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Galleria mellonella Antimicrobial Peptides and Stress Management Gene Expression in Response to Deleterious Events Caused by *Sporothrix brasiliensis*

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Abstract: *Sporothrix brasiliensis* is the most pathogenic species, responsible for the Brazilian cat-transmitted sporotrichosis hyperendemics. In this scenario, investigation of pathogen-host interaction can provide relevant information for future treatment strategies. To this end, the invertebrate *Galleria mellonella* has proven to be a suitable alternative to evaluate the virulence of pathogenic fungi since the insect immune system is similar to the mammalian innate immune response. The aim of this work was to investigate phenotypic and molecular aspects of the immune response of *G. mellonella* throughout the *S. brasiliensis* infection. Hemocyte density and the evolution of the fungal load were evaluated. In parallel, RT- qPCR expression analysis of genes encoding antimicrobial peptides (*Gallerimycin* and *Galiomycin*) and stress management genes (*C7 Contig 15362* and *C8 Contig 1910*) was conducted. Fungal load and hemocyte densities were proportionally increasing simultaneously to the deleterious morphological events and larvae mortality. *Gallerimycin, C7 Contig 15362,* and *C8 Contig 19101* genes were positively regulated ($p<0.05$) at distinct moments of *S. brasiliensis* infection, characterizing a time-dependent and alternately modulated profile. *Galiomycin* gene expression remained unchanged. Our results contribute to the future proposal of potential alternative pathways to treat and, consequently, control *S. brasiliensis* zoonosis, a major public Health in Latin America.

Keywords: sporotrichosis; gene expression; invertebrates; antimicrobial peptides; zoonosis; immune response

1. Introduction

Sporotrichosis is an affection of great importance to public health since human infection can occur through direct contact with contaminated soil organic matter (sapronosis) or, more frequently, by the zoonotic route through the domestic feline [1,2]. In this last scenario, the incriminated fungus is *Sporothrix brasiliensis*, the highest virulent and pathogenic species of the *Sporothrix* genus, with strong evidence of drug resistance [3,4]. The *S. brasiliensis*-zoonotic transmitted sporotrichosis began as an outbreak in the late 1990s in Rio de Janeiro, Brazil, and currently is considered a nationwide hyperendemic [5,6] with reports of recent geographical expansion to other Latin American countries

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[7,8]. Recently, the first three cases of domestic feline-transmitted *S. brasiliensis* outside South America were described in the United Kingdom, Europe [9].

Itraconazole is the drug of choice for human and feline sporotrichosis [10,11]. However, the cat's treatment remains a challenge for the veterinarian, given the limited number of antifungal agents, their high cost and adverse effects, and their commercial presentations. Moreover, little is known about the immune response triggered by the infected hosts, especially the domestic feline [1,11].

In this direction, the expansion of studies that aim to expand information related to the pathogen-host interaction is necessary. To this end, *Galleria mellonella* stands out as an invertebrate model for the investigation of the immune response triggered by distinct fungal pathogens such as yeast of the *Candida* genus [12,13] and *Cryptococcus neoformans* [14]. Among filamentous fungi, *Aspergillus niger*, *Rhizopus* spp. and *Rhizomucor* spp. have also been challenged against the invertebrate biomodel's defense [15–17], as have also the dimorphic *Paracoccidioides lutzii* and *Histoplasma capsulatum* [18,19]. However, few studies were dedicated to the interaction between *G. mellonella* and fungi of the *Sporothrix* genus, all focusing on phenotypic aspects to investigate the host-pathogen interaction [20–22] or *in vivo* drug-response [23].

The *G. mellonella* immune system has close both structural and functional similarities to the mammalian innate immune system [24,25], and is divided into two strongly interconnected pathways: the cell-mediated pathway and the humoral pathway [26]. The cellular response of insects is mediated by hemocytes, phagocytic cells in the hemolymph. These have the function of phagocytosing foreign bodies, capturing and encapsulating them in multicellular structures called nodules or capsules [26–28]; playing roles analogous to those of human macrophages and neutrophils [24,25]. In parallel, the humoral response of insects includes antimicrobial peptides (AMPs), which act directly against invaders. Among the *G. mellonella* AMPs, gallerimycin and galiomycin are the most well-described in the literature [29], with evidence of positive regulation on gene expression in fungal infections, such as *Candida spp.* and *Aspergillus niger* by this insect [17,30,31].

