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Original article

Identification and characterization of *Paracoccidioides lutzii* proteins interacting with macrophages

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ABSTRACT

Paracoccidioidomycosis (PCM), caused by thermodimorphic fungi of the *Paracoccidioides* genus, is a systemic disorder that involves the lungs and other organs. The adherence of pathogenic microorganisms to host tissues is an essential event in the onset of colonization and spread. The host–pathogen interaction is a complex interplay between the defense mechanisms of the host and the efforts of pathogenic microorganisms to colonize it. Therefore, the identification of fungi proteins interacting with host proteins is an important step understanding the survival strategies of the fungus within the host. In this paper, we used affinity chromatography based on surface proteomics (ACSP) to investigate the interactions of pathogen proteins with host surface molecules. *Paracoccidioides lutzii* extracts enriched of surface proteins were captured by chromatographic resin, which was immobilized with macrophage cell surface proteins, and identified by mass spectrometry. A total of 215 proteins of *P. lutzii* were identified interacting with macrophage proteins. *In silico* analysis classified those proteins according to the presence of sites for *N*- and *O*-glycosylation and secretion by classical and non-classical pathways. Serine proteinase (SP) and fructose-1,6-bisphosphate aldolase (FBA) were identified in our proteomics analysis. Immunolocalization assay and flow cytometry both showed an increase in the expression of these two proteins during host–pathogen interaction.

Paracoccidioides spp. is a complex of thermodimorphic fungi, composed of five species in the genus: *Paracoccidioides brasiliensis*, *P. lutzii*, *Paracoccidioides americana*, *Paracoccidioides restrepiensis* and *Paracoccidioides venezuelensis* [1,2]. These are the etiological agents of paracoccidioidomycosis (PCM), an endemic mycosis in Latin America. PCM is initiated by the inhalation of airborne mycelia fragments/conidia of members of the *Paracoccidioides* genus, which are present in soil, and reach pulmonary alveoli when transiting to the yeast form [3].

The adherence of pathogenic microorganisms to the host is an essential step in the beginning of colonization and dissemination from the entry site to other tissues [4]. This host–pathogen interaction is a mutual action between the defense mechanisms of the host and of the pathogenic microorganism's effort to circumvent these defenses [5]. The adhesion process of *Paracoccidioides* spp. to host tissues is a critical step in the development of infection, being mediated by adhesins of the fungal cell surface, which often bind to components of the extracellular matrix (ECM) [6].

Several studies have described adhesion molecules in the *Paracoccidioides* genus. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), located on the surface of many pathogens, is responsible for facilitating colonization and invasion of host tissues by direct interaction with host proteins [7]. In *P. lutzii*, GAPDH is located in the cell wall. It is able to bind to laminin, fibronectin and type I collagen, playing a role in the establishment of the disease [8]. Enolase, localized in *P. lutzii* cell walls, binds to plasminogen, activating it to plasmin, and thus contributing to the virulence of *P. lutzii*, as previously described [9]. In addition, the recombinant protein enolase is able to bind to the surface of macrophages,

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enhancing the protein's role in interaction with host cells and in the promotion of adhesion [9,10]. A 30 kDa adhesin of *P. brasiliensis*, named 14-3-3, was capable of binding to laminin, and the adhesion of yeast cells was inhibited by the pre-treatment of epithelial cells with the recombinant protein [11]. This protein had increased levels in the fungus cell wall during interaction with host cells, which suggests it may be involved in host–parasite interactions [12]. Another protein involved in host–pathogen interaction is elongation factor-Tu (EF-Tu), which is present at the *P. brasiliensis* surface and binds to fibronectin and plasminogen. Furthermore, the protein participates in fungal interaction (attachment/invasion) with pneumocyte cells [13].

During the process of host invasion, lung-resident macrophages are the first lines of defense faced by *Paracoccidioides* spp. *In vitro* experiments showed that yeast cells are phagocytosed by macrophages where they can replicate and survive [14]. Even being phagocytosed, *P. brasiliensis* conidia can bypass the barriers imposed by macrophages, thus allowing their survival and, consequently, replication within these cells [14]. Fungal dissemination is limited by activated macrophages during the early stages of infection due to the production of high levels of TNF-α [15] and nitric oxide (NO) [16] by these cells.

Paracoccidioides spp. interaction with host cells is a crucial step in the evolution of the infection process. The large-scale identification by means of proteomics of the surface-interacting proteins of fungi is a new field with few publications. Proteomic studies were employed to identify plasminogen-binding proteins in members of the *Paracoccidioides* complex [17]. In addition to the previously described enolase [9], we characterized fructose 1,6-biphosphate aldolase (FBA), which binds to plasminogen and converts it to plasmin, potentially increasing the fibrinolytic capacity of the fungus. In that study, the interaction between macrophages and *P. lutzii* was reduced by the addition of the recombinant FBA or polyclonal antibodies directed to the recombinant molecule, decreasing the fungus adhesion and invasion processes [17]. During incubation with macrophages, in an *ex vivo* study model, superoxide dismutase (SOD; PADG_07418), thioredoxins (THX; PADG_02764 and PADG_03161) and cytochrome c peroxidase (CCP, PADG_03163), involved in the oxidative stress response, were induced in *P. brasiliensis* yeast cells [18]. Studies employing proteomic analysis of yeast cells recovered from bronchoalveolar lavage of infected mice revealed a serine proteinase produced and secreted by *P. brasiliensis* during infection of mouse lungs. Polyclonal antibodies against this protein reduced the fungal burden in infected animals, suggesting this protein may act as a virulence factor [19].

As stated above, proteomic studies leading to the identification of potential molecules interacting with the host are still scarce in fungi, and especially in the genus *Paracoccidioides*. The current study allowed a large-scale identification of *P. lutzii* proteins that interact with macrophage surface molecules, as performed by proteomic analysis.

1. Materials and methods

1.1. Paracoccidioides *strain and growth conditions*

*P. lutzii Pb*01 yeast cells (ATCC MYA-826) were maintained in Fava-Netto solid medium at 36 °C, for 7 days. For experiments, fungal yeast cells were cultured for 48 h, at 36 °C and 120 rpm.

1.2. *Biotinylation of macrophage surface molecules*

Protocol previously described [20] was used with modifications. Murine macrophage cells strain RAW 264.7 obtained from the cell bank (Banco de células do Rio de Janeiro BCRJ/UFRJ, access number 0212) were maintained at 37 °C under 5% CO_2, in RPMI 1640 medium (Vitrocell, Brazil) supplemented with 10% [v/v] fetal bovine serum (FBS) and 1% [v/v] non-essential amino acids solution (Sigma Aldrich, Missouri, USA). Macrophages were collected by scraping, and washed three times with ice cold PBS (pH 8.0). Subsequently, they were resuspended at a concentration of 2.5×10^7 cells/mL in PBS, and a solution of N-hydroxysuccinimide-glycol4 polyethylene-biotin (biotin-PEG4-NHS) (Pierce, Rockford, IL) was added at 2 mM and incubated at 4 °C for 30 min. Thereafter, the macrophages were washed three times with PBS and 100 mM of glycine. To confirm the biotinylation of the macrophage surface molecules, an aliquot of the treated cells was incubated with Avidin-fluorescein isothiocyanate (FITC-avidin) (BioLegend, San Diego, CA, USA) for 30 min at 37 °C. Likewise, non-treated cells were used as negative control. Cells were visualized in a fluorescence microscope Axioscope A1 (Zeiss Axiocam MRc-Scope A1, Oberkochen, Germany).

