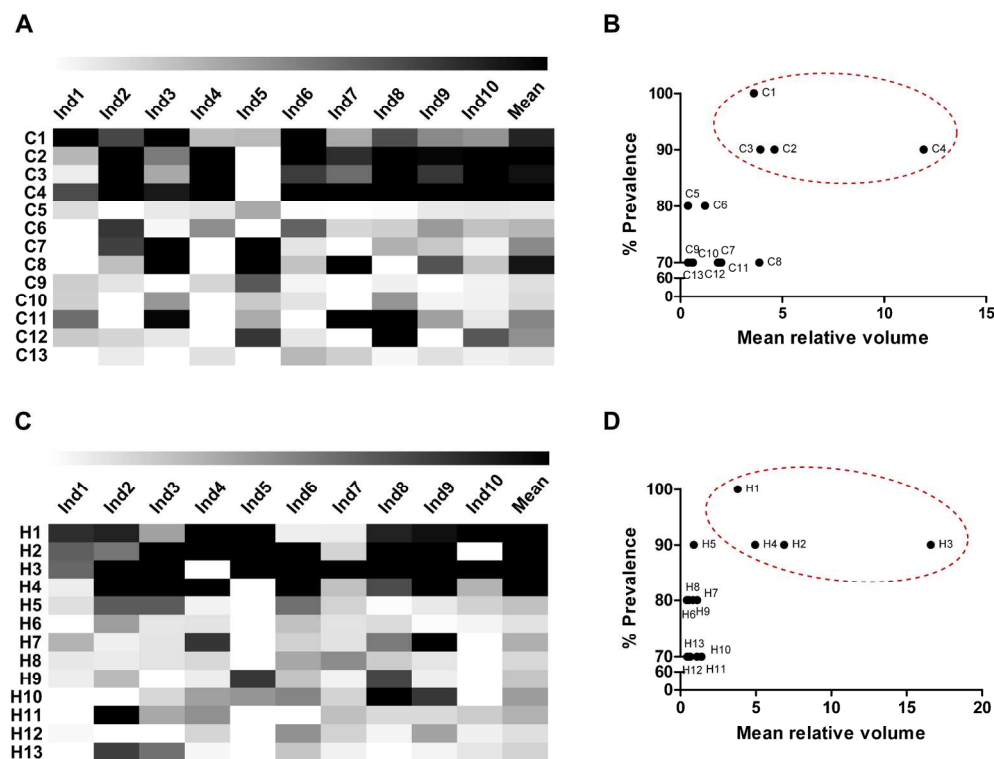


Figure 4. Prevalence and relative volume patterns of immunodominant *Lomentospora prolificans* antigens. Immunomic profiles given by recognition of individual serum samples from the ten immunocompetent donors were analyzed and relative volume values for the most prevalent antigens collected. Heat maps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

167x127mm (300 x 300 DPI)

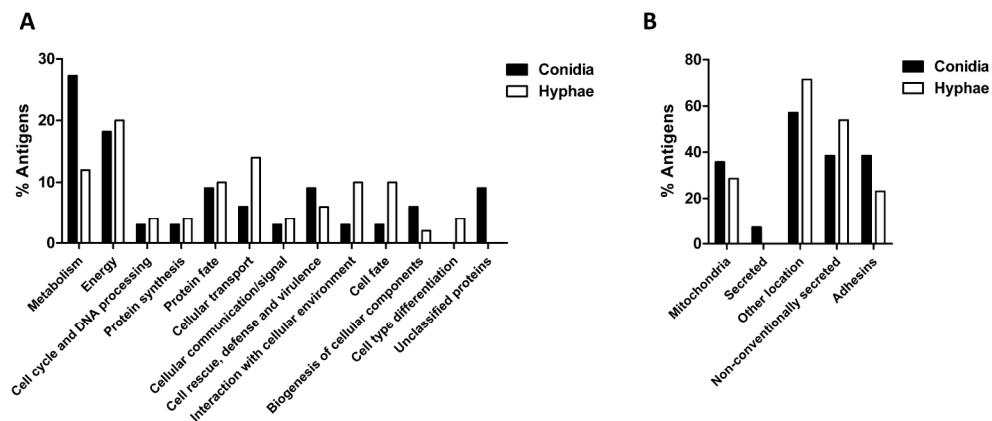


Figure 5. Classification of serum IgG-reactive immunodominant *Lomentospora prolificans* antigens by function and cellular localization. Functional Catalogue (FunCat) was searched to determine the functions carried out by the identified antigens in fungal cells, both conidia and hyphae (A). Similarly,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