In a complementary investigation, other parameters such as the role of stress-managing genes can be evaluated during the immune response of the larvae against pathogens [30]. Among the best-characterized ones are those responsible for the regulation of cytokines (*C7 Contig 15362*), those linked to the phagocytosis and related to protein binding (*C8 Contig 19101*) and those involved in inflammatory responses (*C3- Contig 15265, C4- Contig 290595, C5- Contig 21310, C6- Contig 1327*) [30,32,33].

Data about the cellular and humoral insect defenses during the infectious process can provide new information on the host-pathogen interaction. Furthermore, elucidating mechanisms of *S. brasiliensis* infection in the invertebrate model may contribute to the future detection of new therapeutic targets as well as to the description of promising molecules to control this mycosis. Thus, the objective of the present study was to monitor *G. mellonella's* phenotypic and genotypic responses against *S. brasiliensis*. To the best of our knowledge, the present study is the first to evaluate the defense-related gene expression of *G. mellonella* in response to infection by a dimorphic fungus.

2. Materials and Methods

2.1. Strains and Culture Conditions

The reference strain of *Sporothrix brasiliensis* used in this work was the American Type Culture Collection isolate, ATCC MYA 4823. The yeast cells were maintained by cryopreservation at -80 °C in the mycotheque of the Center for Microorganisms' Investigation (CIM-UFF), until their reactivation for the experiments. For this purpose, the cryotubes were defrosted with subsequent replicas in YPD medium (Yeast Extract Peptone Dextrose), with incubation under shaking at 37°C for 5 days. The microbial growth in the broth was then centrifuged at 2000 xg for 5 minutes, washed with sterile PBS (Phosphate Buffered Saline; Fujifilm Irvine Scientific), and suspended in the same buffer. After these processes, standardized suspensions of $1x10^7$ yeast/larvae were prepared using a hemocytometer [20,21].

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2.2. Galleria mellonella Survival Assays

The invertebrate biomodel investigated was kindly provided from a well-established colony by the Biofilms and Microbial Diversity Laboratory of the Federal University of Rio Grande do Sul. Survival curves were performed with larvae in the final larval stage of development, weighing between 0.2g and 0.3g, with uniform coloration, without spots or signs of melanization [34]. Inoculation of the fungus was subcutaneous, with 10 µL of the inoculum applied in PBS with a Hamilton syringe (701N, Caliber 26, Hamilton Company Reno, USA) in the last left proleg, as recommended [21,34]. Infected larvae were grouped by experimental condition, kept at 37º C in 14 cm Petri dishes, and monitored daily for survival. Lack of movement and extensive melanization of the body were both taken as indicators of animal death. As a control, two groups of animals were selected, one group with only the physical injury of the needle (naive) and the other injected with PBS, to assess mortality from animal handling and mechanical injury. Each group, including the control, consisted of 30 larvae.

2.3. Determination of fungal burden during the survival curve

To monitor the fungal load during the different phases of infection, an experiment with ten (10) inoculated larvae was conducted with the same inoculum ($1x10^7$ yeast/larvae) and under the same temperature conditions, in triplicate, as detailed earlier in the survival curve. Daily, one larva was randomly selected from the pool, which was previously cleaned with ethanol 70% and then the hemolymph was collected. For this purpose, the larvae were sectioned with a sterile scalpel on the lower part and 30 µL of hemolymph were collected from each individual, placed in an Eppendorf® tube containing physiological saline buffer for insects (IPS: 150 mM sodium chloride, 5 mM potassium chloride, 10 mM Tris-HCl, pH 6.9, 10 mM EDTA and 30 mM sodium citrate) in a 1:10 proportion (35). Soon after, serial dilutions were made on Sabouraud agar plates plus Chloramphenicol (Sigma-Aldrich, St. Louis, Missouri, USA), incubated for seven days at 25ºC. After this period, one of the colonies was chosen for observation of confirmatory micromorphology of the genus *Sporothrix* using methyl blue staining. Once the genus was detected, all colony forming units (CFUs) were counted as described by Clavijo-Giraldo and colleagues [21].

2.4. Determination of hemocyte density in hemolymph

Similar to the methodology used for hemolymph collection in the previous item, hemocyte density was estimated from 30 µL of hemolymph diluted in IPS in a 1:10 proportion. The number of hemocytes was counted using a hemocytometer [36]. The experiment was performed in triplicate and the results were expressed in hemocytes/mL. In parallel to the quantitative analysis, qualitative notes of phenomena such as nodule formation as well as yeast adhered to the hemocytes were made.

