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The non-mammalian host *Galleria mellonella* can be used to study the virulence of the fungal pathogen *Candida tropicalis* and the efficacy of antifungal drugs during infection by this pathogenic yeast

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Although *Candida tropicalis* is a frequent cause of invasive fungal diseases, its interaction with the host remains poorly studied. *Galleria mellonella* is a Lepidoptera model which offers a useful tool to study virulence of different microorganisms and drug efficacy. In this work we investigated the virulence of *C. tropicalis* in *G. mellonella* at different temperatures and the efficacy of antifungal drugs in this infection model. When larvae were infected with yeast inocula suspensions of different concentrations (4×10^6, 2×10^6, 10^6 and 5×10^5 cells/larva), we observed a dose-dependent effect on the killing of the insect (50% survival ranging from 1.4 ± 0.8 to 8.8 ± 1.2 days with the higher and lower inocula, respectively). *Candida tropicalis* killed *G. mellonella* larvae at both 30°C and 37°C, although at 37°C the virulence was more evident. Haemocytes phagocytosed *C. tropicalis* cells after 2 hours of infection, although the phagocytosis rate was lower when compared with other fungal pathogens, such as *Cryptococcus neoformans*. Moreover, the haemocyte density in the haemolymph decreased during infection and the yeast formed pseudohyphae in *G. mellonella*. The efficacy of amphotericin B, caspofungin, fluconazole and voriconazole was tested at different concentrations, and a protective effect was observed with all the drugs at concentrations equivalent to therapeutic dose. Fungal burden increased in infected larvae during time of infection and amphotericin B and fluconazole reduced the number of colony-forming units in the worms. Moreover, antifungal treatment was associated with the presence of cell aggregates around infected areas. We conclude that *G. mellonella* offers a simple and feasible model to study *C. tropicalis* virulence and drug efficacy.

Keywords *Candida tropicalis, Galleria mellonella*, phagocytosis, antifungal drugs, virulence

Introduction

Invasive fungal diseases are becoming a life-threatening problem for immunocompromised patients and are commonly caused by yeasts belonging to the genus *Candida*, e.g., *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis*. *Candida tropicalis* causes around 30% of yeast infections due to non-*albicans Candida* species and it is the third or fourth most common species isolated from infections, depending on the geographical region [1–7]. Its incidence is associated with several risk factors, such as cancer, neutropenia, corticosteroid treatment, catheterization and administration of broad spectrum antibiotics and the infections which it causes have a particularly high mortality (around 50%) [2,8]. Although *C. tropicalis* is susceptible to amphotericin B

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(AmB), azoles and echinocandins, a trend of increasing resistance to azoles has been described in the last years. Some recent studies have shown that resistance to voriconazole (VCZ) is more frequent in *C. tropicalis* as compared to *C. albicans* or *C. parapsilosis* [9]. Decreased susceptibility of *C. tropicalis* to fluconazole (FCZ) has been also reported [10] such that in some cases the proportion of isolates with increased resistance to this antifungal was higher than 60% [11]. For these reasons, *C. tropicalis* is becoming a pathogenic yeast of major concern.

Murine models have been used to study virulence of *C. tropicalis* and also to evaluate antifungal efficacy during infection [12]. However, many of these investigations required the use of immunosuppressive agents to fully reproduce the infections [13]. Over the last few years there has been particular interest in developing non-mammalian host models to study microbial virulence to address the bioethical impact of the classical animal experimentation. Among these models, amoebas, nematodes and insects are being used by investigators [14–17]. *Galleria mellonella* (commonly known as the greater wax moth) is a Lepidoptera that has been successfully employed as a model host to study virulence of pathogenic fungi, such as *Cryptococcus neoformans* [18,19], *Candida albicans* [20], and *Aspergillus fumigatus* [21]. In addition, it has also been used to investigate the efficacy of antifungal drugs in the treatment of fungal infections [22–24]. This model is attractive because it can be maintained at different temperatures [25] and some aspects of its immune system display homology with the innate immune response of mammals [26,27]. *Galleria mellonella* has haemocytes, mainly in the haemolymph [28], which present phagocytic activity against bacteria and fungi [19,29,30]. Another important part of its immune system is based on the induction of melanization and encapsulation of forgein particles [28]. The haemocyte density, microbial burden and induction of microbial morphological changes are parameters that have been used to assess the virulence of fungal pathogens in *G. mellonella* [31,32].

Previous reports indicate that *G. mellonella* can be killed by different *Candida* species [17]. In this work, we have extended these studies, and fully characterized the interaction between *C. tropicalis* and *G. mellonella*. Moreover, we have studied if antifungal drugs have any protective role during *C. tropicalis* infection in this model. Our results demonstrate that this non-conventional host is a suitable model to study *C. tropicalis* virulence and to assess the efficacy of antifungal drugs during infection caused by this pathogen.

Materials and methods

Yeast strains and growth conditions

The following *C. tropicalis* strains were used; ATCC 200956, ATCC 750, CL-7119 and CL-7868 (these last two clinical isolates belong to the Yeast Collection of the Mycology Reference Laboratory from the Spanish National Centre for Microbiology). *Cryptococcus neoformans* variety *grubii* (H99 strain, ATCC 20882) was also included as control of phagocytosis. Yeast cells were grown overnight in Sabouraud liquid medium (Becton Dickinson and Company, MD, USA) at 30°C with moderate shaking (150 rpm).

Galleria mellonella *survival assay*

Wax moth larvae killing assays were carried out as described previously [18]. Briefly, groups of 20 larvae (0.3–0.6 grams, R. J. Mous Livebait, The Netherlands) were each inoculated with 10 µl of a 4×10^8, 2×10^8, 1×10^8 or 5×10^7 yeast cells/ml suspension in PBS buffer supplemented with ampicillin (20 µg/ml) to avoid bacterial contamination. Therefore, the resulting the final inoculum concentration for each group was 4×10^6, 2×10^6, 1×10^6 or 5×10^5 yeast cells/larva. The yeast cells were directly instilled into the haemocele of the larva by injection with a 10 µl Hamilton syringe in the last left proleg. The larvae were incubated at 30 or 37°C after inoculation, and survival was monitored every day. The larvae were considered to have died when they did not respond to physical stimulation (slight pressure with forceps). As controls, a group of non-infected larvae and a group of larvae inoculated with PBS-ampicillin were studied in parallel in every infection investigation. For some experiments, *G. mellonella* larvae were also inoculated with yeasts that were heat-inactivated for 45 min at 65°C. Each experiment was repeated at least three times, and representative experiments are presented.

