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Comparison of virulence between *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii* using *Galleria mellonella* as a host model

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Faculdade de Ciências Farmacêuticas; UNESP-Univ Estadual Paulista; Campus Araraquara; Departamento de Análises Clínicas e Núcleo de Proteômica; Laboratório de Micologia Clínica; Araraquara, São Paulo, Brazil

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Paracoccidioidomycosis is a systemic mycosis, endemic in Latin America. The etiologic agents of this mycosis are composed of 2 species: *Paracoccidioides brasiliensis* and *P. lutzii*. Murine animal models are the gold standard for in vivo studies; however, ethical, economical and logistical considerations limit their use. *Galleria mellonella* is a suitable model for in vivo studies of fungal infections. In this study, we compared the virulence of *P. brasiliensis* and *P. lutzii* in *G. mellonella* model. The deaths of larvae infected with *P. brasiliensis* or *P. lutzii* were similar, and both species were able to reduce the number of hemocytes, which were estimated by microscopy and flow cytometer. Additionally, the phagocytosis percentage was similar for both species, but when we analyze hemocyte-*Paracoccidioides* spp. interaction using flow cytometer, *P. lutzii* showed higher interactions with hemocytes. The gene expression of gp43 as well as this protein was higher for *P. lutzii*, and this expression may contribute to a greater adherence to hemocytes. These results helped us evaluate the behavior of *Paracoccidioides* spp in *G. mellonella*, which is a convenient model for investigating the host-*Paracoccidioides* spp. interaction.

Introduction

Paracoccidioidomycosis (PCM) is a systemic mycosis that is caused by thermally dimorphic fungi from the genus *Paracoccidioides*. This infection is endemic in Latin America and has higher incidences in Brazil, Argentina, Colombia and Venezuela.[1] In Brazil, the annual incidence reaches 30 per million inhabitants, and the mortality rate is 1.4 per million per year.[2] This mycosis has many clinical presentations, and retrospective studies have demonstrated that some factors, such as the host's immune response, age, gender and ethnicity, may influence the clinical presentation.[3] The incidence of chronic infection in the lungs and the upper respiratory tract is more frequent in men older than 30. The sub-acute form is commonly found in children and young adults and in afro-descendant women older than 20 years.[3,4] The *Paracoccidioides* spp mycelial form is found in nature and its propagules are the infective forms of the disease. The infection occurs upon inhalation of the propagules, and during this process, the fungus converts into a yeast form. The temperature is essential for this transformation.[1,5]

Genetic studies have revealed the existence of 3 different phylogenetic species in *P. brasiliensis* called S1, PS2 and PS3.[5] A new species was detected by using molecular approaches; the species started as Pb01-like and has been renamed *P. lutzii* .[6,7] In Brazil, a higher incidence of *P. brasiliensis* is found in the south and southeast region and *P. lutzii* predominantly occurs in the western-central part of Brazil.[8]

The *Paracoccidioides* spp. virulence mechanism involves dimorphism,[9] metalloproteins,[10] adhesins, biofilm formation,[11,12] and melanin production,[13] which play important roles in the infection. Fungal-cell interaction is an essential factor in the establishment of the infection, and for this action, the fungus recognizes specific ligands (carbohydrates and proteins) found in the extracellular matrix, thereby initiating infection and its subsequent spread.[14] In *Paracoccidioides* spp, many proteins known as adhesins mediate this process,[11] and those with multiple functions are known as 'moonlighting' proteins.[15]

Murine models are considered to be the gold standard for the study of fungal pathogenesis and for analyzing the efficacy of antifungal treatments. In 1959, initial studies were conducted in which mice were used as infection models for paracoccidioidomycosis.[16] At present, mice are the standard models for mimicking what occurs in the human disease. Many groups use infection by inhalation routes; however, some studies have described a lack of reproducibility caused by the difficulties the yeast have in reaching the lungs.[17-19] Another important aspect to consider is long-term survival curves when using mice as models; specifically, *P. brasiliensis* (Pb18) could require 2 to 8 months for each

*Correspondence to: Liliana Scorzoni; Email: liliscorzoni@yahoo.com.br; Maria José Soares Mendes-Giannini; Email: giannini@fcfar.unesp.br
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experiment.[20] Together with economical, logistical and ethical considerations, these challenges limit the use of mammals in infection experiments.[21,22] In this context, the use of alternative animal models or "non-vertebrate mini-hosts" such as *Galleria mellonella* appears to be an option for studying fungal or bacterial disease.[23-26]

The *Galleria mellonella* model is inexpensive to purchase and does not require specialized facilities for maintenance. The relatively large size of the larvae facilitates both the easy handling and injection of defined inoculums and the use of a large number of samples. Furthermore, in contrast to other invertebrate hosts, *G. mellonella* larvae can be maintained at temperatures of 37°C, which is equivalent to the temperature of mammalian hosts. This model presents structural and functional similarities to the innate immune response of mammals. Additionally, the *G. mellonella* innate immune system has 6 types of cells, including phagocytic cells, and the hemocyte density reflects the pathogenicity of the fungi.[21,27,28] *Galleria mellonella* also possesses genes for pathogen-associated molecular pattern recognition, such as toll-like receptors and the transcription factor nuclear factor-κB, in addition to antimicrobial peptides and proteins, most from the families moricin-like protein genes.[29]

The study of the virulence of different fungi such as *Cryptococcus neoformans*,[30,31] *Fusarium* spp,[32] *Candida* spp,[33,34] *Histoplasma capsulatum* and *P. lutzii*.[35] has been performed in *G. mellonella* successfully. *P. lutzii* was able to kill *G. mellonella*, however was not possible to establish a correlation between cell concentration and larvae death. Besides that, the infection caused larvae melanization and granuloma-like formation in a yeast concentration-dependent manner.[35] However, this model was only evaluated in *P. lutzii* but not in *P. brasiliensis*. The discovery of the *P. lutzii* species leads to new questions about virulence, clinical manifestations and diagnosis.[36,37] Moreover, because of the recent classification of 2 distinct species (*P. brasiliensis* and *P. lutzii*), few studies have compared the virulence and host interactions among them. Given this outlook, the aim of this study was to evaluate the virulence of *P. brasiliensis* and compare it with *P. lutzii* in the *G. mellonella* model. In addition, the hemocyte response to the infection was determined by measuring the hemocytic density, phagocytosis, hemocyte-fungal interactions as well as adhesins genes enolase, gp43, 14-3-3, malate synthase, and triosephosphate isomerase.