1.3. *Immobilization of biotinylated macrophage surface molecules*

Macrophages subjected to biotinylation were lysed on ice with occasional vortexing for 30 min in buffer composed of 16 mM Triton X-100, 20 mM Tris-HCl pH 7.6, 150 mM NaCl, supplemented with a mix of protease inhibitors (GE Healthcare, Piscataway, NJ, USA), and the supernatant containing the biotinylated cell surface proteins was collected. The resin NeutrAvidin agarose (Pierce, Rockford, IL) was packed in a column; the supernatant above was added, incubated at room temperature for 15 min and washed with PBS. A solution of 3.4 M biotin was used to block non-linked agarose resin. Finally, the column was washed with PBS five times.

1.4. *Preparation of* P. lutzii *cell wall enriched protein extract*

Yeast cells of *P. lutzii* were incubated in liquid Fava Netto's medium for 72 h, at 36 °C and subsequently centrifuged. The extract containing *P. lutzii* cell wall enriched protein extract was obtained as described [21], using extraction buffer (50 mM Tris HCl pH 7.8, 69 mM SDS, 10 mM EDTA and 40 mM β-Mercaptoethanol). After centrifugation, the amount of proteins in the supernatant was measured by using the Bradford reagent (Sigma Aldrich, Missouri, USA).

1.5. *Capturing the* P. lutzii *proteins that interact with macrophage surface molecules*

The column containing the resin NeutrAvidin agarose was filled with the supernatant containing biotinylated-macrophage surface proteins. The column was used to capture the *P. lutzii* cell wall enriched protein extract, as described [20]. An equal volume of resin was blocked with 3.3 M biotin solution and placed in a separate column as a negative control. A total of 100 μg of *P. lutzii* cell wall enriched protein extract was added to these columns and incubated at 4 °C for 60 min.

1.6. *Digestion of protein extracts for nano-ESI-UPLC-MS^E acquisition*

Enzymatic digestion of proteins was performed, as described [22], with some modifications. Briefly, 50 μg of protein eluate (previous item) was added to 50 mM ammonium bicarbonate pH 8.5. RapiGEST™ SF Surfactante (0.2% v/v) was used, followed by adding dithiothreitol (DTT) (GE Healthcare, Piscataway, NJ, USA) iodocetamide (GE Healthcare, Piscataway, NJ, USA). Proteins were digested with 10 μL of trypsin 0.05 μg/μL (w/v) (Promega, Madison, WI, USA) at 37 °C for 16 h. The peptides were incubated with 5% (v/

v) trifluoracetic acid solution (Sigma-Aldrich, St. Louis, MO, USA), followed by centrifugation at 18,000 *g* at 4 °C, for 30 min. The supernatants were dried in a speed vacuum (Eppendorf, Hamburg, Germany). All obtained peptides were suspended in 80 μL of a solution containing 20 mM ammonium formiate and 250 fmol/μL of PHB (Rabbit Phosphorylase B) (Waters Corporation, Manchester, UK) (MassPREPTM protein).

Nanoscale LC separation of tryptic peptides was performed using a nanoACQUITY™ system (Waters Corporation, Manchester, UK) equipped with a nanoEase™ 5 μm × BridgeTM BEH130C18 300 μm × 50 mm precolumn; trap column 5 μm, 180 μm × 20 mm and BEH130C18 1.7 μm, 100 μm × 100 mm analytical reversed-phase column (Waters Corporation, Manchester, UK). The peptides were separated using a gradient of 3%, 10.8%, 14%, 16.7%, 20.4% and 65% (v/v) acetonitrile, with a flow rate of 2000 μL/min. The lock mass was used for calibration of the apparatus, using a constant flow rate of 0.5 μL/min at a concentration of 200 fmol protein GFP ([Glu^1]-Fibrinopeptide B human (Sigma-Aldrich, St. Louis, MO, USA). Mass spectrometry analysis was performed on a Synapt G1 MS™ (Waters Corporation, Manchester, UK) equipped with a nanoelectronspray source and two mass analyzers: a quadrupole and a time-of-flight (TOF) operating in TOF V-mode. Data were obtained using the instrument in the MS^E mode, which switches the low energy (6 V) and elevated energy (40 V) acquisition modes every 0.4 s. Samples were analyzed from three biological replicates.

1.7. Data processing and protein identification

The mass spectrometer data that were obtained from the LC-MS^E analysis were processed and searched using the ProteinLynx Global Server version 2.4 (Waters Corporation, Manchester, UK). The processed spectra were searched against *P. lutzii* protein sequences (Broad Institute; http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html) together with random sequences, as negative control. The mass error tolerance for peptide identification was under 50 ppm. The protein identification criteria also included the detection of at least 2 fragment ions per peptide, 5 fragments per protein and the determination of at least 1 peptide per protein; the identification of protein was allowed with a maximum 4% false positive discovery rate in at least three technical replicate injections.

Protein tables generated by ProteinLynx Global Server (PLGS) were merged, and the dynamic range of the experiment was calculated using the software program MassPivot v1.0.1 [22]. The peptide and protein tables were compared using Spotfire® v8.0 (TIBCO Software Inc.) software, and suitable graphics were generated for all data.

1.8. In silico *analysis*

In silico analysis was performed using the Signal P 4.0 program (http://www.cbs.dtu.dk/services/SignalP/) to predict signal peptides (D-*score* ≥ 0.45). For prediction of secretion by non-classical routes, the Secretome P program was used (http://www.cbs.dtu.dk/services/SecretomeP/- NN-*score* ≥ 0.5). Subcellular localization was predicted using WolfPSORT (http://www.genscript.com/psort/wolf_psort.html). The TMHMM 2.0 version program (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict transmembrane regions. To identify proteins with a putative GPI anchor, the big-P Fungal Predictor program was used (http://mendel.imp.ac.at/gpi/fungi_server.html).

NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) was used to predict *N*-glycosylation sites (score value ≥ 0.5). For *O*-glycosylation sites, the NetOGlyc 3.1 program (http://www.cbs.dtu.dk/services/NetOGlyc/) was used (G-*score* ≥ 0.5). The prediction of adhesin-like proteins was performed using the Faapred tool (http://bioinfo.icgeb.res.in/faap) [23] (e-value ≥ −0.8). The identified proteins were grouped into functional categories according to MIPS Functional Catalogue Database (FunCatDB), available on the Pedant database (http://pedant.gsf.de/genomes.jsp?category=fungal).

1.9. Production of recombinant protein, obtaining polyclonal antibodies and western blot analyses

cDNA cloning and serine proteinase (SP) protein production (GenBank accession number AAP83193) were performed, as previously described [24]. The protein species corresponding to SP fused to glutathione S transferase (rSP) was used to obtain polyclonal antibodies in mice, as described [24]. Recombinant SP fused to GST was identified by using peptide mass spectrometry analysis, as described above, to confirm the identity of the protein. The anti-fructose, 1,6 biphosphate aldolase (FBA) polyclonal antibodies were previously obtained using the purified protein, as described [17].