Haemocytes density determination in the haemolymph

Groups of 10 larvae were inoculated with 4×10^6 yeast cells and then incubated at 37°C for 2 and 7 h; 50 µl of haemolymph were collected in the same volume of Insect Physiological Saline buffer (IPS; 150 mM sodium chloride, 5 mM potassium chloride, 10 mM Tris-HCl pH 6.9, 10 mM EDTA and 30 mM sodium citrate) [32]. After a 1:10 dilution in IPS, haemocytes density was enumerated using a haemocytometer. Groups inoculated with PBS and non-infected larvae were included as control. Results were expressed as haemocytes/ml.

In vivo *phagocytosis assay*

Candida tropicalis ATCC 750 and ATCC 200956, as well as *C. neoformans* var. *grubii* H99 strain were grown in liquid Sabouraud medium as described above, and a suspension of 10^9 cells/ml was stained with 10 µg/ml of

Calcofluor White (Sigma Aldrich, St Louis, MO, USA) for 30 min at 37°C, and 10 µl were injected in each larvae. Haemolymph was recollected in IPS buffer after 2 h and then centrifuged at 78 *g* for 4 min. The pellet was placed on slides, covered with coverslips and immediately observed in a Leica DMI 3000B fluorescence microscope. Images were captured using the imaging software Leica applications suite (LAS) 3.3.1. The percentage of haemocytes containing yeast cells was calculated. For reproducibility, groups of 10 larvae were included in each condition and the experiment was repeated on different days.

Filamentation

Candida tropicalis ATCC 200956 and ATCC 750 were grown as described above. Larvae were inoculated with 2×10^6 cells and incubated at 37°C for 24 h. They were then macerated on nylon cell strainers (100 µ pore size, Becton Dickinson) in 1 ml of IPS. The liquid was collected, centrifuged and suspended in 1 ml of the same buffer. Samples were stained with Calcofluor White as described above, and yeast morphology was observed with a Leica DMI 3000B fluorescence microscope. The length of the blastoconidia and filaments was measured using the LAS 3.3.1 software. As control, larvae at time zero (immediately after inoculation) were also macerated, and the size of the yeast measured.

Antifungal drugs

Amphotericin B (AmB, Sigma Aldrich Quimica, Madrid, Spain), caspofungin (CAS, Merck & Com, Inc, NJ, USA), fluconazole (FCZ, Pfizer SA, Madrid, Spain) and voriconazole (VCZ, Pfizer SA) were used to evaluate their protective effect during infection.

Efficacy of amphotericin B, caspofungin, fluconazole and voriconazole in G. mellonella *infected with* C. tropicalis

Larvae killing assays were carried out at 37°C as previously described above, using a dose of 2×10^6 yeast cells/larva. Antifungals were administered by inoculation at three different concentrations. For AmB and CAS we used 1, 2 and 4 µg/gram of larvae. The concentrations of FCZ were 1.1 and 5 µg/larva and for VCZ the levels were 1.1 and 5.5 µg/larva. Parallel groups of uninfected larvae treated with the same concentrations of antifungals were also included to eliminate possible drug toxicity and other effects of the drugs as factors contributing to the observed results. Survival was monitored every 24 h.

Fungal burden determination

Fungal burden was determined by CFU counts at different times after inoculation. For this purpose, three groups of 60 larvae were selected, all of which received 2×10^6 cells/larva of *C. tropicalis* ATCC 750 strain. While one group remained untreated, the other two were treated with AmB (2 µg/gram of larvae) or FCZ (5 µg/larvae). Once inoculated and treated, 5 larvae were taken from each group, washed with ethanol and cut into small pieces with a scalpel. The material was suspended in 2 ml of PBS-ampicillin and transferred to 50 ml conical tubes containing glass beads. The tissue was homogenized gently with a vortex for a few seconds. The homogenate was washed twice with the same buffer, and finally suspended in 6 ml. Each sample was serially diluted twices and 50 µl of the resulting dilutions were inoculated onto Sabouraud (Oxoid)-cloramphenicol (Sigma) agar plates. The plates were incubated during 48 h at 37°C, and CFUs enumerated, with results expressed as the average and standard deviation.

Histological study of larvae tissue

To evaluate the presence of *C. tropicalis* inside tissue of *G. mellonella*, three larvae per group were fixed for 24 h in 4% buffered formalin and dehydrated with increasing concentrations of ethanol (70%, 80%, 90%, 96% and 100%). The samples were then treated with xylene and paraffin embedded. Tissue sections of 5 microns were stained with periodic acid Schiff (PAS) and sections examined with a Leica DMI 3000B fluorescence microscope. Samples from non-infected larvae were included as control.

Statistics

Killing curves were plotted and estimation of differences in survival (log rank and Wilcoxon tests) analyzed by the Kaplan-Meier method using Graph Pad Prism 5 software (La Jolla CA, USA). P value < 0.05 was considered significant. Differences in phagocytosis, haemocyte density and CFUs were estimated using Student's t-test and P value < 0.05 was considered significant.