Results

Virulence of *P. brasiliensis* and *P. lutzii* in *G. mellonella*

This study provides the first description of a *P. brasiliensis* and *P. lutzii* virulence comparison in *G. mellonella*. The larvae were infected with 3 different concentrations of both species (5×10^5, 1×10^6 and 5×10^6 cells/larva) with the objective of finding the concentration that was able to kill them within 7 days of starting the experiment (**Fig. 1**). This time is considered appropriate for the use of the *G. mellonella* model because of the length of the insect's life cycle. The 1×10^6 cells/larva concentration of *P. brasiliensis* was able to kill more larvae than the same concentration for *P. lutzii* but in the end of the experiment almost 60% of the larvae were alive. The 5×10^6 cells/larva concentration was selected because the majority of larvae were dead in within the observation period. An analysis of the survival curve for both *Paracoccidioides* species, for the 5×10^6 cells/larva concentration, showed that the time necessary to reach 50% killing ($TD_{50\%}$) ranged from 3 to 4 days for *P. brasiliensis*, and the $TD_{50\%}$ ranged from 4 to 5 days for *P. lutzii*. However, the survival curve profiles were very similar, which suggested that *P. brasiliensis* and *P. lutzii* have similar virulence levels ($p = 0.3051$) in this model.

Histopathology analysis

The histopathology of uninfected and infected *Paracoccidioides* spp. larvae was also performed to study the infection by this yeast. Larvae were collected after one hour and 4 days of infection and stained with periodic acid Schiff (PAS). After one hour of infection, *P. brasiliensis* (**Fig. 2C**) and *P. lutzii* (**Fig. 2D**) were visualized in the periphery of the *G. mellonella* tissues. After 4 days of infection, *G. mellonella* tissues of larvae infected with *P. brasiliensis* (**Fig. 2E**) and *P. lutzii* (**Fig. 2F**) were destroyed when compared with uninfected larvae (**Fig. 2A, B**), and both fungal species were able to multiply inside the larvae. The cellular response to the invasion, encapsulation and granuloma, in addition to the humoral phenomenon, that is, the melanization, were observed in the histopathology (**Fig. 3**).

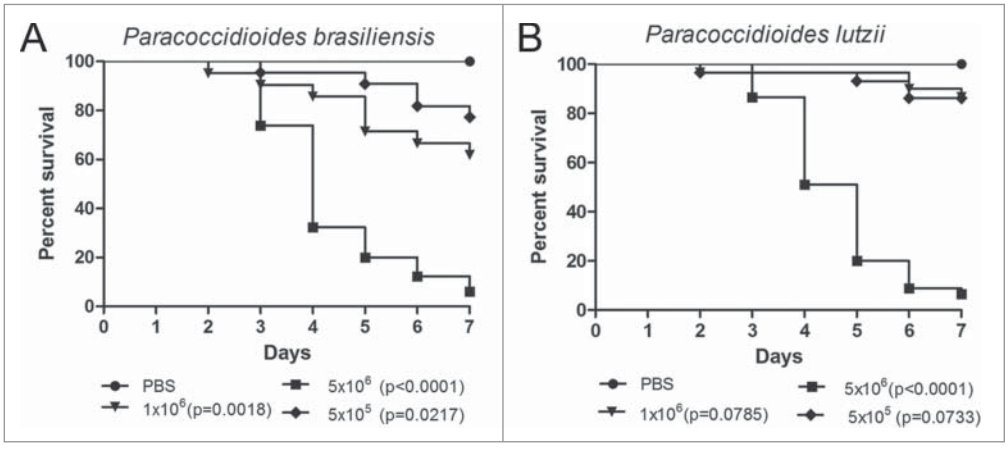

Figure 1. Survival curves of *G. mellonella* infected with *P. brasiliensis* (**A**) and *P. lutzii* (**B**) at different concentrations; PBS-infected larvae were used as controls and statistical significance ($p < 0.05$) is relative to the PBS control.

Hemocyte density after infection by *Paracoccidioides* spp

Changes in the hemocyte density are one important factor to observe in the larvae response when analyzing virulence. Larvae were infected with 5×10^6 cells/larvae and incubated at 37°C for one and 3 hours and measure the hemocyte density of *G. mellonella* after these periods of infection by *Paracoccidioides* species by using 2 methodologies, namely microscopy and flow cytometry (**Fig. 4**). For microscope estimation was used a hemocytometer under a brightfield microscope. According to the microscopy analyses, there was a hemocyte reduction of 5 and 8 times in both species at 1 and 3 h after infection, respectively (**Fig. 4A**). In the flow cytometry analyses, the hemocyte density after infection with *P. brasiliensis* or *P. lutzii* decreased 3 times for both incubation times (**Fig. 4B**). The results indicate that infections with 5×10^6 cells/larva of *P. brasiliensis* and *P. lutzii* reduced the density of hemocytes compared with the non-infected larvae during the analyzed time points. Additionally, the hemocyte density in the larvae infected with *P. brasiliensis* was similar to that with *P. lutzii*.

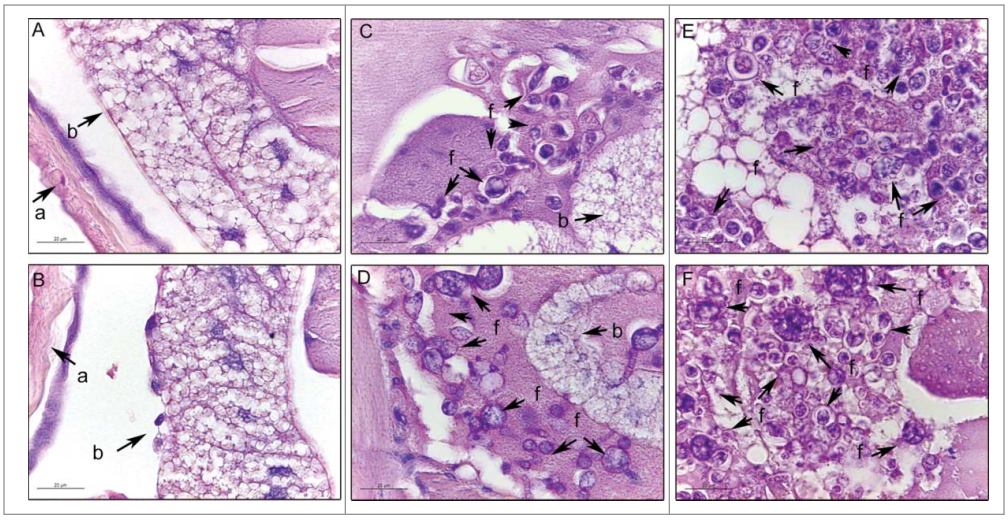

Figure 2. Histology of *G. mellonella* stained with PAS. Uninfected larvae (**A, B**); larva infected with *P. brasiliensis* after 1 hour (**C**) and after 4 days (**E**); larva infected with *P. lutzii* after 1 hour (**D**) and after 4 days (**F**). Amplification 1000x. Arrows indicates *P. brasiliensis* or *P. lutzii*. Structures annotated: (a) cuticle; (b) adipose bodies; (f) fungal cells.