2.5. Analysis of gene expression

2.5.1. RNA extraction and cDNA sythesis

For the RNA extraction procedure, 20 control larvae with only the physical injury and 20 larvae inoculated with $1x10^7$ *S. brasiliensis*/larvae were selected. The assays to investigate the dynamics of gene expression were performed at three moments of the survival curve: on days one, five, and eight (D1, D5, and D8) after infection by *S. brasiliensis*. For each incubation time (D), six (06) larvae from each group were randomly selected, cooled on dry ice, and conditioned in microtubes for total RNA extraction from the tissue. For this purpose, the larvae were macerated using a sterile pestle, and the extraction was performed with SV Total RNA Isolation System kit (Promega Corporation, Madison, WI, USA), following the manufacturer's recommendations. The total RNA was transcribed into complementary DNA (cDNA) using GoScript Reverse Transcription System Kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. In the cDNA synthesis, besides the reverse transcriptase, the DNase enzyme was also used to eliminate possible contaminating DNA in the sample. The cDNA was then quantified in the Quantus Fluorometer

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(Promega Corporation, Madison, WI, USA) using the QuantiFluor One dsDNA System kit (Promega Corporation, Madison, WI, USA). Finally, samples were diluted to the final use concentration of 2.5 ng/µl.

2.5.2. Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)

Quantification of gene expression was performed by RT-qPCR using the Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, Carlsbad, CA). The gene expression levels related to the production of Gallerimycin and Galiomycin AMPs in addition to *C7 Contig 15362* and *C8 Contig 19101* genes were evaluated at the three time points of infection. The gene *β-actin* was used as a normalizing reference control to monitor the amount of a housekeeping gene not involved in immune response, using the same cDNA preparations. The primer sequences used are specified in Table 1. The group of non-inoculated larvae was used as a control, which was compared with the group inoculated with the fungal pathogen. The samples and controls were tested in triplicate and all reactions were performed in 96-well plates with a final volume of 15 µl, being: 7.5 µl of Power SYBR™ Green PCR Master Mix, 300 nM of each primer, 7.5 ng of cDNA and DEPEC water (InvitrogenTM, Carlsbad, CA, USA) to complete the volume.

Table 1. Genes quantified during the fungal infection process of *Galleria mellonella* by *Sporothrix brasiliensis*. The references used as well as the primer sequence.

Gene	NCBI Genbank References	Sequence (5′ – 3′)	Annealing temperature
Galiomycin	AY528421.1 [37]	**F-** TCCAGTCCGTTTTGTTGTTG **R-** CAGAGGTGTAATTCGTCGCA	60ºC
Gallerimycin	AF453824.1 [37]	**F-** GAAGATCGCTTTCATAGTCGC **R-** TACTCCTGCAGTTAGCAATGC	60ºC
C7 Contig 15362	*Contig 15362* [30]	**F-** CGAGCTAAAGACAGGCGATT **R-** TCACCTGCGGTTGAATCATA	58ºC
C8 Contig 19101	*Contig 19101* [30]	**F-** ATTGCTAGCCAGGTTCAGGA **R-** AGCTATTTGGCGGAAACTCA	60ºC
β-actin	[38]	**F-** GGACTTGTACGCCAACACAG **R-** CCACATCTGCTGGAATGTCG	55ºC

The reaction was performed in the 7500 Fast Real-Time PCR System (Applied Biosystems, Framingham, MA, USA) with the following conditions: initial heating at 50ºC for 2 minutes, denaturation at 95ºC for 2 minutes, followed by 40 cycles of denaturation at 95ºC for 15 seconds, annealing and extension at temperatures ranging from 55º-60ºC for 1 minute, depending on each primer (Table 1). In the end, a melting curve was performed to verify the specificity of the reaction - 95ºC for 15 seconds, 60º for 1 minute, followed by 30 seconds at 95ºC. The mean of the threshold cycle (cT) values, measured in triplicate, were used to calculate the expression of the target genes. The results were obtained as relative gene expression values (based on the formula: $2^{-\Delta\Delta CT}$) compared to the reference gene expression, the *β-actin*, resulting in a value equal to 1.