Results

Virulence of C. tropicalis *in* G. mellonella *depended on the strain, temperature, and inoculum size*

To evaluate the virulence of *C. tropicalis* in *G. mellonella*, we first analyzed the survival of larvae infected with different strains and different concentrations of yeast cells at two temperatures (30 and 37°C). As shown in Fig. 1, all strains killed the larvae, indicating that *G. mellonella* is a

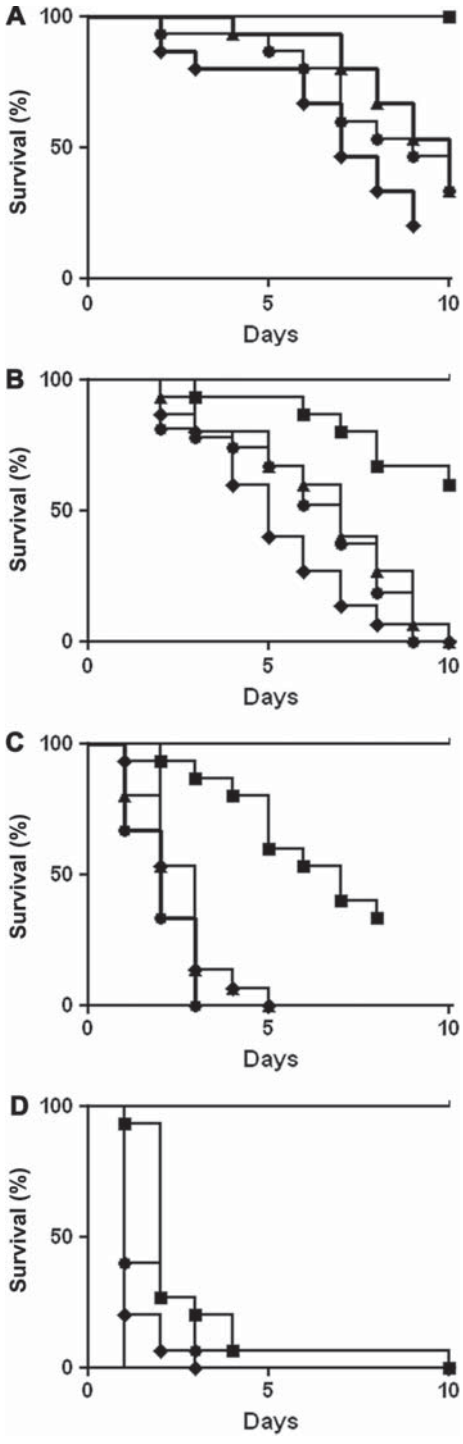

Fig. 1 *Galleria mellonella* killing by different *Candida tropicalis* strains depends on the inoculum. Groups of 20 *G. mellonella* larvae where infected as described in Material and Methods with different *C. tropicalis* strains, and survival was monitored every day. Survival experiment was performed at 37°C. (●) ATCC 750, (■) ATCC 200956, (▲) CL-7119, (◆) CL-7868. (A) 5×10^5, (B) 10^6, (C) 2×10^6, (D) 4×10^6 cells per larva, respectively.

feasible host model to study various aspects of the virulence of this fungal pathogen. We found inter-strains differences in the virulence and that the 50% survival mark varied according to inoculum size. With the low inoculum levels (5×10^5), 50% of larvae died after 8.8 ± 1.2 days, except for those instilled with ATCC 200956 which were not affected by the fungus (100% survival) during the time of the experiment (10 days). When the inoculum concentrations were increased to 10^6 cells/larva, 50% survival time decreased (6.3 ± 1.2 days), except for strain ATCC 200956 which seemingly had little effect on survival. With the higher inoculum levels of 2×10^6 and 4×10^6, the time required for 50% kill of larvae decreased significantly, i.e., 3.8 ± 2.2 and 1.4 ± 0.8 days, respectively (Fig. 1).

These studies included two clinical isolates and two ATCC strains of *C. tropicalis*. Clinical isolates and ATCC 750 were more virulent in the model system than ATCC 200956 strain (Fig. 1).

Virulence of *C. tropicalis* was dependent upon the temperature at which the larvae were incubated (Fig. 2). At 30°C the strains were less virulent than with larvae incubated at 37°C. The 50% survival at 30°C and 37°C with a 2×10^6 inoculum of ATCC 750 strain was 7 and 3 days, respectively; for ATCC 200956 strain it was 10 and 7 days, and for the CL-7119 strain it was 5 and 3 days. However, although the difference in the survival of the larvae inoculated with CL-7868 strain was minimal (4 and 3 days at 30°C and 37°C, respectively), 100% death occurred at 10 days at 30°C as opposed to 5 days at 37°C.

In all infected larvae, a typical dark color due to the accumulation of melanin was observed after a few minutes of the infection. For this reason, and to ensure that larvae death was not due to factors other than the viability of the fungi, we evaluated the survival of caterpillar inoculated with heat-killed yeasts. As shown in Fig. 3, heat-inactivated yeast cells did not kill *G. mellonella* larvae. In contrast, viable yeast cells induced a rapid killing of the larvae ($P < 0.0001$), indicating that death of the worms was due to *C. tropicalis* infection. Interestingly, heat-inactivated cells induced melanization of the larvae as was found with the viable yeast cells.

Candida tropicalis *was phagocytosed by* G. mellonella *haemocytes*

Phagocytosis plays an important role in the innate immune response of *G. mellonella* [33]. Therefore, we studied if *C. tropicalis* yeast cells were phagocytosed by larvae haemocytes, and compared the results with the phagocytosis observed with other fungal pathogens that have been described to be efficiently phagocytosed by haemocytes, such as *C. neoformans. Candida tropicalis* was phagocytosed by *G. mellonella* after 2 h of infection (Fig. 4A).

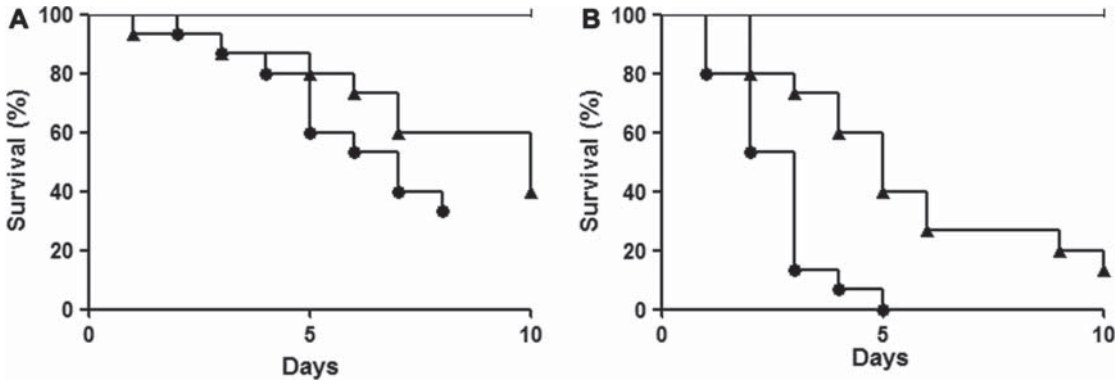

Fig. 2 Virulence of *Candida tropicalis* depends on the incubation temperature of the larvae. Larvae were infected with 2×10^6 *C. tropicalis* yeast cells, and then incubated at 30 or 37°C (A) ATCC 200956 strain, (●) 37°C; (▲) 30°C, (B) CL-7868 strain, (●) 37°C and (▲) 30°C, (−) PBS control. The difference between temperatures was significant in both strains ($P < 0.0001$).