1.10. Immunofluorescence assay

Macrophages were cultured in RPMI medium containing bovine fetal serum 10% (v/v), MEM non-essential amino acid solution (Sigma Aldrich, Missouri, USA) and IFN-γ (1U per mL) at 36 °C and 5% CO_2, until complete confluence. Immunofluorescence assay of macrophages with yeast fungal cells was performed as described [18]. Macrophages (10^5 cells/mL) were incubated with 5×10^5 *Pb*01 yeast cells/mL for 4 h at 37 °C, with 5% CO_2 to allow adhesion. As control, *P. lutzii* cells were maintained without macrophages, under the same incubation conditions. After 4 h, macrophages were lysed with cold water for 10 min, centrifuged at 5000 *g* for 5 min at 4 °C. The recovered fungal cells were fixed with methanol 100% for 2 h at − 80 °C and washed three times with PBS. *P. lutzii* cells were incubated with polyclonal antibodies anti-SP or anti-FBA (1:100) or pre-immune serum (1:100) as control for 1 h at room temperature. Then, cells were washed three times with PBS and incubated with anti-mouse IgG coupled to fluorescein isothiocyanate (FITC) 1:500 diluted, for 1 h at room temperature. The labeled fungal cells were washed with PBS three times to remove the FITC excess. The images were obtained using an Axioscope A1 microscope (Carl Zeiss AG, Germany) and photographed at bright field, and at 450–490 nm.

1.11. Flow cytometry

The interaction assay was performed as described above. After 4 h, cells were washed three times with PBS to remove unbound fungal cells, fixed with methanol 100% for 2 h at −80 °C and washed three times with PBS to remove methanol excess. Then, the cells were collected by scraping and blocked for 30 min with PBS containing BSA (3% [w/v]) and Tween 20 (0.2% [v/v]) at room temperature. Afterwards, the cells were washed three times with PBS to remove block buffer excess and were centrifuged 5000 *g* for 5 min at 4 °C. The cells were incubated with polyclonal antibodies anti-SP or anti-FBA (1:100) for 1 h at room temperature, washed three times with PBS, and incubated with anti-mouse IgG coupled to fluorescein isothiocyanate (FITC, 1:500 diluted), for 1 h at room temperature. The labeled fungal cells were washed with PBS three times to remove the FITC excess. The excitation wavelength was 488 nm and emitted light was collected with a 530/30-nm filter. A total of 10,000 cells per sample was acquired by the Guava easyCyte instrument (Merck Millipore, Darmstadt, Germany). Analyses were performed in the gate of fungal cells (FSC × SSC) and data were shown as mean fluorescence intensity (MFI) of SP or FBA expression.

2. Results

2.1. Biotinylation of macrophage surface molecules

To gain new insights into the interactions between the proteins of *P. lutzii* cell wall enriched extract and the cells of its host, the RAW 264.7 surface molecules were labeled using the reagent PEG4-NHS-biotin. The PEG4-NHS-biotin does not readily dissolve in polar solutions, and it does not permeate the cell membrane. Thus, only the surface molecules of macrophages would be labeled with biotin [20]. To confirm the biotinylation of macrophage surface molecules, the pre-treated and untreated cells were incubated with avidin-fluorescein isothiocyanate (FITC-avidin). A significant fluorescence was detected in the biotinylated cells. This was not detected in the control cells, confirming that the macrophage surface molecules were indeed biotinylated (Fig. S1).

2.2. Confirmation of P. lutzii proteins captured by biotinylated macrophage surface molecules

NeutrAvidin, which was used to immobilize the biotinylated macrophage surface molecules, is a chemically-modified version of avidin [25]. The NeutrAvidin agarose resin with immobilized macrophage surface molecules was used to capture *P. lutzii* surface proteins by means of affinity chromatography. After binding, the captured proteins were eluted using a high-salt concentration buffer. To verify the presence of proteins in the eluted extract, one-dimensional SDS-PAGE gel was performed, as depicted in Fig. S2. The presence of proteins in the eluate was confirmed. These results indicated that *P. lutzii* cell wall enriched protein extract were obtained upon interaction with macrophage proteins.

2.3. Identification and characterization of P. lutzii proteins that interact with macrophage surface proteins

The resulting NanoUPLC-MS^E protein and peptide data generated by PLGS analysis are shown in Fig. S3. The experiments resulted in the identification of 3922 peptides. A total of 15% of the peptides were identified by a missed trypsin cleavage. An in-source fragmentation rate of 11% was observed (Fig. S3A). The accuracy of the *m/z* fragment matches in the database is shown in Fig. S3B. A total of 87% of the peptides were assigned, with up to 5 ppm m/z of error. Fig. S3C shows the abundant dynamic range of the identified proteins; the distribution of protein concentrations comprised three orders of magnitude.

Liquid chromatography-mass spectrometry (LC-MS/MS) was used to separate and identify the collected proteins. In addition, a negative control eluate performed by NeutrAvidin agarose resin blocked only with biotin solution was used to assess possible contamination with background proteins. It was also analyzed by LC-MS/MS, and non-contaminating proteins were detected. A total of 215 *P. lutzii* proteins that interact with macrophage surface proteins were identified (Table S1). The proteomic data were compared with the murine database and no protein was identified. This clearly confirmed that precipitated proteins were exclusively from *P. lutzii*. The functional classification of identified proteins, according to MIPS Functional Catalogue Database (FunCatDB), is shown in Fig. 1. The most representative functional categories are metabolism (14.4%) and protein synthesis (27.4%).

Regarding transmembrane domains, 30 proteins (13.89%) of *P. lutzii*, out of 215, had at least one transmembrane domain (Table S1, Fig. 2A). Two proteins of *P. lutzii* (conserved hypothetical protein - PAAG_00898 - and aminolevulinate synthase - PAAG_00397) are predicted to contain GPI-anchor (Table S1, Fig. 2A).

The analysis revealed that 77 *P. lutzii* proteins (35.65%) showed *N*-glycosylation sites, and 181 proteins (83.8%) showed *O*-glycosylation sites (Table S1, Fig. 2A). With regard to proteins classified as adhesins, the Faapred program identified 42 proteins (19.44%) with adhesion characteristics in *P. lutzii* (Table S1, Fig. 2A).

Several proteins are transported to the cell surface of organisms to be integrated into the cell wall or to be exported to the extracellular medium [26]. In order to predict secreted proteins, Signal P 4.0 and Secretome P 2.0 were employed. In *P. lutzii*, from 215 identified proteins, 7 (3.25%) had a signal peptide, and 126 (58.6%) were predicted to be secreted by alternative routes, totaling 133 proteins (Table S1, Fig. 2A).

2.4. P. lutzii proteins that bind to macrophage surface proteins

For further analysis, we considered as additional criteria only those proteins that presented signal peptides and/or predicted non-classical secretion. A total of 133 proteins that showed signal peptides or non-conventional secretion were identified in the *P. lutzii* cell wall enriched protein fractionof *P. lutzii* during interaction with macrophage surfaces (Table 1, Fig. 2A). Functional classification of these proteins is shown in Fig. 2B.