2.6. Statistical Analyses

Survival analyses of *G. mellonella* infected with fungal pathogens were estimated using the Kaplan-Meier survival curve, in which the log-rank test was used to compare the groups in terms of survival. The evolution of fungal load over days and the relationship between hemocyte count and CFU were analyzed using simple linear regression models. Adherence to the Normal distribution of the variables was done using the Shapiro-Wilks test. Kruskal Wallis test and Dunn's post hoc test were used to check the dynamics of gene expression as a function of day for each experimental group. The Mann-Whitney test was used to compare the relative quantification of genes on days D1, D5, and

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D8, as a function of the non-inoculated and inoculated groups. The significance level adopted was 5%, and the software used was R, version 4.1.2.

3. Results

3.1. Galleria mellonella Survival Assays

The survival curve showed that larvae inoculated with 1x10^7 yeast of *S. brasiliensis* started to die on day 4, while the majority of all naive larvae and those inoculated with PBS only remained unaltered ($p < 0.001$; Kaplan-Meier), reaching the pupal stage in the last days of the curve. The results are represented in Figure 1A.

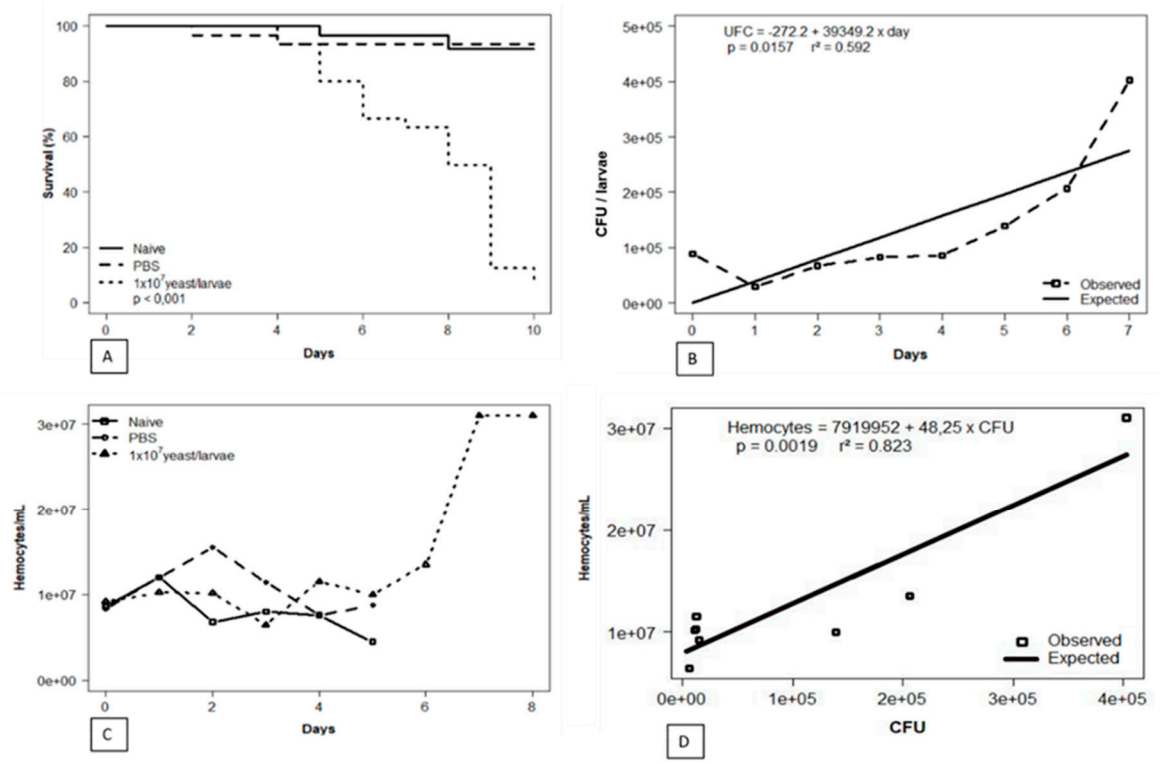

Figure 1. Phenotypic investigation of the infection dynamics in the invertebrate host *Galleria mellonella* against the fungal pathogen *Sporothix brasiliensis.* **(A)** Survival curve performed up to the tenth day of infection. **(B)** Monitoring the fungal load from the insect's hemolymph during the infection process. **(C)** Hemocyte count performed from insects in the control groups and the group infected with *S. brasiliensis.* **(D)** Parallel drawn between insect cellular immune response versus fungal load along the survival curve.