As described above, hemocyte estimation was evaluated by using a traditional hemocytometer under a brightfield microscope and compared with the flow cytometer results. For this, *Paracoccidioides* spp suspension of 5×10^8 cells/mL was stained with 100 μM CFDA-SE for 30 minutes at 37°C and hemocytes samples of infected and uninfected larvae were stained with 0.165 μM Alexa Fluor® 647 phalloidin conjugates. The development of the flow cytometry assay for hemocyte estimation provide faster results and the possibility of analyze a greater amount of samples, moreover, because of the double stain the differentiation of fungi or hemocyte cells are precise. Both methodologies were comparable by Pearson coefficient (**Fig. 5**). Correlation coefficients for the hemocyte density in *G. mellonella* that were injected with PBS were 0.83 at 1 h ($p=0.0002$) and 0.80 at 3 h ($p=0.0006$), and those injected with *P. brasiliensis* were 0.86 for 1 h ($p<0.0001$) and 0.95 at 3 h ($p<0.0001$); those injected with *P. lutzii* were 0.96 for 1 h ($p<0.0001$) and 0.90 at 3 h ($p<0.0001$). This result suggested that a good correlation was found between the hemocyte density obtained by flow cytometry and by the standard method of counting cells on a hemocytometer (microscopy) in larvae that were infected with PBS or *Paracoccidioides* species at the different time points.

Paracoccidioides spp. - hemocyte interactions

The hemocyte-fungal interactions were evaluated by flow cytometry methodology. *Paracoccidioides* spp suspension of 5×10^8 cells/mL was stained with 100 μM CFDA-SE as described above and larvae were infected. After 1 and 3 h of infection, hemocytes samples of infected and uninfected larvae were stained with 0.165 μM Alexa Fluor® 647 phalloidin conjugates. The double-stained population (the CFDA-SE-positive and phalloidin-positive population) was examined for its hemocyte-fungal interactions using flow cytometry, and the percentage

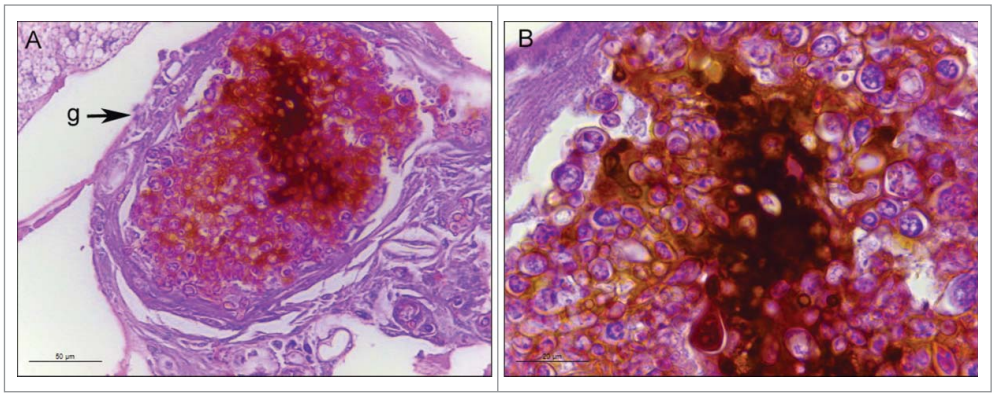

Figure 3. The cellular and humoral response to infection with *P. brasiliensis* after 3 days. Granuloma structure, amplification 400X (**A**); melanization and encapsulation process, amplification 1000x. (**B**). Similar structures were observed during *P. lutzii* infection. Structure annotated: (g) granuloma-like structure.

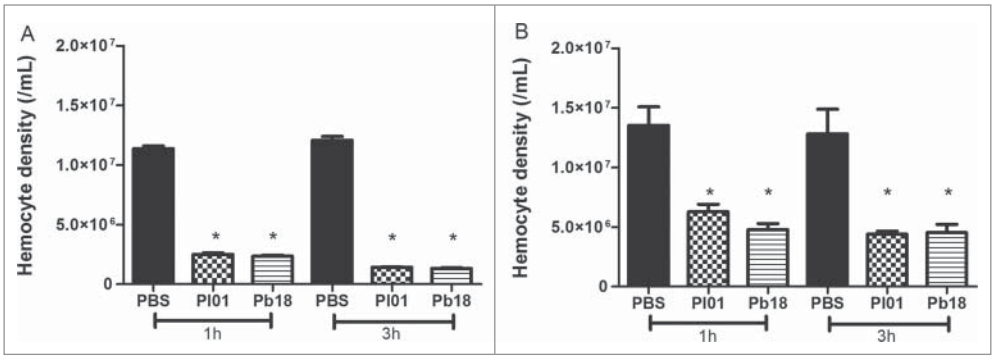

Figure 4. Hemocyte density, as obtained by microscopy (**A**) and flow cytometry (**B**), in *G. mellonella* larvae infected with *P. brasiliensis* (Pb18) and *P. lutzii* (PI01) when assessed after 1 and 3 h. The asterisks indicate statistical significance ($p < 0.05$) relative to the PBS control.

of double-stained cells in the population is shown in **Figure 6**. An important difference was observed in the hemocyte-fungal interaction profile between the fungal species. The *P. brasiliensis*-hemocyte interactions were 13 % and 14 % after 1 and 3 h, respectively (**Fig. 6A**); however, the *P. lutzii* interaction was 49 % and 48 % after 1 and 3 h, respectively (**Fig. 6B**). To evaluate the efficiency of the innate immune system against these species and consequentially the ability to escape from them, hemocytes phagocytosis was evaluated. The *Paracoccidioides* species were stained with 10 μg/mL Calcofluor white and larvae were infected with stained cells at 5×10^6 cells/larvae and incubated for 3 h at 37°C. After the infection hemocytes samples of infected larvae were stained with 0.165 μM Alexa Fluor→ 647 phalloidin conjugates and images were taken and a percentage of hemocytes containing yeasts was calculated using fluorescence microscopy. In this assay, 5 % of the hemocyte cells from *G. mellonella* were able to phagocytize *P. brasiliensis* or *P. lutzii* (**Fig. 7**), these results demonstrated that innate immune system of *G. mellonella* recognize equally these species.