Considering the criteria for secretion as mentioned above, proteins belonging to several functional categories are putatively involved with fungal adhesion to macrophages. A large number of

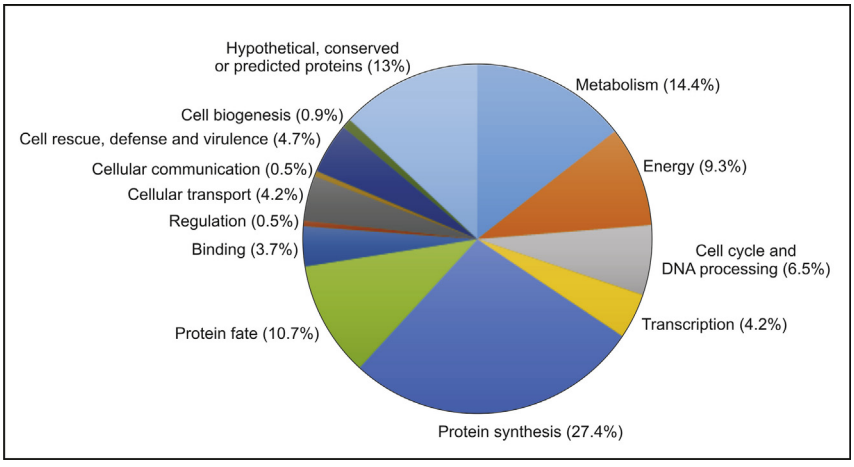

Fig. 1. Functional classification of identified proteins. The classification was performed according to MIPS Functional Catalogue Database (FunCatDB).

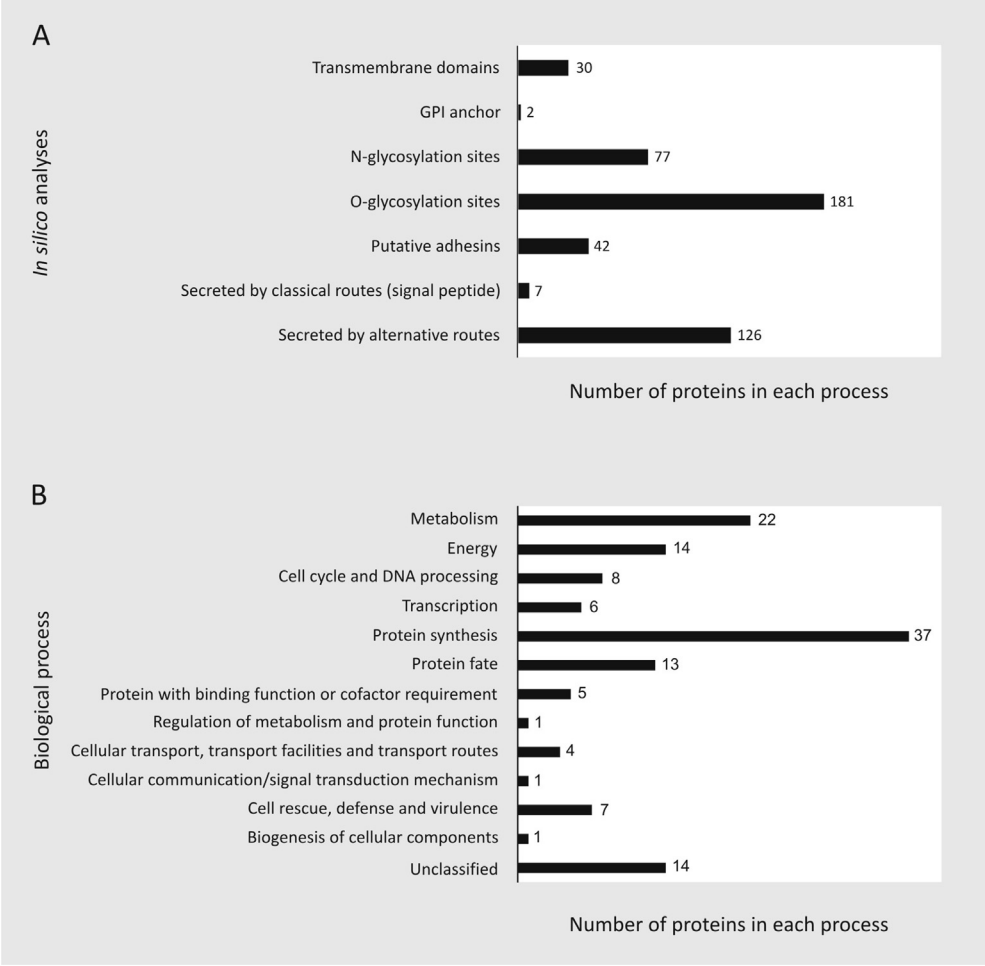

Fig. 2. ***In silico*** **analysis of** ***P. lutzii*** **proteins that interact with macrophage surface. A:** The protein sequences were examined for the presence of transmembrane domains using TMHMM 2.0 version program (http://www.cbs.dtu.dk/services/TMHMM/). The big-PI program (http://mendel.imp.ac.at/gpi/fungi_server.html) was used to search for protein GPI-anchoring sites. Using the NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc) and NetOGlyc (http://www.cbs.dtu.dk/services/NetOGlyc/) programs, protein glycosylation sites could be determined. The prediction of adhesin-like proteins was performed using the Faapred tool (http://bioinfo.icgeb.res.in/faap). In addition, the signal peptide search was performed using SignalP (http://www.cbs.dtu.dk/services/SignalP/). SecretomeP (http://www.cbs.dtu.dk/services/SecretomeP/) was used to identify non-classical protein secretion signals. **B:** The database MIPS FunCat2 was used to perform the classification of biological processes. In this graph, only the proteins that showed signal peptide or non-conventional secretion were presented.

proteins related to protein synthesis, folding and modification were characterized as macrophage-binding molecules. Also, enzymes associated with the metabolism of amino acids were characterized as macrophage-binding molecules. Furthermore, glycolytic/gluconeogenesis and TCA cycle enzymes were described. Proteins related to cell rescue defense and virulence were also characterized as macrophage-binding molecules.

2.5. Heterologous expression of serine proteinase, identification of the recombinant protein by LC-MS/MS and polyclonal antibodies

SP (GenBank accession number AAP83193) was identified by proteomic analysis as a macrophage-binding protein of *P. lutzii*. *In silico* analysis showed that this protein can be secreted by a classical pathway, as it presented signal peptide. It also presented *O*-glycosylation and *N*-glycosylation sites. The protein was selected to perform cloning and expression in a heterologous system, as previously performed [24]. SP was induced for expression in bacteria, as depicted in Fig. S4A.

LC-MS/MS was performed to identify the recombinant protein. The protein species corresponding to SP fused to glutathione S transferase (SP-GST) was excised from the gel, digested, and peptide mass spectrum was generated (Fig. S4B). The protein has a score value of 229,393 and a sequence coverage percentage of 5.43%.