3.2. Fungal load during the infection

Parallel to the survival curve, daily collection followed by hemolymph serial dilutions and subsequent plating were performed to obtain CFUs. Colonies whose macromorphology was compatible with the genus *Sporothrix* were further evaluated by micromorphological analyses, evidencing thin, hyaline, septate, branched hyphae, and conidia arranged in flower-like structures. Figure 1B shows the evolution of the fungal load along the survival curve, with increasing values from 6x10^3 up to 4x10^5 CFU/larva ($\bar{x}$ = 1,01 x10^5; σ ±1.4x10^5). A direct correlation between the increase of *S. brasiliensis* yeasts over the days was observed (p= 0.0157).

3.3. Sporothrix brasiliensis-Galleria mellonella Interaction

Daily hemocyte counting allowed the detection of increasing insect cellular defense generated by the fungus, verified by values from 6.4x10^6 up to 3.0 x10^7 hemocytes/mL along the curve ($\bar{x}$ = 1,28 x10^7; σ ± 7.6 x10^6). The hemocyte count of the larvae from control groups (naive and PBS) was

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performed until D5 since afterward, both began to develop into pupae. In contrast, larvae infected with *S. brasiliensis* showed slower development in the applied experimental conditions (Figure 1C).

The hemocyte count associated with the determination of the fungal load along the survival curve allowed the evaluation of the *Sporothrix brasiliensis-Galleria mellonella* interaction dynamics. Progression of infection by the fungal pathogen triggered significant, proportional growth in insect hemocyte density (p = 0.0019; r2 = 0.823; Figure 1D).

3.4. Gene Expression

3.4.1. Antimicrobial peptides

For the gene encoding the AMP Gallerimycin dynamics, an early higher expression was observed, since the first day after infection (D1; Figure 2A), compared to naive larvae (p<0.0001; Mann-Whitney test). The higher expression in S. brasiliensis infected larvae remained on the subsequent days D5 (p> 0.05) and D8 (p = 0.0011; Mann-Whitney test). In parallel, comparing the intragroup Gallerimycin gene expression, a significant reduction was observed in the infected larvae (p = 0.0055; Kruskal-Wallis test), contrarily from the observed for the control group (p = 0.9968; Figure S2A; Supplementary).