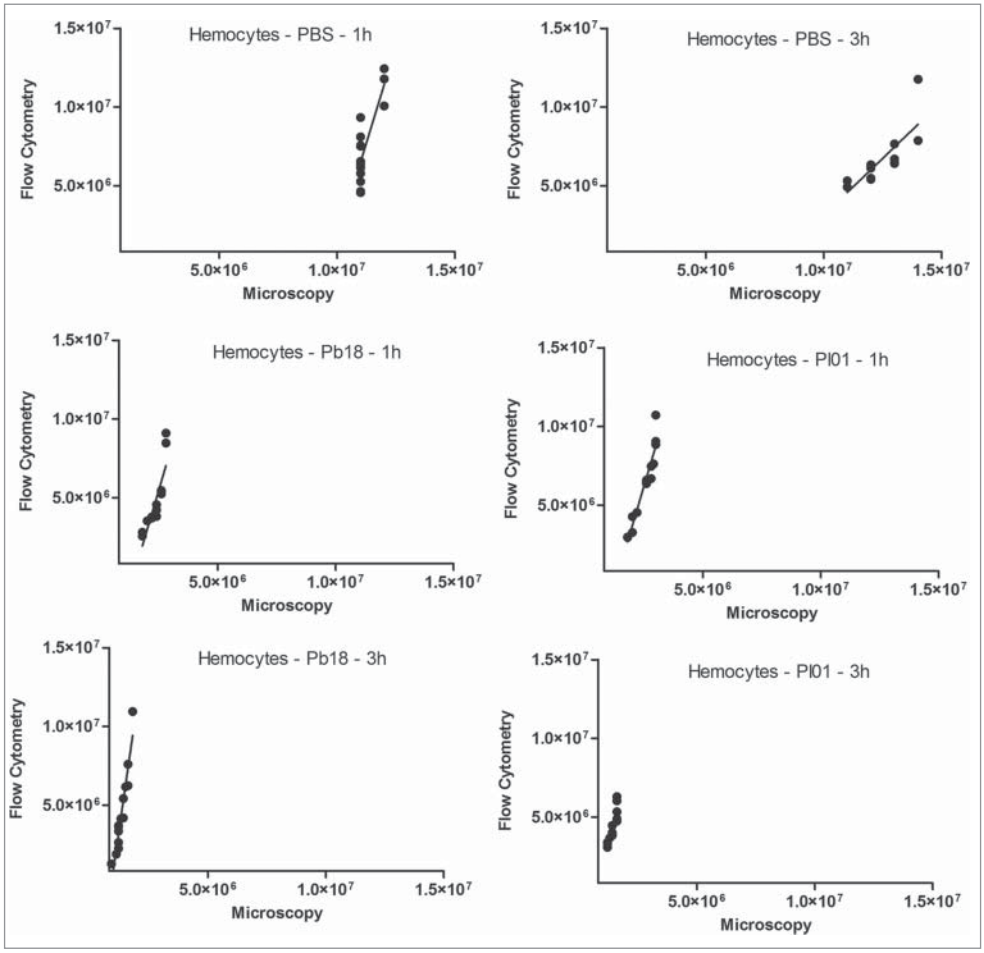

Figure 5. Linear regression and scatter plots for hemocyte counts (/mL) obtained by flow cytometry and microscopy (manually) in *G. mellonella* larvae injected with PBS, *P. brasiliensis* (Pb18 - 5×10^6 cells/larva) or *P. lutzii* (PI01 - 5×10^6 cells/larva) that were assessed after 1 and 3 h.

Paracoccidioides spp. adhesin gene expression

Adhesion is an important factor in establishing an infection. The gene expression of important adhesins in *Paracoccidioides* spp, namely enolase, gp43, 14-3-3, malate synthase, and triosephosphate isomerase, was analyzed after the infection in *G. mellonella*. For this, after one hour of infection the hemolymph from infected and uninfected larvae was collected and *Paracoccidioides* spp. cells were recovered by centrifugation, RNA was extracted and real-time PCR was performed. The *P. lutzii* expression of gp43 and triosephosphate isomerase were higher than they were in *P. brasiliensis* (13-folds ($p = 0.0011$) and 2.5-folds ($p = 0.0018$), respectively). This higher gene expression could explain the potential adhesion by *P. lutzii* to the hemocytes. For enolase, 14-3-3 and malate synthase, the expressions for both species were similar (**Fig. 8**).

Gp43 importance during *Paracoccidioides* spp- hemocyte interaction

Because of the importance of gp43 in the pathogenicity of *Paracoccidioides* spp, a Western blot assay was performed to verify the presence of this adhesin and compare it with the gene expression results. For this purpose, larvae protein extracts were obtained 3 days after infection with *P. brasiliensis*, or *P. lutzii* and control proteins were collected from *Paracoccidioides* spp., uninfected *G. mellonella*, and purified gp43. The presence of a 43 kDa fraction was observed in samples taken from *G. mellonella* that had been infected with *P. lutzii* and *P. brasiliensis* (**Fig. 9**). The antibody used in this work was obtained against the *Paracoccidioides* cells, and thus the test could detect other proteins in addition to gp43. In extracts from uninfected *G. mellonella* the presence of this protein was not detected.