Mouse polyclonal antibodies obtained against SP specifically identify SP in Western blot analysis. The specificity of the antibodies anti-SP is shown in Fig. S4C-D, confirming previous results [24].

2.6. Biological assays to validate the fungal protein function in the pathogen-host interaction

We selected SP (PADG_04168) and FBA (PADG_01995) for further analysis of *Pb*-macrophage interaction. Previous studies in our laboratory demonstrated that these proteins are important during *in vitro* and *in vivo* infection models with *Paracoccidioides* spp. [17,19]. The immunofluorescence assays to SP and FBA showed an increase in the fluorescence of *P. lutzii* cells treated with anti-SP or anti-FBA after interacting with macrophages, when compared to the control (Fig. 3A). Flow cytometry analysis confirmed that both SP and FBA were detected in higher amount in the fungal surface

Table 1
Paracoccidioides lutzii secreted proteins that interact with macrophages.

Accession number[a]	Protein description and biological process[b]	Signal P Score ≥0,5[c]	Secretome P (NN-score)[d]	fmol[e]
	Metabolism			
	Amino acid metabolism			
PAAG_00468	4-aminobutyrate aminotransferase	–	0.58	31.14
PAAG_01194	2-oxoisovalerate dehydrogenase subunit beta	–	0.73	180.01
PAAG_01144	Aspartate aminotransferase	–	0.57	74.50
PAAG_07786	Acetyl-CoA acetyltransferase	–	0.62	96.02
PAAG_02354	Serine 3-dehydrogenase	–	0.60	41.76
PAAG_08163	Fumarylacetoacetase	–	0.62	56.23
PAAG_08313	L-PSP endoribonuclease family protein (Hmf1)	–	0.85	147.05
PAAG_01310	2-oxoisovalerate dehydrogenase subunit alfa	–	0.72	53.83
PAAG_04559	2-methylcitrate dehydratase	–	0.60	83.34
PAAG_02603	Aspartate aminotransferase	–	0.58	69.18
PAAG_03978	3-hydroxyisobutyrate dehydrogenase	–	0.60	55.10
	Nucleotide/nucleoside/nucleobase metabolism			
PAAG_05611	Deoxyuridine 5′- triphosphate nucleotidohydrolase	–	0.55	134.59
	C-compound and carbohydrate metabolism			
PAAG_01534	Pyruvate dehydrogenase E1 component subunit beta	–	0.73	174.79
PAAG_00050	Pyruvate dehydrogenase complex dihydrolipoamide acetyltransferase	–	0.56	118.14
PAAG_00850	Glucosamine-fructose-6 phosphate aminotra	–	0.71	117.61
PAAG_00856	Isocitrate dehydrogenase subunit 1	–	0.59	120.62
PAAG_04233	2-nitropropane dioxygenase	–	0.75	64.18
PAAG_07729	Isocitrate dehydrogenase subunit 2	–	0.85	85.68
PAAG_01831	SCF E3 ubiquitin ligase complex F-box protein grrA	–	0.62	30.41
PAAG_07634	Small GTPase RhoA	–	0.58	223.60
	Lipid, fatty acid and isoprenoid metabolism			
PAAG_02664	3-ketoacyl-CoA thiolase	–	0.67	41.14
PAAG_05249	Aldehyde dehydrogenase	–	0.56	193.23
	Energy			
	Glycolysis and gluconeogenesis			
PAAG_01995	Fructose-1,6-bisphosphate aldolase 1	–	0.51	172.75
PAAG_00771	Enolase	–	0.60	397.56
PAAG_08468	Glyceraldehyde-3-phosphate dehydrogenase	–	0.53	484.07
	Tricarboxylic-acid cycle			
PAAG_01463	Succinyl-CoA ligase subunit beta	–	0.50	107.66
PAAG_00053	Malate dehydrogenase	–	0.66	199.93
	Electron transport and membrane-associated energy conservation			
PAAG_05735	NADH-ubiquinone oxidoreductase 49 kDa subunit	–	0.69	109.57
PAAG_07672	Ubiquinol-cytochrome c reductase subunit 7	–	0.55	190.12
PAAG_06093	Cytochrome c1, heme protein, mitochondrial	–	0.85	58.50
	Respiration			
PAAG_04931	Electron transfer flavoprotein subunit beta	–	0.71	56.64
PAAG_04570	ATP synthase D chain, mitochondrial	–	0.51	171.22
PAAG_04838	ATP synthase subunit 4	–	0.71	82.08
PAAG_08037	ATP synthase subunit beta	–	0.62	149.52
PAAG_08057	Cytochrome c oxidase polypeptide V	–	0.79	167.77
PAAG_00173	Electron transfer flavoprotein subunit alpha	–	0.64	100.00
	Cell cycle and DNA processing			
	DNA processing			
PAAG_02773	Ubiquitin-conjugating enzyme variant MMS2	–	0.91	111.85
PAAG_06511	Mitochondrial genome maintenance protein mgm101	–	0.71	60.98
	Cell cycle			
PAAG_01347	Actin cytoskeleton protein VIP1	–	0.76	69.80
PAAG_03031	Tubulin beta chain	–	0.53	106.86
PAAG_00623	Kinetochore protein spc24	–	0.85	28.99
PAAG_07773	Cyclin-dependent kinases regulatory subunit	–	0.84	166.05
PAAG_08917	Histone H2a	–	0.75	140.25
PAAG_03129	Dynein light chain	–	0.70	106.89
	Transcription			
	RNA synthesis			
PAAG_07099	Histone H3.3	–	0.67	326.17
PAAG_02537	Small nuclear ribonucleoprotein Sm D2	–	0.90	106.41
PAAG_04814	Nucleic acid-binding protein	–	0.70	122.19
PAAG_08223	Small nuclear ribonucleoprotein Sm D3	–	0.65	90.07
	RNA processing			
PAAG_04511	ATP-dependent RNA helicase SUB2	–	0.74	170.82
	RNA modification			
PAAG_03941	G4 quadruplex nucleic acid binding protein	–	0.53	69.57
	Protein synthesis			
	Ribosome biogenesis			

Table 1 (*continued*)