We did not find differences in the capacity of engulfing yeast cells among *C. tropicalis* strains used in this investigation. However, the percentage of haemocytes that phagocytosed *C. neoformans* was higher than those that engulfed *C. tropicalis* cells ($P < 0.05$, Fig. 4B).

Haemocytes density after infection with C. tropicalis

Changes in haemocytes density in the haemolymph have been reported in *G. mellonella* after infection with microbial pathogens [31], so we studied if *C. tropicalis* had any effect on this immune response. Challenge of *G. mellonella* with *C. tropicalis* ATCC 750 resulted in a decrease in the concentration of haemocytes, as observed after 2 and 7 h of infection. Statistically, significant differences in haemocytes density were observed between larvae inoculated with *C. tropicalis* and PBS. The number of haemocytes in non-infected larvae was similar to those inoculated with PBS ($P > 0.05$, Fig. 5).

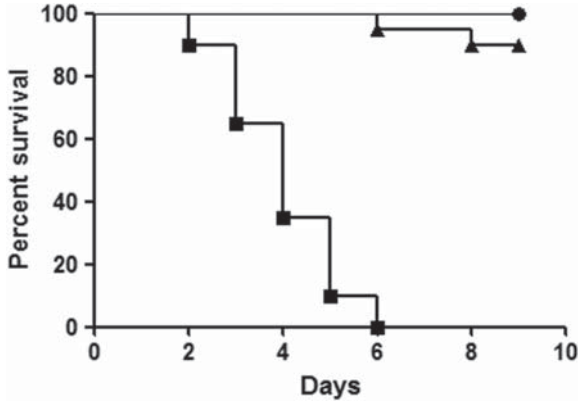

Fig. 3 *Galleria mellonella* infected with heat-killed cells. Cells from *C. tropicalis* ATCC 750 strain were heat-inactivated as described in *Materials and methods*. Then, they were used to infect a group of 20 *G. mellonella* (2×10^6 cells per larva). Parallel groups were injected with live cells using the same dose, and PBS as control (●) PBS, (■) ATCC 750 alive cells, (▲) ATCC 750 HK cells. Virulence experiment was performed at 37°C.

Candida tropicalis *induced pseudohyphae formation during infection*

Morphological transitions (pseudohyphae and hyphae) have been described and related with the virulence of *Candida* spp. [34]. Consequently, we investigated if any of these changes occurred during infection in *G. mellonella*. In this experiment, we used *C. tropicalis* strains which differed in their virulence in this model (see Fig. 2). We did not observe any statistical difference in the size of blastoconidia among the four strains prior to inoculation. During infection ATCC 750, CL-7119 and CL-7868 formed pseudohyphae at both 30°C and 37°C. In contrast, ATCC 200956 was unable to form these structures in *G. mellonella* (Fig. 6).

Amphotericin B, caspofungin, fluconazole and voriconazole protected G. mellonella *during* C. tropicalis *infection*

To evaluate if *G. mellonella* can be used as an *in vivo* model to test the efficacy of antifungals during *C. tropicalis* infection, we treated infected larvae (2×10^6 cells per larva) with AmB, CAS, FCZ and VCZ and performed survival experiments. Larvae treated with AmB (2 and 4 μg/gram/larva) were protected from infection (Fig. 7A, $P = 0.0001$ compared with the untreated group). In the same manner, CAS protected the larvae at the three concentrations tested (Fig. 7B, $P = 0.0001$, compared with the untreated group). Fluconazole protected the larvae at concentrations of 5 μg/larva (Fig. 7C $P = 0.0012$) but not at 1.1 μg/larvae (Fig. 7C, $P = 0.2$). Finally, VCZ afforded protection at 5.5 μg/larvae (Fig. 7D, $P = 0.0002$) but not at 1.1 μg/larvae (Fig. 7D $P = 0.6$). None of the