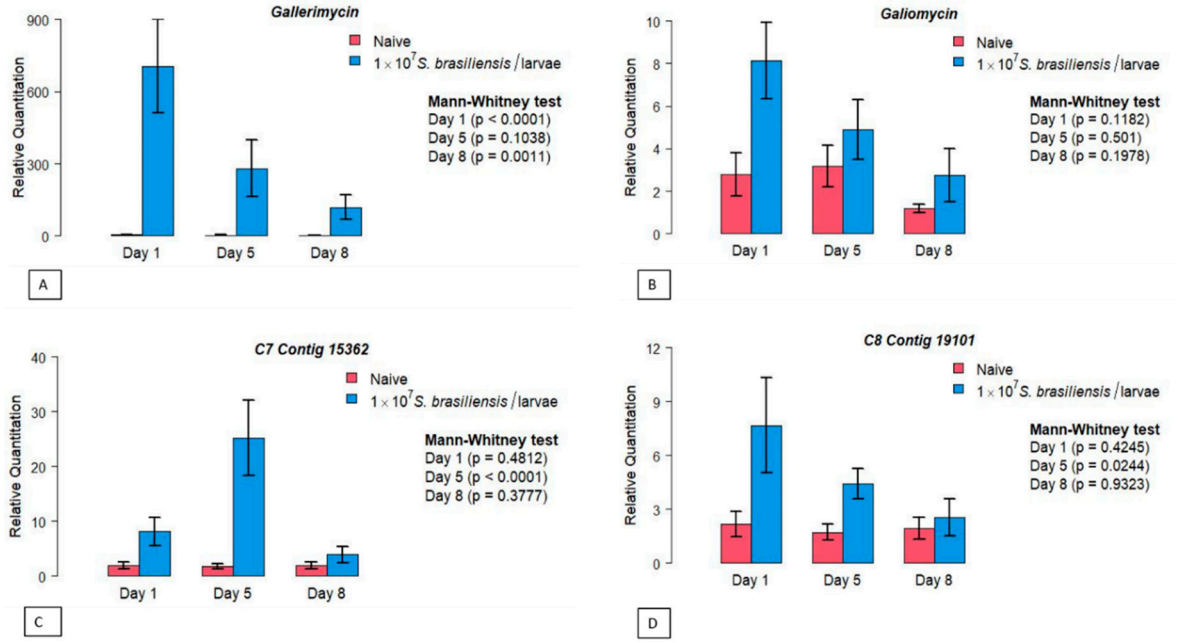

Figure 2. Investigation of the gene expression profile of the invertebrate host *Galleria mellonella* in infection by the pathogenic fungus *Sporothix brasiliensis*. Relative quantification of genes encoding antimicrobial peptides **(A)** Gallerimycin, **(B)** Galliomycin. And the stress manager genes **(C)** *C7 Contig 15362* and **(D)** *C8 Contig 19101*. The units on the Y axis were calculated based onthe $2^{-\Delta\Delta CT}$ method and are expressed as mean. Each gene was normalized and compared to the expression of control (naive) insects using the *β-actin* reference gene. The Mann-Whitney test was used to compare the relative quantification of genes and a $p \leq 0.05$ value was considered significant.

Differently, the quantification of the expression of the gene encoding the Galiomycin AMP revealed no significant differences between the two experimental groups, as well as after the intragroup analysis (p>0.05; Mann-Whitney test; Figures 2B and S2B).

3.4.2. Stress managing genes

The C7 Contig 15362 mRNA expression analysis differs between the investigated groups at D5 post-inoculation (p<0.0001; Mann-Whitney test). After this period, it decreases and at D8 shows its

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lowest values (Figure 2C). Analysis of the intragroup gene expression showed a significant difference between the three evaluated periods ($p = 0.0024$; Kruskal-Wallis test; Figure S2C), with higher levels of mRNA production at D5. This difference was not observed in non-infected larvae between the evaluated days ($p > 0.05$; Supplementary).

The expression of C8 Contig 19101 showed an initial, non-significant increase in mRNA expression, since the first day after S. brasiliensis inoculation (D1; $p>0.05$). However, at D5 an important increase in C8 Contig 19101 gene expression was detected compared to naive larvae (Figure 2D). In the intragroup comparison, C8 Contig 19101 mRNA expression did not vary significantly between D1 x D5 x D8 ($p>0.05$; Kruskall-Wallis test; Figure S2D).

4. Discussion

Although sporotrichosis is a disease of great importance in public health, numerous aspects of the pathogen-host relationship are yet to be elucidated, mainly for the main host of this zoonosis, the domestic feline [1,39]. As a matter of fact, such an investigation requires an appropriate experimental model. For decades, the murine model has been used as the *in vivo* gold standard model for pathogenicity mechanisms of distinct microorganisms [40], including *Sporothrix* spp [41]. However, recently, the scientific community has been showing ethical and social concerns, applied to the rationalization of animal model use [29,42,43].

Therefore, aiming at obtaining an alternative approach to mammalian models, several invertebrate models of infection have been studied, with emphasis on *Galleria mellonella* [44,45]. In spite of the previous use of this insect for the investigation of different fungal and bacterial infections [19,46], studies concerning the *Sporothrix* genus pathogenicity are still limited. As a matter of fact, only four previous studies investigated *S. brasiliensis-G. mellonella* binomial, based on the description of phenotypic aspects [20–23]. Therefore, this is the first study to draw a parallel between *G. mellonella* inate immunity-related gene expression and deleterious events during *Sporothrix* infection.

The results obtained on the survival curve of *S. brasiliensis* infection *in G. mellonella* standardization showed that the ideal yeast inoculum ($1x10^7$ yeast/larva) and temperature (37°C) were partially similar to those described by Clavijo-Giraldo and collaborators [21]. For more efficient killing by the fungal pathogen, these authors concluded that temperatures closer to the natural mammalian host are required to maintain all virulence attributes expressed by the yeast morphology. However, a less concentrated inoculum of $1x10^5$ yeast/larvae was sufficient for these authors to obtain an appropriate survival curve. It is believed that this difference may be related mainly to the lineage of the larva used or even uncontrolled environmental conditions. On the other hand, Freitas and co-authors [20] used the same fungal load of $1x10^7$ yeast/larva, since when testing lower concentrations ($1x10^4$ yeast/larva and $1x10^6$ yeast/larva), they did not observe mortality in the survival curve. In addition, the temperature of 37°C was also recommended for the experiment, corroborating in both aspects with the results obtained here.