Accession number[a]	Protein description and biological process[b]	Signal P Score ≥0,5[c]	Secretome P (NN-score)[d]	fmol[e]
	Metabolism			
PAAG_06367	30S ribosomal protein S17P	–	0.55	113.48
PAAG_05017	40S ribosomal protein S10-A	–	0.60	246.26
PAAG_08634	40S ribosomal protein S12	–	0.61	62.00
PAAG_05704	40S ribosomal protein S13-1	–	0.83	101.55
PAAG_01433	40S ribosomal protein S14	–	0.54	316.21
PAAG_01435	40S ribosomal protein S16	–	0.57	55.18
PAAG_01413	40S ribosomal protein S17	–	0.74	86.43
PAAG_03513	40S ribosomal protein S18	–	0.55	388.93
PAAG_03322	40S ribosomal protein S20	–	0.75	387.05
PAAG_08955	40S ribosomal protein S3aE	–	0.71	158.24
PAAG_03816	40S ribosomal protein S4	–	0.67	70.36
PAAG_05484	40S ribosomal protein S5	–	0.79	187.66
PAAG_07182	40S ribosomal protein S7	–	0.88	107.66
PAAG_00430	60S ribosomal protein L2	–	0.79	133.20
PAAG_00952	60S ribosomal protein L20	–	0.75	260.59
PAAG_08888	60S ribosomal protein L4-A	–	0.69	263.58
PAAG_06569	60S ribosomal protein L43	–	0.70	53.94
PAAG_03019	60S ribosomal protein L6-B	–	0.76	176.34
PAAG_06743	60S ribosomal protein L23e	–	0.75	123.69
PAAG_09083	TCTP family protein	–	0.51	301.23
PAAG_00238	Ubiquitin-60S ribosomal protein L40	–	0.78	182.12
PAAG_00205	60S ribosomal rotein L24	–	0.58	141.88
PAAG_00385	40S ribosomal protein S23	–	0.60	124.46
PAAG_00548	60S ribosomal protein L5	–	0.63	114.00
PAAG_01939	60S ribosomal protein L27-A	–	0.77	117.77
PAAG_00347	60S ribosomal protein L9-B	–	0.82	53.98
PAAG_00724	Ribosomal protein 11 large subunit	–	0.65	88.63
PAAG_03827	60S ribosomal protein L21-A	–	0.85	101.97
PAAG_04965	60S ribosomal protein L31	–	0.65	75.38
PAAG_05337	40S ribosomal protein S22	–	0.82	234.10
PAAG_05778	40S ribosomal protein S19	–	0.81	143.65
PAAG_06536	Ubiquitin	–	0.83	53.24
PAAG_07385	60S ribosomal protein L23a	–	0.60	135.54
PAAG_07847	40S ribosomal protein S26	–	0.61	234.32
PAAG_05590	Protein transporter SEC23	–	0.82	363.07
	Translation			
PAAG_03028	Elongation factor 1-beta	–	0.60	123.63
PAAG_02921	Elongation factor Tu	–	0.77	173.44
	Protein fate (folding, modification, destination)			
	Protein folding and stabilization			
PAAG_05142	10 kDa heat shock protein, mitochondrial	–	0.57	1248.25
PAAG_00986	Disulfide isomerase Pdi1	0.77		127.12
PAAG_00739	Peptidyl-prolyl-cis-trans isomerase B	0.64		169.45
PAAG_07509	Peptidyl-prolyl-cis-trans isomerase ssp1	–	0.72	45.95
PAAG_05788	Peptidyl-prolyl-cis-trans isomerase A2	–	0.75	157.94
PAAG_06255	Mitochondrial co-chaperone GrpE	–	0.77	42.87
PAAG_05679	Heat shock protein	–	0.08	902.88
	Protein targeting, sorting and translocation			
PAAG_03772	Mitochondrial import inner membrane translocase subunit tim9	–	0.81	9.30
PAAG_05643	Endoplasmic reticulum and nuclear membrane	–	0.53	234.19
PAAG_07490	Monothiol glutaredoxin-5	–	0.63	87.53
	Protein modification			
PAAG_00204	Ubiquitin-conjugating enzyme	–	0.52	126.75
	Protein/peptide degradation			
PAAG_04168	Aqualysin-1 (serine proteinase)	0.73	–	64.29
PAAG_01962	Proteasome 26S subunit	–	0.64	41.05
	Protein with binding function or cofactor requirement (Structural Or Catalytic)			
	Metal binding			
PAAG_04391	Progesterone binding protein	–	0.70	45.21
PAAG_03717	Myosin regulatory light chain cdc4	–	0.54	66.21
	Nucleotide/nucleoside/nucleobase binding			
PAAG_06288	Vacuolar ATP synthase subunit B	–	0.50	38.43
PAAG_08093	GTP-binding protein ypt3	–	0.55	140.40
	Complex cofactor/cosubstrate/vitamine binding			
PAAG_01321	Oxidoreductase 2-nitropropane dioxygenase family	–	0.70	237.20
	Regulation of metabolism and protein function			
	Regulation of protein activity			
PAAG_08518	Ras-2	–	0.71	17.69
	Cellular transport, transport facilities and transport routes			

(*continued on next page*)

Table 1 (*continued*)

Accession number[a]	Protein description and biological process[b]	Signal P Score ≥0,5[c]	Secretome P (NN-score)[d]	fmol[e]
	Metabolism			
	Transport facilities			
PAAG_08082	Plasma membrane ATPase	–	0.71	71.38
	Transport routes			
PAAG_05960	NIPSNAP family protein	–	0.76	41.30
PAAG_06994	Endoplasmic reticulum vesicle protein	0.87	–	36.03
PAAG_04328	Endosomal cargo receptor (Erp3)	0.91	0.88	104.67
	Cellular communication/signal transduction mechanism			
	Cellular signaling			
'PAAG_02458	GTP-binding protein ypt7	–	0.69	102.20
	Cell rescue, defense and virulence			
	Stress response			
PAAG_00871	30 kDa heat shock protein	–	0.8	335.11
PAAG_01262	Hsp70-like protein	0.86		240.81
PAAG_03216	Mitochondrial peroxiredoxin PRX1	–	0.58	28.04
PAAG_06175	Peroxisomal matrix protein	–	0.93	101.57
PAAG_06811	Heat shock protein STI1	–	0.51	87.32
	Detoxification			
PAAG_02364	Thioredoxin	–	0.85	71.01
PAAG_03292	Cytochrome c peroxidase	–	0.81	281.91
	Biogenesis of cellular components			
	Cytoskeleton/structural proteins			
PAAG_06192	ARP2 3 complex 20 kDa subunit	–	0.87	91.14
	Unclassified			
PAAG_01501	Hypothetical protein	–	0.54	54.94
PAAG_02539	Hypothetical protein	–	0.62	73.50
PAAG_01926	Hypothetical protein	–	0.56	24.72
PAAG_07960	Hypothetical protein	0.75	–	339.76
PAAG_08058	Hypothetical protein	–	0.51	394.79
PAAG_03299	Hypothetical protein	–	0.72	63.68
PAAG_06036	Hypothetical protein	–	0.50	130.03
PAAG_05019	Hypothetical protein	–	0.50	69.76
PAAG_01079	Hypothetical protein	–	0.59	231.13
PAAG_01591	Hypothetical protein	–	0.92	68.75
PAAG_07246	Hypothetical protein	–	0.614	376.87
PAAG_07957	Hypothetical protein	–	0.801	107.96
PAAG_06539	Hypothetical protein	–	0.86	10.22
PAAG_06954	OTU domain containing protein 6B	–	0.78	7.29

[a] Accession – accession number of matched protein from *Paracoccidioides* Broad Institute database (https://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/).

[b] Description – proteins annotation from *Paracoccidioides* database or by homology in NCBI database (http://www.ncbi.nlm.nih.gov/) and biological process according to the classification of FunCat (https://www.helmholtzmuenchen.de/en/ibis/resourcesservices/services/funcat-the-functional-catalogue/index.html).

[c] Prediction secretion according to SignalP 4.0 server, the corresponding number for the D-score must equal or exceed the value of 0.340 (D-score ≥ 0,340) (http://www,cbs,dtu,dk/services/SignalP/).