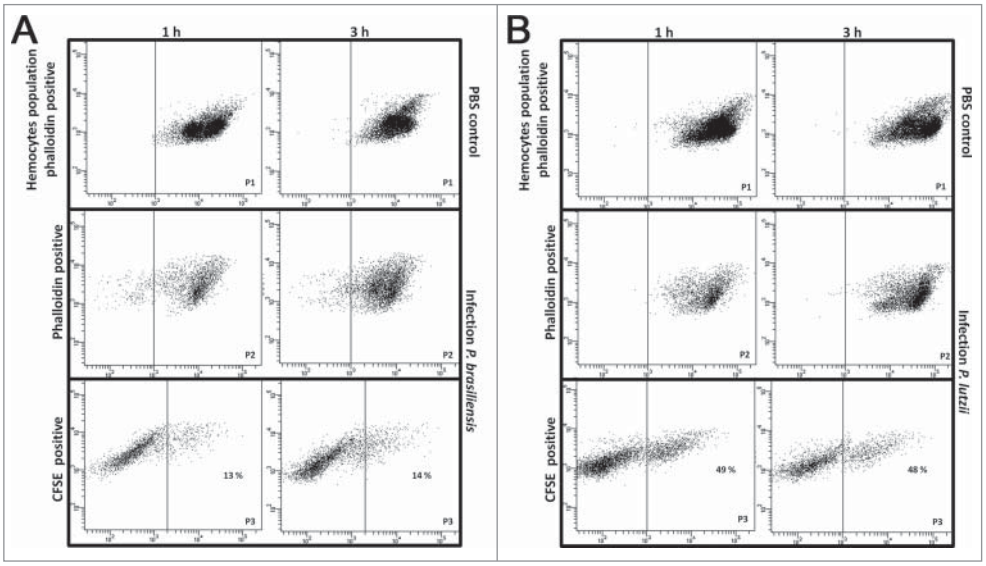

Figure 6. Hemocyte-fungal interaction obtained by flow cytometry after infection with *P. brasiliensis* (**A**) and *P. lutzii* (**B**). In gate P1, the hemocyte population is phalloidin positive with non-infected larvae; in gate P2, the hemocyte population is phalloidin-positive with infected larvae; and gate P3 holds the doubly stained population (hemocyte-stained phalloidin and fungal-stained CFDA-SE) that are considered hemocyte-fungal interactions that were obtained by flow cytometry after infection with *P. brasiliensis* (**A**) and *P. lutzii* (**B**). Differences in the hemocyte-fungal interaction between the fungal species ($p < 0.05$).

Discussion

Ethical questions are restricting the use of experimental vertebrate animals.[38] In this context, the use of alternative animal models such as amoebae (*Acanthamoeba castellanii* and *Dictyostelium discoideum*), nematodes (*Caenorhabditis elegans*), insects (*Drosophila melanogaster* and *Galleria mellonella*) and recently embryonated chicken eggs has emerged in recent years, and they provide good alternatives for studying fungal pathogenesis.[39-43]

In the last decade, *G. mellonella* has become more accepted as an alternative animal model of infection.[22,44] The structural and functional similarities between the immune responses of insects and mammals permits the insects to be used to predict the likely response of mammals to a variety of pathogenic microorganisms.[45] The hemolymph has analogous functions to blood and contains cells called hemocytes. There are at least 6 types of hemocytes and plasmatocytes, and granulocytes are the most abundant phagocytic cell types. Furthermore, antimicrobial peptides are also responsible for killing invading microorganisms.[46,47]

In this study, we used *G. mellonella* to establish a comparison between the virulence of 2 *Paracoccidioides* species. The survival curve profiles show that *Paracoccidioides* spp caused the concentration-dependent death of *G. mellonella*. Likewise, a correlation between the yeast cell number of *P. lutzii* and the time to death of *G. mellonella* was observed in a previous study, although the tested concentrations (10^1 to 10^6 cells/larva) were different from those in the present study.[35] However, the death time of larvae infected with 10^6 yeast cells was lower than that in our study. One explanation for this finding may be related to the different preparations of the inoculums. The cells of the fungus are very irregular in size and shape, and we believe that the use of inoculums with standard cell sizes was fundamental for the experiment.

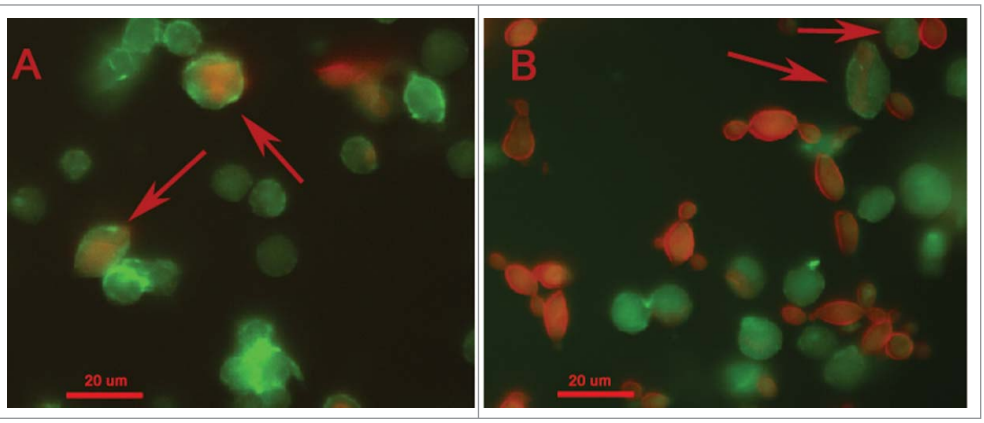

Figure 7. Phagocytosis of *Paracoccidioides* spp by hemocytic cells after 3 h of infection with 5×10^6 cells/larva from *P. brasiliensis* (**A**) and *P. lutzii* (**B**). The arrow indicates the phagocytosis.

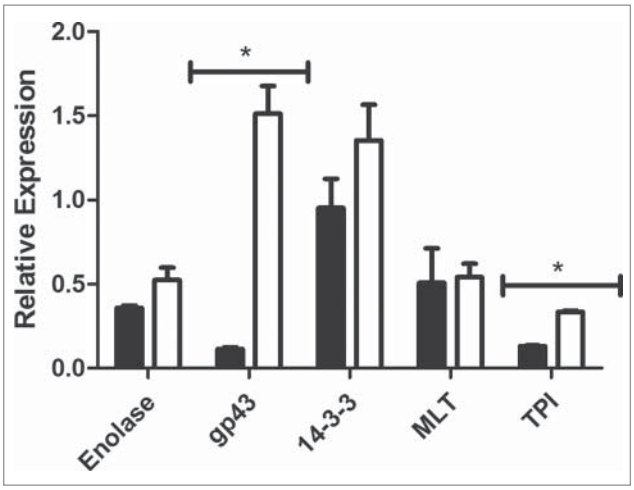

Figure 8. Relative gene expression of enolase, gp43, 14-3-3, triosephosphate isomerase and malate synthase in *P. brasiliensis* (black) and *P. lutzii* (white), (*) $p < 0.05$.