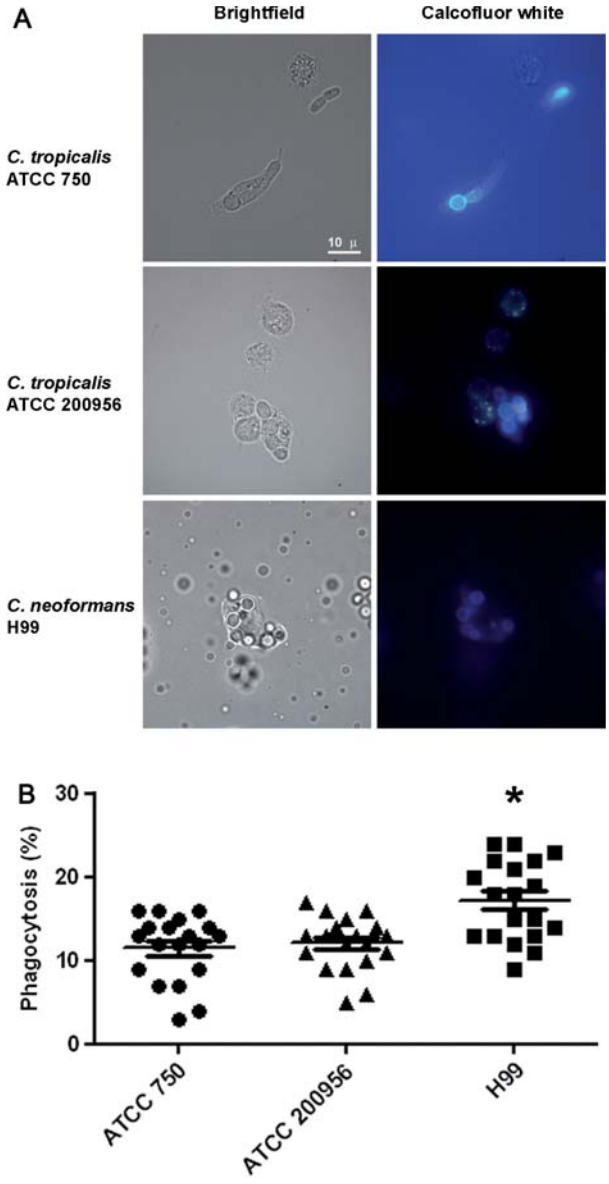

Fig. 4 *Candida tropicalis* and *Cryptococcus neoformans* were phagocytosed by larvae haemocytes. Larvae were infected with 10^9 yeast cells/ml of *C. tropicalis* (ATCC 750 and ATCC 200956 strains) and *C. neoformans* var *grubii* (H99 strain), previously stained with Calcofluor white. The larvae were incubated at 37°C for 2 hours. Then, the haemolymph was obtained in IPS buffer. (A) Representative images of infected haemocytes are shown. Scale bar upper left panel denotes 10 µm and applies to the rest of the micrographs. (B) Quantification of the phagocytosis percentage. Difference between *C. neoformans* variety *grubii* H99 and both *C. tropicalis* strains was significant ($*P < 0.05$).

drugs were toxic to the larvae at the concentration used (result not shown).

We evaluated the fungal burden to investigate the effect of the antifungals during infection. In non-treated larvae,

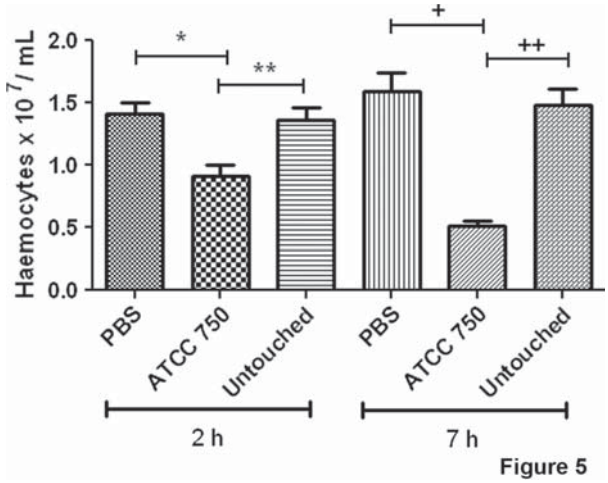

Fig. 5 Haemocyte density in *Galleria mellonella* decreases after challenge with *Candida tropicalis*. Haemocyte density in the haemolymph was calculated as described in *Materials and methods* after 2 and 7 hours of infection with ATCC 750 strain at 4×10^6 cells per larvae. Parallel groups of non infected larvae or larvae injected with PBS were carried out as control. Graph shows the average and standard deviation of 10 larvae per group. Statistical differences are highlighted ($*P = 0.0004$, $**P = 0.0028$, $+P < 0.0001$, $++P < 0.0001$).

the CFUs increased over time as compared to the initial inoculum ($P < 0.05$ in all cases). In addition, with the exception of day 0, significant differences ($P < 0.05$) were observed in the number of CFUs recovered from larvae treated with AmB or FCZ and the untreated larvae group (Fig. 8).

Histological study of larvae tissue

We performed histophatology studies to better characterize the development of the infection in *G. mellonella*. We observed tissue sections on the second and fourth day after the initiation of infection. Yeast cells and filaments were observed in the tissue, both in the treated and untreated larvae, primarily in clusters. However, in untreated larvae, there were a higher number of infected areas compared to the larvae treated with AmB or FLZ. Moreover, when infected larvae were treated with antifungals, the yeasts were surrounded by tissue structures that were absent in untreated larvae (Fig. 9).

Discussion

Candida tropicalis is becoming a pathogen of concern because of the significant increase in its incidence, high association with poor patient outcomes and its ability to develop resistance to some antifungals, such as azoles. Despite its importance, little is known about the virulence of this pathogen and about its interaction with the