177x74mm (300 x 300 DPI)

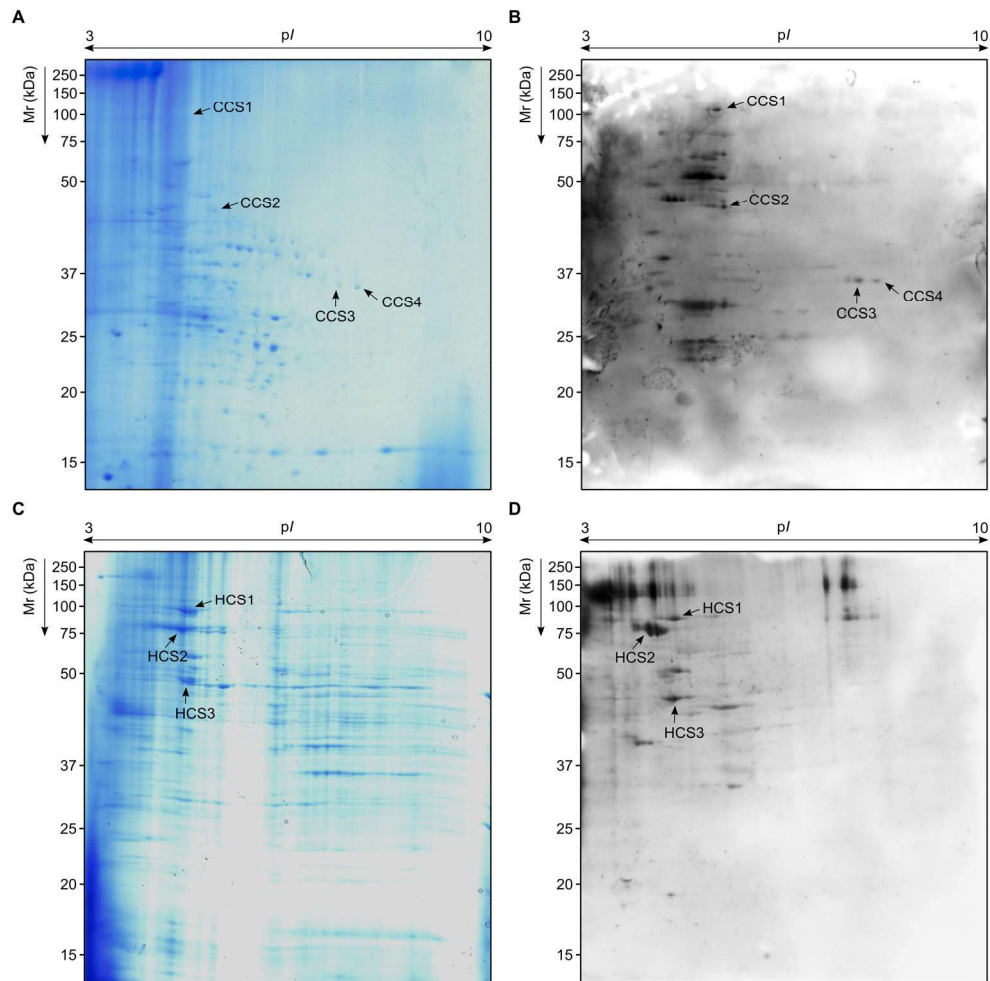


Figure 6. Representative 2D proteomes and immunoblots of *Lomentospora prolificans* cell surface proteins (CSP). Conidial (A) and hyphal (C) CSP extracts were obtained by chemically shaving entire cells and separated by 2D SDS-PAGE. Serum IgG-reactive proteins of conidia (B) and hyphae (D) were detected using Western blot and interesting spots were identified by LC-MS/MS (refer to Table 3).  
182x180mm (300 x 300 DPI)