Paracoccidioides brasiliensis caused larval death faster (3-4 days) than *P. lutzii* (4-5 days). Furthermore, the histopathology of infected larvae at 1 hour and 4 days after the infection showed

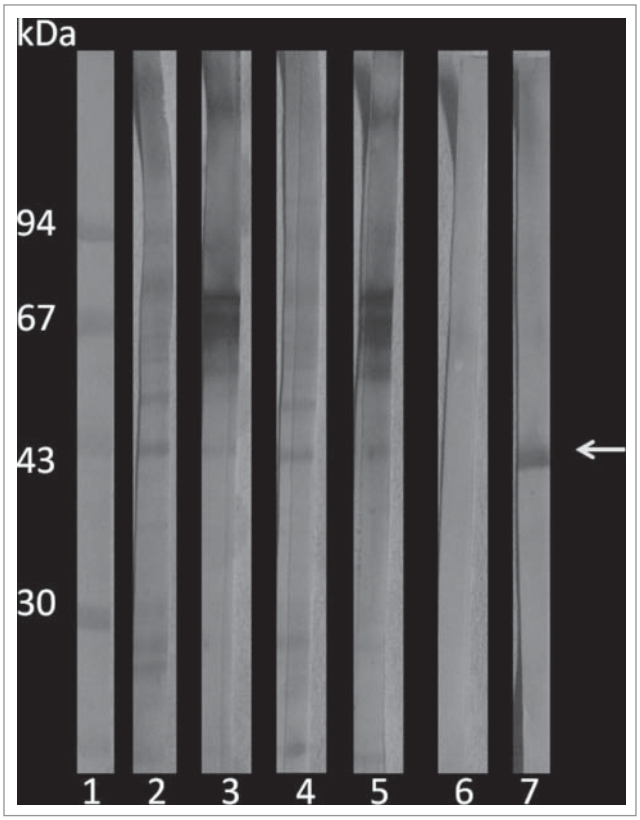

Figure 9. Western blot of *G. mellonella* infected with *P. brasiliensis* or *P. lutzii*. 1) molecular weight marker; 2) *P. lutzii*; 3) *G. mellonella* infected with *P. lutzii*; 4) *P. brasiliensis*; 5) *G. mellonella* infected with *P. brasiliensis*; 6) *G. mellonella* extract infection; and 7) purified gp43. The arrow indicates the gp43.

that both species were able to multiply inside the larvae and destroy the *G. mellonella* tissue. Moreover, encapsulation, melanization and granuloma-like structures were observed in the histopathology. The presence of granulomas were described during the paracoccidioidomycosis in murine model affecting the lung and spleen.[48] The effect of melanization on the elimination of pathogens is widely discussed, and the activation of the pro-phenol oxidase cascade produces substances that are very toxic to microorganisms and also helps in phagocytosis, aiding in the survival of invertebrates.[49] The encapsulation occurred when the cells were unable to phagocytize and thus tried to control the invasion by creating a cell barrier.[50]

By using 2 techniques, we demonstrated that the infection of *Paracoccidioides* spp. causes a decrease in the hemocyte density. After just one hour of infection, a significant reduction in the hemocyte density was observed in the infection with *P. brasiliensis* or *P. lutzii* compared with the control. The same reduction was observed after 2 more hours of incubation. Moreover, no significant difference was observed between the 2 tested species. Numerical differences between the techniques (flow cytometry and hemocytometer count) were observed; however, the hemocyte reduction profile remained the same. The hemocyte density is an important factor for evaluating virulence because low survival after infection is related to low hemocyte density.[51] In addition, the hemocyte density can vary during the infection, and a decrease was observed during the first hours of the infection.[52]

Another important aspect of this work is the standardization of hemocyte counting. We developed a practical flow cytometry protocol with double staining to determine the hemocyte density and the percentage of hemocyte-fungal interaction. The statistical analyses show a good correlation between the traditional hemocytometer estimation and the results obtained by flow cytometry. Thus, flow cytometry may be considered a reliable method for hemocyte counting because it is faster, more practical and gives precise results.

Phagocytosis was evaluated during the early stages of infection (3h), for both species, 5% phagocytosis was found, however, *P. brasiliensis* showed lower interactions with hemocytes when compared with the *P. lutzii* infection, which would indicate a better escape mechanism by this species from phagocytosis. The choice for 3h of post-infection time for phagocytosis assay is because of the significant reduction of hemocyte density, phagocytosis could be difficult to observe after more hours of infection. Other studies have demonstrated in *vitro* phagocytosis of *P. brasiliensis* around 7% after 6 h of infection and 6-12% after 24 h of infection depending on the type of macrophage studied.[53,54] Even though, in this model we have similar phagocytosis index. Additionally, the literature has been shown the absence of digestive and killing capacities of PMNs from paracoccidioidomycosis patients (susceptible individuals) in unambiguous contrast of cells from healthy (resistant) individuals.[55]

Studies showed significant similarity between the rates of *in vitro* phagocytosis of opsonized *C. albicans* by neutrophils and hemocytes. In the same way that it occurs for neutrophils, phagocytosis by hemocytes also stimulates the respiratory burst pathway involving NADPH oxidase activity and superoxide

production for efficient microbial killing.[56] Other similarities between neutrophils and hemocytes were demonstrated by treating the hemocytes with cytochalasin b and nocodazole, leading to an increase in the susceptibility of the *G. mellonella* larvae to the infection caused by a disruption of hemocyte function, which is the same way that they affect mammalian neutrophils. Earlier studies have described noticeable neutrophil infiltrates in *Paracoccidioides* lesions of experimental animal models and also in tissue samples from patients. Neutrophils were present in *P. brasiliensis* microabscesses and in loose granulomas from oral lesions.[57] Nevertheless, they are usually not sufficient to eliminate the fungus cells completely.