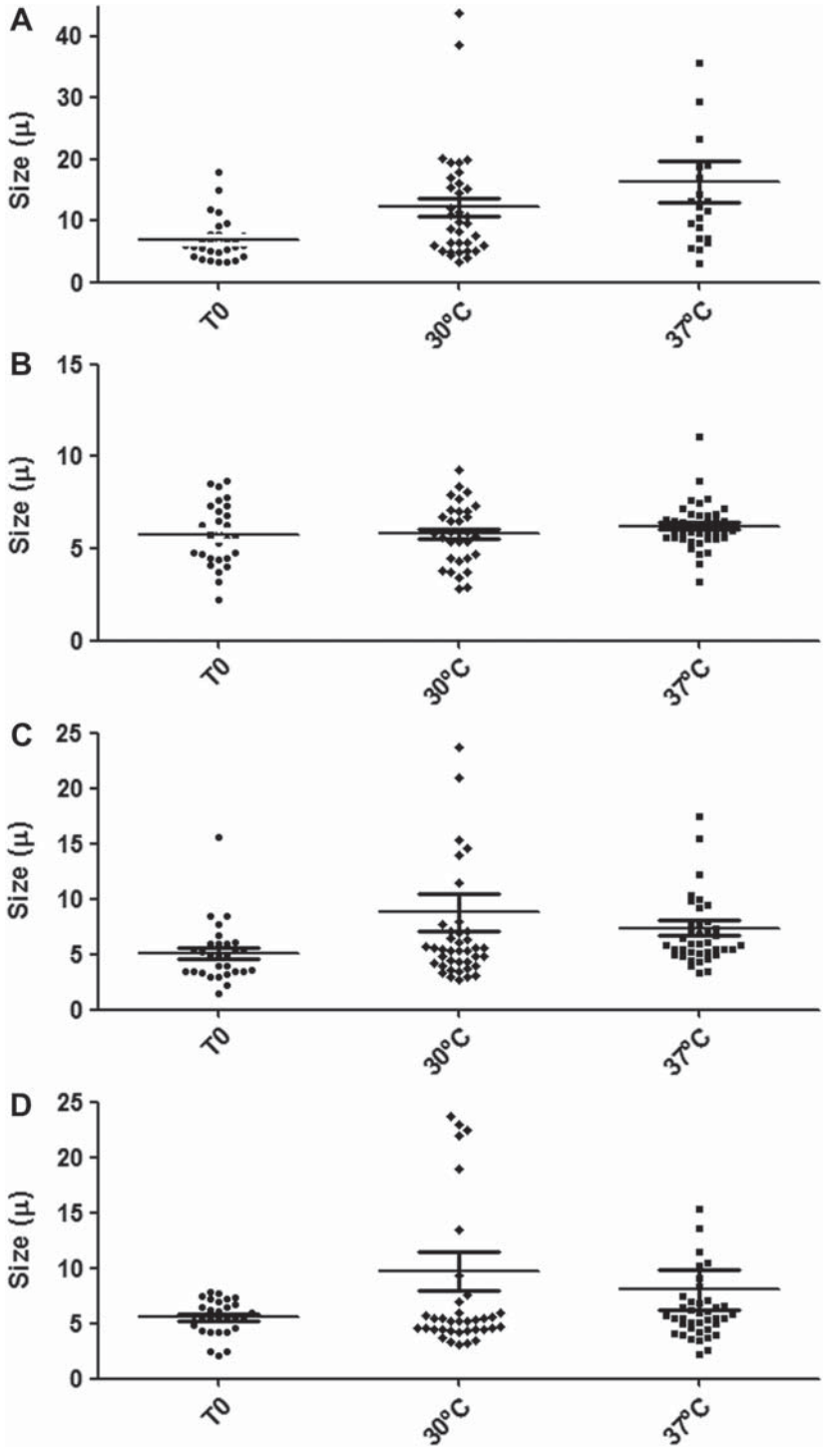

Fig. 6 Distribution of the length of the pseudohyphae of *Candida tropicalis* in *Galleria mellonella*. (A) ATCC 750. (B) ATCC 200856. (C) CL-7119 and (D) CL-7868 strains were inoculated in *G. mellonella* (2×10^6 cells per larva), and the length of the cells at time zero (●) and after 24 hours of infection at 30°C (◆) and 37°C (■) was measured.

host. Mammal models (such as mice, rats, guinea pigs or monkeys) can be used to study microbial pathogenesis, but are associated with animal welfare and bioethical issues. For this reason, we have investigated the virulence of *C. tropicalis* in an invertebrate system. The use of these non-mammalian models to study microbial pathogenesis

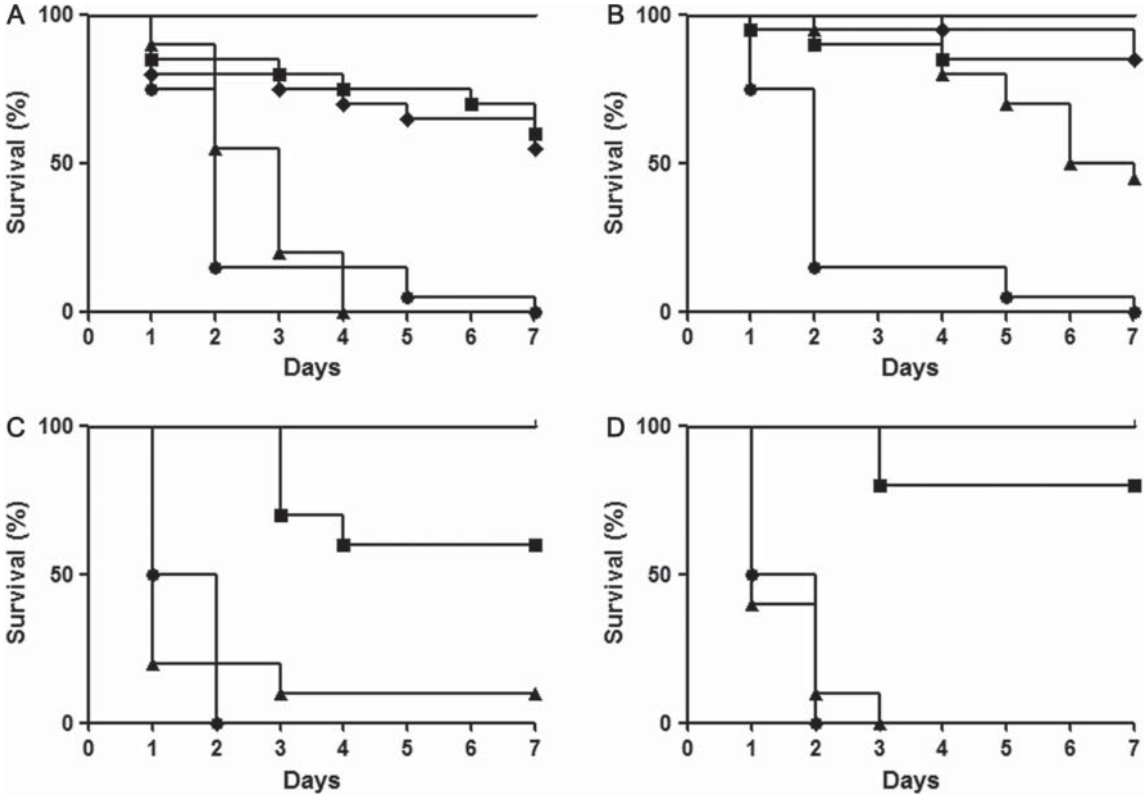

Fig. 7 Efficacy of antifungal drugs in *Galleria mellonella* infected with *Candida tropicalis*. Larvae were treated with different doses of AMB, CAS, FCZ or VCZ after the inoculation with 2×10^6 cells of *C. tropicalis* ATCC 750. In some groups, the larvae were treated with 10 µl of PBS or different drug concentration alone, instead of *C. tropicalis*, as a procedural control and drug toxicity on the larvae, respectively. (A) AmB at 0 (●), 1 (▲), 2 (■) and 4 (◆) µg/gram of larva. (B) CAS at 0 (●) 1 (▲), 2 (■), 4 (◆) µg/ gram of larva. (C) FCZ at 0 (●), 1.1 (▲) and 5 µg/larva, (D) at VCZ 0 (●), 1.1 (▲), 5.5 (■) µg/larva, respectively. (-) PBS control.

has gained its application and importance over the last years [35].