Daily monitoring of *S. brasiliensis* load during the infection curve provided progressive increasing values ranging from $1.2x10^4$ to $4x10^5$ CFU per animal. An average fungal load ranging from $1.8x10^5$ to $2.4x10^5$ CFU per animal was previously described [22] after 24 hours of infection. Gandra and colleagues [31] observed that by inoculating $1x10^7$ *Candida albicans*/larvae, CFUs ranged from $1.0x10^8$ to $1.0x10^{12}$ at 6, 24, and 48 hours. These data reinforce the suitability of *G. mellonella* as a model of yeast infection since it differentially mirrors *Candida* and *Sporothrix* fungal loads increase, such as previously described for mammalian hosts [47,48].

It is noteworthy to mention the proportional increase of hemocytes in response to *S. brasiliensis's* higher fungal loads, beginning on the fifth day of the curve. As a matter of fact, on the fifth day the major phenotypic event registered was the beginning of larvae death. Thus, it is possible to hypothesize that larvae morbidity is a result of higher fungal loads as the immune system, although proportionally stimulated reaching greater hemocyte recruitment, fails [26,49].

Lozoya-Pérez and collaborators [22] described a similar *S. brasiliensis-G. mellonella* approach, at a single-specific point of the survival curve. CFU and hemocyte counting from the insect's hemolymph were obtained 24 hours after yeast inoculation, previously grown in distinct culture

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media. CFU values between $1.8x10^5$ and $2.4x10^5$ were found and 3.9 and 9.2 $\times 10^6$ hemocytes/mL, depending on the culture medium used. Such values were lower than those found in the present study ($6.4x10^6$ to $3.0x10^7$ hemocytes/mL). This difference may be related to the 100x higher *S. brasiliensis* inoculated in the present study protocol.

In spite of the increasing amount of literature involving the use of *G. mellonella* as an invertebrate biomodel, little is known about its defense strategies against *S. brasiliensis*. Even though the present study was able to establish a comparison with the previous work concerning the phenotypic events of the *Sporothrix-G. mellonella* interaction [21,22,50] we are limited to few previous publications approaching the molecular aspects of the *S. schenckii* infection [50]. Actually, gene expression data in such experimental scenario is limited to the genus *Candida* [31,44].

To the best of our knowledge this is the first study to investigate *G. mellonella* phenotypic and molecular events triggered by the infection with the most virulent *Sporothrix* species, *S. brasiliensis*. In contrast, a considerable number of published works is available on the *G. mellonella* humoral response to *Candida* species. These quantify the expression of AMPs promoter genes with distinct purposes, among them the description of the larval immune response profiles under different infection protocols and the evaluation of potential antimicrobial drugs. By respecting the differences inherent to each fungal species, these findings were used as parameters in order to discuss the present study findings.

Given the previous works highlighting the importance of the *G. mellonella* AMPs encoding genes *Gallerimycin* and *Galiomycin*, during both filamentous and yeast fungal infections [29,31] this study seeked to quantify these genes expression after the infection by the dimorphic fungus *Sporothrix brasiliensis*. While *Gallerimycin* presented an expressive increase in gene expression along the survival curve, *Galiomycin* remained inaltered. These results suggest that the AMP Gallerimycin plays a relevant role in the insect's response to *S. brasiliensis* infection, as previously described during *Candida* spp. infection [31,51]. Contrarily, Dekkerová-Chupáčová and co-authors [12] observed that the inoculation of $2x10^5$ yeast/larvae of *C. albicans* and *C. dubliniensis* into *G. mellonella* larvae triggered an expressive increase in AMP coding genes, especially *Galiomycin*.

The differential relevance of the *G. mellonella Galiomycin* gene expression during *Candida albicans and dubliniensis* versus *S. brasiliensis* infections may be explained by the fact that the first is a well know microbiota yeast member interacting with vertebrates during millions of years of evolution while *Sporothrix* species evolved from saprophytic mycelial fungi recently adapted to parasitism by termodimorphism. Therefore, *Sporothrix* species may be in the process of gene expression adapting to the animal organic matter [2,52].