The hemocyte fungal interaction was studied in this work as an important key to discuss the difference between the virulence of both fungi. Differences in the virulence between *Paracoccidioides* spp strains have been reported, and they are likely related to antigenic differences and the adhesion process.[58,59] In this study, we observe that *P. lutzii* is able to interact with hemocytes 3.7-folds more than *P. brasiliensis.*

Different proteins with roles in fungal-cell adhesion are well-described for *P. brasiliensis*, and most of them participate in the glycolytic pathway, tricarboxylic acid cycle and glyoxylate cycle, and because of their multiple functions, these proteins are known as 'moonlighting' proteins.[15] In this work, we analyze the gene expression of 5 important adhesins from *Paracoccidioides* spp, namely enolase, gp43, 14-3-3, triosephosphate isomerase and malate synthase. A significant difference of expression for gp43 and triosephosphate isomerase was observed in *P. lutzii* after the infection of *G. mellonella*. Because of the importance of gp43, we investigated its expression by Western blot assay during the *G. mellonella* infection, and we tested if its presence can explain the higher hemocyte adhesion found for this species. Although gp43 production is detected *in vitro* within less than one hour of incubation,[60] we use 3 days of infection to detect this protein because of the low amount of yeast present in the sample. The 43 kDa glycoprotein was the first to be described as an adhesin and to be able to bind to laminin,[61,62] and it was more recently shown to bind both fibronectin and laminin. In *Paracoccidioides* spp., certain additional adhesins have also been described and may play an important role in pathogenesis.[59,63-67]

The importance of gp43 was studied.[68] by silencing the PbGP43 gene. No morphology, vitality or fungal growth was affected by the reduction in expression of gp43 gene. However, the expression of this protein was reduced *in vitro* and thus affected the recovery of yeasts from macrophages that were adhered to or phagocytized; and it was reduced *in vivo* from the lungs of infected mice. In addition to being described as an adhesin, the 43 kDa glycoprotein is the primary diagnostic antigen of *P. brasiliensis*.[69] Recent studies have demonstrated that gp43 is highly specific for PCM caused by *P. brasiliensis* and false negative for *P. lutzii*.[70,71] By analyzing the peptide sequence of gp43 protein in both species, an 81 % similarity was found; however, the molecular models show differences between them. Specifically, the N-glycosylation site is absent, and the glucanase site and its enzymatic activity are preserved in gp43 from *P. lutzii*.[36] The *Galleria* model has the same behavior as the *in vivo* model, and gp43 from *P. lutzii* may be expressed in the same way as it is in human disease. Moreover, gp43 appears to play a role in adhesion processes during the first hours of infection.

Triosephosphate isomerase (TPI) is a glycolytic enzyme that is present in the cytoplasm, and it is responsible for the conversion of dihydroxy acetone phosphate to glyceraldehyde-3-phosphate. Its gene was also more expressed in the *G. mellonella P. lutzii* infection. The importance of triosephosphate isomerase in paracoccidioidomycosis was first described when it was determined to be a protein that can react with the sera of PCM patients.[72] Moreover, when *P. lutzii* cells were treated with anti-TPI polyclonal antibody, an inhibition of the interaction with epithelial cells was observed. TPI plays an important role in host cell binding and could be considered an adhesion.[66] Triosephosphate isomerase is also one of the molecules responsible for interactions between other microorganisms. This binding may occur between the TPI of *S. aureus* and the mannotriose present in the capsule of *C. neoformans*, and the consequence of the binding is the death of *C. neoformans*.[73] Both microorganisms could be found in the nasopharynx, with *S. aureus* in healthy individuals. The hypothesis is that the presence of *S. aureus* in human nasal cavities may prevent the entry of *C. neoformans*,[74] and immunoelectron microscopy studies confirm that this cytoplasmic enzyme was localized on the cell surface and interacted with the mannotriose of the *C. neoformans*.[75] More studies are necessary to explain the differences between both species in the *G. mellonella* model as well as in human infections.

When studying virulence, it is necessary to consider that multifactorial events belong to the host and to the microbe; moreover, the likely phenomena of latency, colonization, commensalism and death could occur during host-microbe interactions.[76,77] In this study, we evaluated the virulence of *P. brasiliensis* and *P. lutzii* in a *G. mellonella* model by using survival curves, hemocyte density, phagocytosis, hemocyte interaction with *Paracoccidioides* spp yeast cells and adhesin expression. The parameter that differentiates the virulence of both species was yeast-hemocyte interaction, in which we found that *P. lutzii* has better abilities to adhere to the hemocytes than *P. brasiliensis*, and we attributed this capacity to the higher expression of important adhesins (gp43 and triosephosphate isomerase). However, some studies have addressed the expression of gp43 during *P. lutzii* infection in humans,[36] and in *G. mellonella*, it seems to be important to the adhesion process. These results were very important to understand the differences and similarities in both species better. Furthermore, *G. mellonella* was demonstrated to be a suitable model to study this fungus because of the ethical issues, cost, easy rearing and manipulation of larvae and reduced time, and the parameters analyzed in this study could be useful in the evaluation of different *in vivo* assays.

Material and Methods

Strains and growth conditions

Paracoccidioides brasiliensis (São Paulo, Brazil) (Pb18) and *P. lutzii* Pb01-like strain 01 (ATCC MYA-826/ Goiania, Brazil)

(Pl01) in the yeast phase were used. Both species were maintained in Fava-Netto medium at 37°C and subcultured every 4-5 days. After this period, the fungal mass was transferred to a Brain Heart Infusion (BHI) broth supplemented with 1 % glucose (BHI suppl).[78] for 3-4 days at 37°C at 150 RPM to obtain exponential-phase cells.[79,80] The *Paracoccidioides* spp strain stock was recently reisolated from cell culture.

Inoculum standardization

The *Paracoccidioides* spp growths were washed 3 times with PBS buffer after their cell clumps were separated by repeated passages through an 18-gauge needle (Becton Dickinson, Brazil) into a 5 mL syringe (Becton Dickinson, Brazil). The suspension was filtered through 40 μm nylon cell strainers (Corning, USA) to obtain cells of homogeneous sizes. The cell concentrations were estimated by using a hemocytometer. The inoculum was prepared in PBS plus 20 mg/L of ampicillin to prevent bacterial contamination.

***Galleria mellonella* rearing and larvae manipulation**

The larvae were fed with wax and pollen and maintained at 25°C.[81] until reaching 150-200 mg in weight. Larvae without color alterations and with adequate weights were selected, placed in Petri plates and incubated at 37°C overnight under protection from light. The insect pro-leg regions were cleaned with 70% ethanol before the experiments. Using a syringe (Hamilton, USA), 10 μL of inoculum was injected through the last left proleg of the larvae. Each experiment was repeated at least 3 times.