Galleria mellonella is gaining wide acceptance as a non-mammalian model to study microbial virulence and in particular, fungal pathogenesis [14,20,21]. *G. mellonella* offers the following significant advantages: (i) it is an inexpensive model, (ii) it is easy to manipulate to inoculate with defined doses of both the pathogen and antibiotics), (iii) virulence experiments can be performed at different temperatures which allows for the investigation of pathogenesis at physiological and environmental temperatures, as well as to permit the analysis of the virulence of thermosensitive mutants, (iv) it can be used to study the *in vivo* efficacy of antifungals, and (v) there is a correlation between the virulence of a microorganism in *G. mellonella* and in mammalian models [20,30].

In this work, we have demonstrated that *G. mellonella* can be used to evaluate the pathogenicity of *C. tropicalis*. The virulence of *C. tropicalis* in *G. mellonella* is inoculum-dependent which correlates with data found with other fungal pathogens, such as *C. albicans* and *C. neoformans* [17,18]. We found with *C. tropicalis*, as previously reported, that inoculation of clinical isolates into *G. mellonella* produced faster mortality rates than when larvae were inoculated with strains maintained in laboratory collections for several years [17]. Studies in mice have also shown differences in pathogenicity among *C. tropicalis* clinical isolates [12]. Although the larval killing required high fungal doses, the virulence observed was pathogen-specific as no infection was noted after inoculation with heat-killed yeast cells. The use of either live or heat-killed yeast cells induced larvae melanization, a mechanism of the innate immune system. However, this response was not sufficient to achieve insect protection from infection.

Larvae killing occurred faster at 37°C than at 30°C, which is a similar situation described in studies with *C. neoformans* [18]. We believe that this is a consequence of both an enhanced virulence of the yeast and an impairment of the immune response of the larvae at physiological temperatures [28].

Morphological transitions of *Candida* spp. are related with their pathogenicity [36,37]. The mechanisms by which

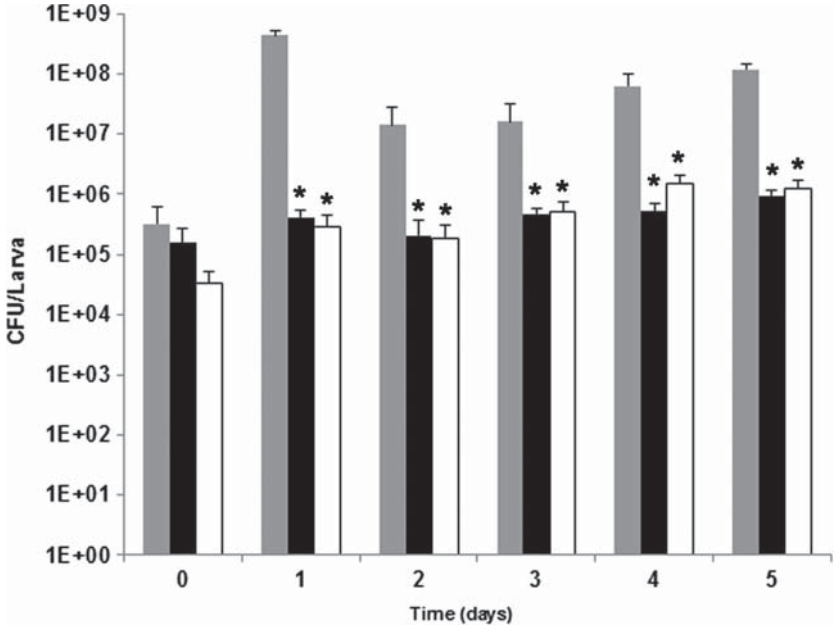

Fig. 8 Fungal burden during *Galleria mellonella* infected with *Candida tropicalis*. CFUs of larvae infected with 2×10^6 cells of *C. tropicalis* ATCC 750 strain were estimated as described in *Materials and methods*. In some cases, larvae were treated with AmB (2 μg/gram of larva) or FCZ (5 μg/larva). Statistical differences between untreated and treated larvae are highlighted ($*P < 0.05$). Gray bars, untreated larvae; black bars, larvae treated with FCZ; and white bars, larvae treated with AmB.

hyphae and pseudohyphae contribute to virulence include high adherence to biological surfaces, increased dissemination and evasion of phagocytosis [38]. In addition, the immune system responds differently when presented with yeast cells and filaments [39,40]. The role of *C. tropicalis* pseudohyphae during infection in *G. mellonella* remains unknown, but it could offer an advantage to escape from haemocytes. In this study we found an association between low virulence and strain ATCC 200956's inability to form filaments. We also demonstrated that *C. tropicalis* is engulfed by haemocytes as noted with other fungal pathogens, such as *C. albicans* or *C. neoformans*. In the case of *C. tropicalis*, pseudohyphae were observed inside the phagocytic cells. These findings suggest that the ability to induce pseudohyphae could provide a mechanism to escape from haemocytes. In accord with these results, we noted that injection of *C. tropicalis* into *G. mellonella* reduced the haemocyte density in the haemolymph. Changes in haemocyte density following microbial challenge have been observed previously [31]. It has been shown that high haemocyte concentration correlate with high survival rates while low survival is associated with low haemocyte numbers [41]. The decrease in haemocyte density could be explained by different mechanisms, as for example pseudohyphae formation by the yeasts after phagocytosis could explode the haemocytes, resulting in a reduction in the number of visible phagocytic cells. In addition, *C. tropicalis* could induce a process known as nodulation which consists in the appearance of clumps of haemocytes and yeast cells [26].