[d] Secretion prediction according to SecretomeP 2.0 server, the corresponding number for the SecP- score must equal or exceed the value of 0.5 (SecP score≥0,50) (http://www,cbs,dtu,dk/services/SecretomeP/).

[e] Protein quantification in NanoUPLC-MS^E.

during *P. lutzii* interaction with macrophages (Fig. 3B). These results reinforce that those proteins are ligands of macrophages and may be important during the infection process.

3. Discussion

In our study, the identification of proteins capable of interaction with macrophages was obtained by affinity chromatography and shotgun proteomics (LC-MS/MS), to investigate the interactions between host and fungus on a large scale. First, the affinity chromatography method, which uses the agarose resin with immobilized surface molecules from the host cells as bait, was applied to capture the specifically interacting proteins from the cell wall enriched protein extract of *P. lutzii*. Our results showed that when treated with biotin, the macrophage surface molecules presented a significant green fluorescence after incubation with FITC-avidin, while no specific green fluorescence was observed in the negative control (Fig. S1). The same result was obtained in biotinylated human laryngeal epithelial cell line Hep-2 in experiments with *Streptococcus suis* [20]. The *P. lutzii* cell wall enriched protein extract captured by affinity chromatography were eluted with a very high salt concentration. As the binding affinity between avidin and biotin is extremely high, the high-salt buffer does not affect the binding between the immobilized biotinylated macrophage surface proteins and NeutrAvidin agarose resin. Thus, only the *P. lutzii* proteins were eluted (Fig. S2).

We identified 215 proteins of *P. lutzii* that interact with macrophage surface proteins. In a parallel experiment, the eluate from the negative controls was also analyzed by LC-MS/MS. This experiment identified only one protein, showing that *P. lutzii* proteins identified in experimental conditions are in fact those that interacted with the surface of macrophages and not with contaminating background proteins (data not shown).

With respect to cellular location, several proteins identified in our analysis were predicted as primarily localized in the cytoplasm, followed by mitochondria and the nucleus (Table S1). Examples of these proteins predicted to be present in the cytoplasms include elongation factor 1-α, members of HSP70 proteins, the HSP90

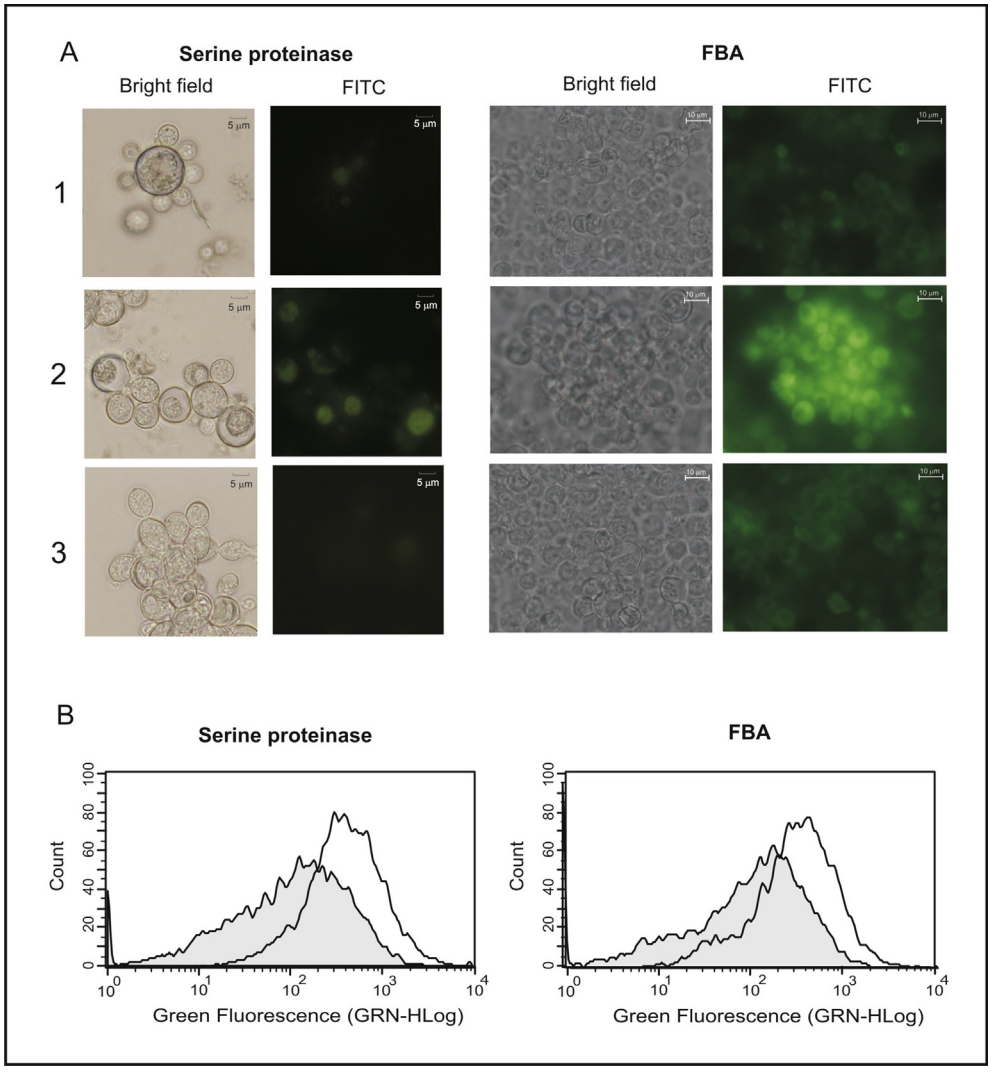

Fig. 3. Immunofluorescence and cytometry assays with polyclonal antibodies anti-SP and anti-FBA upon *P. lutzii* yeast cells, incubation with RAW 264.7 macrophages. A: Immunofluorescence assay was performed with control *P. lutzii* yeast cells (1) and with *P. lutzii* yeast cells after 4 h of interaction with macrophages (2). Macrophages were disrupted and yeast cells were incubated with the polyclonal antibodies raised to SP or FBA. The cells were incubated with the anti-mouse antibody FITC. The images were obtained at bright field and at 470/440 nm wavelength (FITC). Control with pre-immune serum was also performed and photographed in the same conditions (3). **B:** Flow cytometry was performed as described above. Control yeast cells are shown in gray graphic and yeast cells upon 4 h of interaction with macrophages are shown in white graphic. A total of 10,000 cells per sample was acquired by instrument Guava easyCyte (Merck Millipore, Darmstadt, Germany).

family, and elongation factor 2, while ADP/ATP carrier protein is present in mitochondria, and histones are in the nucleus. Interestingly, despite being located in intracellular compartments, all these proteins were on the surface of *P. lutzii* interacting with macrophages. Studies on different microorganisms have shown the presence of those intracellular proteins associated with the cell wall. Elongation factor 1-α is located in the cell wall of *Candida albicans* and *Saccharomyces cerevisiae* [26,27]. In *P. lutzii*, elongation factor 1-α is a secreted protein when the fungus is infecting macrophages [28]. Proteins related to stress response and virulence, such as heat shock proteins (HSPs), were also identified on the wall of *C. albicans* by immunolocalization [29]. These proteins may perform functions related to the biosynthesis, secretion, and mounting of cell wall components, as well as to the structure of the cell wall itself [29]. Although histones are commonly associated with DNA, the presence of these proteins in the cell wall of *Histoplasma capsulatum* has been reported [30]. In *Mycobacterium leprae*, some histones have been demonstrated as binding to laminin in the peripheral nerve, facilitating invasion of Schwann cells [31]. Elongation factor 2 and the carrier protein ADP/ATP were described on the surface of *C. albicans* [27]. As we have seen, there are many reports about cytosolic proteins associated with fungal cell walls.