Survival assay

Sixteen larvae per group were inoculated with different inoculum concentrations of *Paracoccidioides* spp. (5×10^5, 1×10^6 and 5×10^6 cells/larva) for the survival assay. The control groups were larvae that were injected with PBS and untouched larvae. The larvae were incubated at 37°C, and this death was monitored daily for 7 days by visual inspection, which was done by checking for a lack of movement after touching the larvae with forceps.

Histopathology analysis

Uninfected and infected larvae were collected after one hour and 4 days of infection and processed as described by Scorzoni el al, 2013.[82] for a periodic acid Schiff (PAS) stain. Three tissue sections were observed per larva with a Leica DMI3000 microscope.

Determination of hemocyte density by flow cytometry

A *Paracoccidioides* spp suspension of 5×10^8 cells/mL was stained with 100 μM 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE/Sigma Aldrich, USA) for 30 minutes at 37°C and washed 3 times with PBS at 5000 rpm for 5 minutes. *Galleria mellonella* larvae were infected with the previously stained suspension and incubated at 37°C for one and 3 hours. A control group was set up by inoculating PBS. After the infection period, hemolymph samples (10 μL) were collected and stained with 50 μL of 0.165 μM Alexa Fluor® 647 phalloidin conjugates (Invitrogen) diluted in cold 4 % paraformaldehyde (Sigma Aldrich, USA) plus 0.25 % Triton X-100 (Amersham Biosciences, USA) and incubated for 20 minutes. The hemocyte density was determined by flow cytometry (BD FACSCanto) by acquiring 60 seconds on medium acquisition mode. In this mode, 60 μL was acquired, and the correlation was made in milliliters. A dot plot SSC-A (side scatter) by phalloidin (APC) was made to analyze the hemocyte number in control PBS (gate P1) and after the infection (gate P2). The hemocytes from larvae injected with PBS were compared with the infected ones.

Determination of hemocyte density using a hemocytometer

Larvae were infected with 5×10^6 cells/larvae and incubated at 37°C for one and 3 hours. After the infection period, hemolymph samples were collected and diluted at 1:20. A control group was set up by inoculating with PBS. The hemocyte density was estimated by using a hemocytometer under a brightfield microscope. The control was compared with PBS injected larvae.

Hemocyte-fungal interaction assay by flow cytometry

The hemocyte-fungal interactions were evaluated by flow cytometry methodology. Double-positive fluorescent-stained cells (with phalloidin and CFDA-SE) were used to observe fungal cells that adhered to or phagocytosed the hemocytes. Stain control was used on the fungal cells and hemocytes alone, under unlabeled and labeled conditions, with both stains and one stain alone. All cytometry data were analyzed using the BD FACS Diva software.

Phagocytosis assay

The yeast cells were stained with 10 μg/mL Calcofluor white (Sigma Aldrich, USA) for 30 min at 37°C. After that, 10 larvae were infected with stained cells at 5×10^6 cells/larvae and incubated for 3 h at 37°C. The hemolymph samples were collected and the hemocytes were stained as described for the flow cytometry assay. The hemolymph samples of each larva were placed in a 96-well plate (made of a plate glass bottom and black borders; Glass Bottom/GE), and the images were acquired with In Cell Analyzer 2000-GE at an amplification of 60x. One hundred hemocytes from each larva were counted and the percentage of hemocytes containing yeasts was calculated.

***Paracoccidioides* spp gene expression of adhesins by real-time PCR**

Genes encoding for enolase, gp43, 14-3-3, triosephosphate isomerase and a malate synthase protein described as adhesin in *Paracoccidioides* spp. were analyzed by real-time PCR. For this analysis, 60 larvae were infected with 5×10^6 cells/larvae of *P. brasiliensis* or *P. lutzii* and incubated for one hour at 37°C, and uninfected groups of larvae and *P. brasiliensis* or *P. lutzii* without pass-through infection were used as controls. The hemolymph from each group was collected, and the *Paracoccidioides* spp cells were recovered by centrifugation and washed twice with PBS. RNA was extracted with RNeasy (Qiagen). First-strand cDNA synthesis was performed with reverse transcriptase (RevertAidTM H Minus Reverse Transcriptase, Fermentas, Canada). Real-time PCR was performed with Maxima® SYBR Green/ROX qPCR

Master Mix (2X) (Fermentas, Canada) using Applied Biosystems 7500 cycler equipment. The relative expression was calculated by using $2^{-\Delta\Delta CT}$ values with 60S ribosomal L34 as the housekeeping gene; this constitutive gene is widely described as a control in different studies on paracoccidioidomycosis.[83,84]

Protein Extraction and Western Blot assay

To perform a Western blot, 16 *G. mellonella* larvae at 3 days of infection were used with *Paracoccidioides* spp., along with 16 larvae that were not infected and *Paracoccidioides* cells. The protein extraction was performed first, which involved adding 3 mL of 10 mM Tris-HCl pH 6.8 and protease inhibitor mix to each sample. The samples were then lysed with glass beads and vortexed. The material was centrifuged at 5000 rpm for 1 hour at 4°C. After centrifugation, the supernatant was collected and the proteins were precipitated with 10 % TCA in 90 % acetone. The precipitated proteins were diluted in 10 mM Tris-HCl pH 6.8 and quantified by Bradford assay. One hundred micrograms of each sample was applied to an SDS-PAGE gel to separate the proteins. The proteins were subsequently transferred to a nitrocellulose membrane. The membrane was incubated with a polyclonal antibody that was obtained against a total *P. brasiliensis* extract [85] and the secondary antibody was rabbit anti-mouse IgG conjugated to peroxidase. The reaction was developed with chromogen: diaminobenzidine (DAB) substrate diluted in PBS that was added to hydrogen peroxide. For controls, we used the protein extract of uninfected *G. mellonella*, purified gp43 and *Paracoccidioides* spp protein.

Statistical Analysis

Graphs and statistical analyses were performed with GraphPad Prism 5 (La Jolla CA, USA). Survival curves were analyzed by Log-rank (Mantel-Cox) test. The hemocyte density data were analyzed by ANOVA with Tukey's post-test. A comparison of the hemocyte density by using a flow cytometry and a hemocytometer was performed with a Pearson correlation, and the real-time PCR was analyzed by t-test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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