We also evaluated the efficacy of four important antifungal drugs used for the treatment of infections caused by *Candida* spp., i.e., AmB, CAS, FCZ and VCZ. Our results showed that *G. mellonella* was useful in the evaluation of the efficacy of CAS and AmB at concentrations equivalent to the therapeutic doses used in human. Fluconazole and VCZ were evaluated at concentrations equivalent to therapeutic (5 and 5.5 μg/larva, respectively) or subtherapeutic (1.1 μg/larva) doses used in human. Consequently, our results confirm previous findings indicating that this model can be used as an *in vivo* system to evaluate toxicity and efficacy of new antimicrobial agents [22–24]. Our investigation also demonstrated that antifungal efficacy is not correlated with fungal clearance in *G. mellonella*. In larvae treated with FCZ we observed a fungistatic effect. Although AmB is known for its fungicidal action, this characteristic was not observed in this study. However, this polyene prolonged the survival of *G. mellonella* larvae after infection with *C. tropicalis*. The lack of information about distribution and stability of the antifungals in *G. mellonella* makes it difficult to interpret our results. Further studies focused on determining the pharmacokinetics of this drug in *G. mellonella* could be useful to understand the effectiveness of these drugs in this model.

We conclude that *G. mellonella* is an alternative model for the study of host-pathogen interactions with

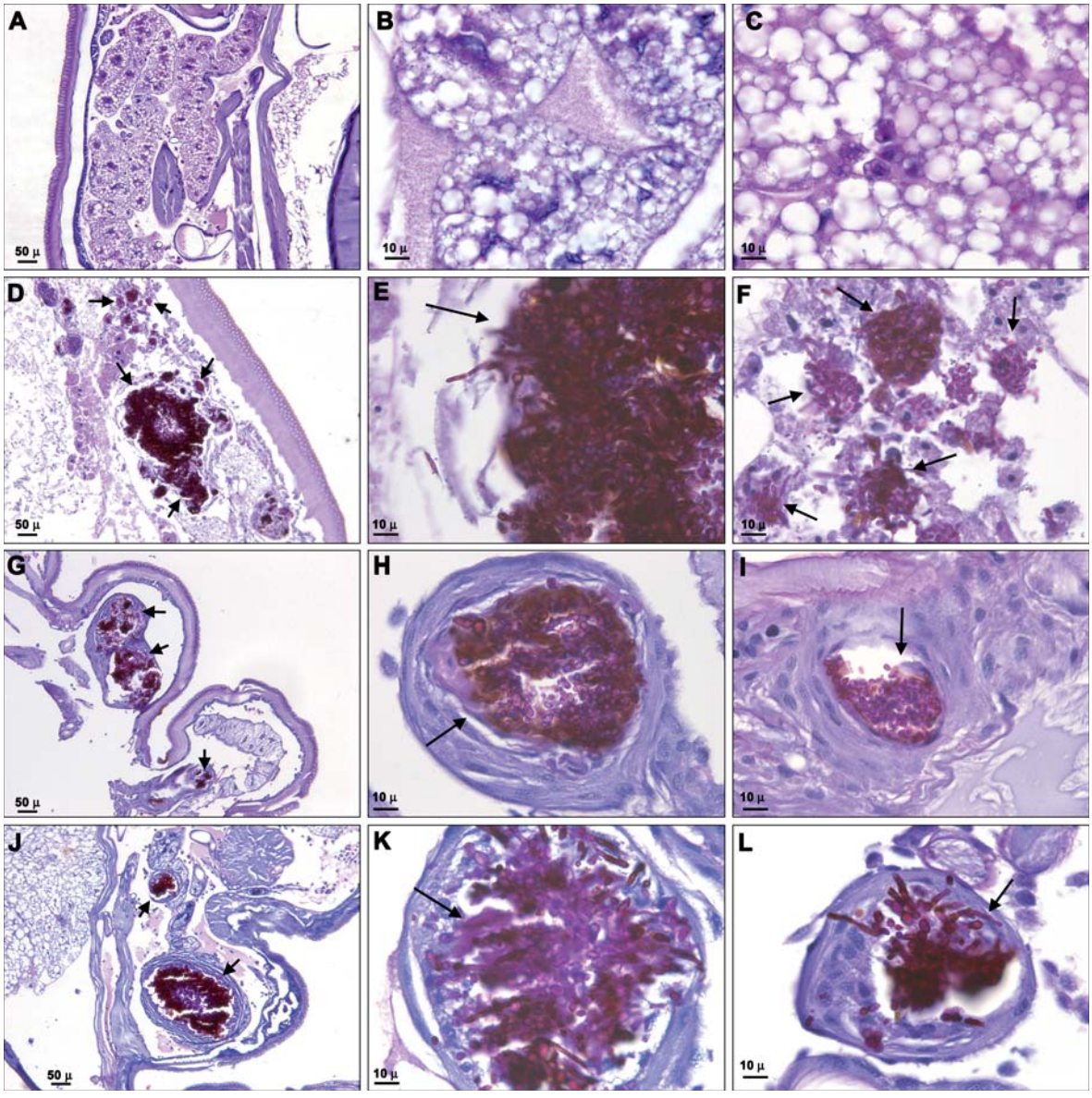

Fig. 9 Tissue sections of *Galleria mellonella* infected with *Candida tropicalis* and treated with amphotericin B or fluconazole. *Galleria mellonella* larvae were infected with 2×10^6 cells of ATCC 750 strain, and treated with AmB (2 µg/gram of larva) or FLZ (5 µg/larva). Uninfected larvae were carried out in parallel as controls. After 4 days of infection, the larvae were processed for histopathology (see *Materials and methods*). The panels show PAS staining of the tissue sections. (A), (B) and (C), uninfected control; (D), (E) and (F), infected larvae; (G), (H) and (I), infected and treated with AmB; (J), (K), (L), infected and treated with FLZ. The images show low (A, D, G and J) and high (B, C, E, F, H, I, K, L) magnification panels. Arrows indicate infected areas.

C. tropicalis and the *in vivo* efficacy of antifungals against this yeast.

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