We observed a high rate of proteins that may be secreted by a non-classical pathway in *P. lutzii* (59.26%) (Table 1, Fig. 2). Corroborating these data, previous work described that 52.5% of the total extracellular proteins identified in *P. lutzii* mycelium and yeast cells were predicted to be secreted by non-classical pathways [28]. Several unconventional secretion pathways are suggested and may explain how proteins reach the cell surface. In *S. cerevisiae* for example, the glycolytic enzyme enolase is secreted via a SNARE-dependent secretory pathway [32]. Proteins can be exported by direct translocation across plasma membranes such as fibroblast growth factor 2, or through the involvement of intracellular transport intermediates, as shown in the case of acyl-CoA binding protein [33]. It is believed that those proteins without a signal peptide may be involved in multiple processes, including cell wall dynamics and interaction with host components, as well as virulence of pathogens [26].

Another non-classical route for protein transportation outside the plasma membrane is through exosomes, which derive from intracellular multivesicular bodies [34]. Proteomic analysis of extracellular vesicles led to the identification of several proteins in *Cryptococcus neoformans* [35] and in *H. capsulatum* [36]. In *P. brasiliensis*, proteomic analyses identified 72 proteins that were considered to be commonly transported by extracellular vesicles [37]. Here, we have identified 46 proteins, which were previously described as secreted by vesicles in *P. brasiliensis* [37]. Among these proteins we can cite some involved in carbohydrate metabolism (GAPDH, enolase, FBA and TPI), translation (elonagation factors and several ribosomal proteins), response to stress (heat shock proteins and 14-3-3-like protein), oxidation-reduction (superoxide dismutase, disulfide isomerase) and other biological processes, suggesting that some of the proteins that interacted with macrophages were brought into the extracellular space through vesicles.

Our analysis also identified a large number of proteins with predicted glycosylation sites, both *N*-glycosylation and *O*-glycosylation (Tables S1, Fig. 2A). We were interested to observe that most of the proteins – over 80% – identified in this work are predicted to possess *O*-glycosylation sites. *O*-glycosylation allows the attachment of a glycan to a hydroxylysine, hydroxyproline, serine, or threonine [38]. Studies have shown that glycosylation can contribute to protein secretion [39], and in the case of membrane glycoproteins, those glycans can mediate a cell's communication with the outside space [40]. Thus, we can suggest that glycosylation, and hence the secretion of those proteins to the surface, allows interaction with macrophages. In addition, it has been reported that most fungal adhesins have a modular structure consisting of *N*-glycosylation, *O*-glycosylation and glycosylphosphatidylinositol (GPI) anchors [41], suggesting these posttranslational processes are important to ensure that proteins perform their adhesin function properly.

We also identified proteins with enzymatic properties, encompassing several metabolic pathways (Tables S1 and 1). Present among the identified proteins are GAPDH, enolase and FBA, all of which participate in the glycolytic pathway and are presumed to be involved in the process of interaction between *Pararcoccidioides* spp. and host cells [8,9,17,42]. These enzymes exert enzymatic activity and alternative cellular functions in *P. lutzii* and were described in the cytoplasm and at the cell wall of the yeast cells [8,9,17]. Cell wall-associated GAPDH binds to extracellular matrix components such as fibronectin, laminin, and type I collagen, and mediates the attachment and internalization of the *P. lutzii* yeast cells to host tissues. In addition, pneumocytes treated with recombinant GAPDH and *P. brasiliensis* yeast cells treated with anti-GAPDH polyclonal antibodies inhibited adherence and internalization of *P. brasiliensis* in *in vitro* cultured cells, indicating GAPDH could contribute to the adhesion of the fungus to host tissues, thereby potentially playing a role in the establishment of disease [8]. Studies have shown that enolase and FBA located at the *P. lutzii* cell surface are capable of recruiting plasminogen and activating the fibrinolytic system, which in turn can degrade the extracellular matrix of the host by promoting the spread of the pathogen [9,17]. Previous works demonstrated that enolase transcripts increased during *in vivo* infection in mice [9] and bound extracellular matrix components such as collagen type I and fibronectin [10]. In addition, the data reinforce the role of those molecules in interaction with host cells, suggesting their contribution to the pathogenesis of the fungus. Furthermore, it has been demonstrated that FBA is secreted by *P. lutzii* [28], an aspect that reinforces our data. In fact, the addition of recombinant FBA and/or anti-rFBA antibodies reduced fungus–macrophage interaction, thus reinforcing the notion that FBA has a relevant role to play in the adhesion, invasion, and dissemination of members of the *Paracoccidioides* complex.

SP and FBA, identified in our proteomic analysis as macrophage-binding proteins in *P. lutzii*, were used for additional confirmatory analysis. Previous data of transcriptional analysis of *P. lutzii* yeast cells, derived from infected mouse livers, depicted the induction of the transcript encoding *P. lutzii* SP [43]. Additionally, the protein is induced and secreted during nitrogen deprivation [24]. The infection of mouse lungs by *P. brasiliensis* demonstrated that SP was upregulated and secreted in the lung tissue during infection [19], reinforcing the protein's role in the fungus' interaction with the host. FBA, although a cytoplasmic protein, has already been described at the surface of bacteria playing a role in cell adhesion and invasion [44]. In addition, in pathogenic fungi such as *C. neoformans*, FBA binds host molecules and depicts an adhesion function beyond its glycolytic activity [45]. In *P. lutzii*, FBA was previously characterized, and shown to be an antigenic molecule, reactive with sera of PCM patients [46]. Proteomic analysis of the *P. lutzii* secretome identified plasminogen-binding proteins, such as FBA, which may participate in the processes of cell adhesion and tissue invasion/dissemination of *Paracoccidioides* during infection [17]. In our study, both proteins, SP and FBA, presented increased expression when *P. lutzii* cells were incubated with macrophages, suggesting involvement of these proteins in the interaction with host cells during the adhesion process, and its participation in the virulence of the fungi.

Notably, it is important to reinforce the fact that the methodology used in this work can detect proteins which may play a role during infection in the host. Previously described proteins which allow the interaction of *P. lutzii* with host cells, such as GAPDH, enolase and FBA, were also detected. On the other hand, several proteins that have not previously been associated with the infection process were here detected. These findings introduce new perspectives for the study of the role of these molecules in the pathogenicity of *P. lutzii*, as well as in other species of the genus.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2019.03.002.

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