In addition to positive regulation, time-dependent regulation was observed, and *Gallerimycin* evidenced a significant increase on days D1 and D8. Such a result partially corroborates with that found by Dekkerová-Chupáčová and co-authors [12]. They noted that the maximum up-regulation of both genes was shown at 24 h post-infection, but as early as one hour into the infection, a positive regulation of expression was already observed. More specifically, the gene encoding *Gallerimycin* was up to 1.3-fold higher (1 h post-infection) and 3.2-fold higher (24 h post-infection) in larvae infected with *C. albicans* compared to *C. dubliniensis.* Whereas for *Galiomycin* we observed up to 3.7-fold (1 h post-infection) and 7.1-fold (24 h post-infection) relative up-regulation in *C. albicans*-infected larvae. The up-regulation of both genes decreased at 48 h post-infection by these two species.

In this regard, the difference in gene expression analysis intervals was based on the metabolic characteristics of the fungal species. *Candida* spp. demonstrates considerably faster growth both *in vitro* and *in vivo*, in the invertebrate model itself, compared to *Sporothrix* spp. [13]. Moreover, given the scarcity of information regarding the gene expression of *G. mellonella* against *Sporothrix* spp., the choice of day (D) for gene expression analysis was defined according to the defense phenomena of the larvae observed in response to the fungus, such as melanization, mobility changes, and the onset of mortality.

Concerning the results observed in the expression of stress management genes, *C7 Contig 15362* and *C8 Contig 19101* showed a significant difference in gene expression between the experimental groups on the fifth day (D5) of infection. Drawing a parallel with the study conducted by Melo and

collaborators [30] evaluating *G. mellonella* gene expression after *C. albicans* infection versus different antifungal therapies, in 24 and 48 hours, it was also possible to observe a positive regulation of both genes in the infected, untreated larvae. A relevant point is that in this case *C8 Contig 19101*, was about 4 times overexpressed than C7 *Contig 15362* gene.

In the present study, it was possible to observe that the expression of the genes studied is not only time-dependent but also alternately modulated. Since, when comparing the genes responsible for encoding AMPs with those of stress management, this significant increase D-day varied. *C7 Contig 15362* and *C8 Contig 19101* are considered stress management genes and showed a significant increase in expression on day 5 post-infection. Interestingly, this moment coincides with the onset of mortality and deleterious phenotypic changes observed during the survival curve, as well as with the beginning of the expressive increase in defense cells and fungal load. In contrast, genes encoding AMPs, especially *Gallerimycin*, showed an immediate response verified by the intense mRNA encoding as early as the first post-infection day. This finding is compatible with the participation of the AMPs, previously characterized as an early element of humoral immunity against the infectious process [53,54]. For this same fact, different authors determine evaluation protocols in hours, both in the investigation of the infectious process by *Candida* spp. [12,31,51] and by *Aspergillus niger* [17].

This work is the first to evaluate the gene expression related to the defense of the insect *G. mellonella* in response to infection by a dimorphic fungus. Most authors have dedicated themselves to the investigation of yeasts and, among these, of the genus *Candida* [12,30,31]. Among the filamentous fungi, previous investigation considered *Aspergillus niger* [17] and *Fusarium oxysporum* [55].

The possibility that filamentations occurred during the infectious process in *G. mellonella* cannot be excluded. Thus, the comparative discussion of some of the parameters evaluated here is limited. It is important to add the fact that previously the presence of hyphae of *S. brasiliensis* has been reported, both *in vitro* in feline phagocytes [56] and either during human or animal parasitism (manuscript in preparation). It is known that the fungal cell wall undergoes profound transformation altering, during the dimorphic transition, β-1-3-glucans to α-glucans posing an immediate challenge to the host immune response (PAMPs) [17,57]. However, only studies that consider distinct periods within the survival curve will be able to answer whether the verified gene expression was exclusively yeast-triggered and or yeast-targeted.

Another acknowledged limitation is the absence of data regarding gene expression in the intervals between the defined days. However, because this is a pioneer investigation in the area, new perspectives and methodologies can be generated and improved in order to overcome the scarcity of data in the literature and elucidate the aspects of this relevant pathogen-host interaction. The present work opens new doors of investigation on the pathogenesis by *Sporothrix* spp. as well as offers a better characterized model for the research of potential antifungal drugs effective for the control of this important zoonosis.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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