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RESEARCH ARTICLE

Evaluation of *Galleria mellonella* larvae as a model for elucidating the virulence factors of the human pathogenic fungi *Candida albicans* and *Aspergillus fumigatus*

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ABSTRACT

Background: Testing microbial virulence in mammals is a prerequisite before applying therapeutic agents to humans. However, conducting experiments in mammalian models is expensive and requires ethical considerations. Therefore, insects have been widely used as an alternative model to study human microbial virulence. **Aim:** This work aimed to evaluate the use of *Galleria mellonella* larvae as a model for testing the virulence factors of the human pathogenic fungi, *Candida albicans,* and *Aspergillus fumigatus*. Fungal-induced influences on mortality and various immunological and histological parameters were also evaluated. **Materials and Methods:** The hemocyte density, melanin formation, phenoloxidase (PO) activity, and cytotoxic effects were evaluated and assessed as indicators of fungal virulence. **Results:** Our findings, collected from survival tests and time post-infection, revealed interspecific differences, which were more pronounced with *C. albicans* than with *A. fumigatus*, showing an increased burden of infected larvae over time. The differences may be partially elucidated by disparities in growth rate and the production of hydrolytic enzymes of the tested fungi. Challenged larvae with either fungal species exhibited a notable reduction in circulating hemocytes and hemolymph protein content, as well as a significant increase in melanin formation, phagocytosis, and PO activity, along with pronounced cytotoxic effects in the cuticle and midgut. No significant differences were found between the tested fungi, although lower rates in the immunological and cytological parameters were induced in the *A. fumigatus*-injected larvae during the same study period. These alterations are associated with the virulence of fungal species. **Conclusion:** We can conclude that *G. mellonella* larvae constitute a simpler and more suitable model for studying the virulence of fungi.

Keywords: *Aspergillus fumigatus*, *Candida albicans*, Cytotoxicity., *Galleria mellonella*, hemocytes, Melanin, Animal model, Phagocytosis, Phenoloxidase, Virulence

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INTRODUCTION

In humans, the yeast *Candida albicans* causes a common fungal disease known as candidiasis, which may present in several forms, such as invasive mycoses and mucocutaneous infections (Brown *et al*., 2012). Organ donors are more likely to get *Aspergillus fumigatus* if they have a history of lung cancer, cystic fibrosis, tuberculosis (TB), or asthma, or if they are on immunosuppressant medication just before donating a kidney (Vonberg and Gastmeier, 2006).

The urgent need for alternate therapeutic techniques to handle the challenging management of invasive fungal infections has been heightened by the introduction of new fungal pathogens and their resistance to existing antifungal medicines. Susceptibility testing is carried out using animal models to assess fungal infections and the effectiveness of antifungals. However, using mammals as models, especially mice, is limited due to ethical and cost considerations (Borman, 2018).

Several studies have lately focused on non-mammalian host models for microbial virulence research, to make large-scale screening studies easier (Trevijano-Contador and Zaragoza, 2014). Several studies have also shown that *G. mellonella*, an invertebrate, may be used as a model organism to investigate bacterial pathogenicity and antibiotic effectiveness (Rady *et al*., 2020; Chen and Keddie, 2021; Abo-Kersh and Barakat, 2023). *Galleria* larvae are a popular and well-known infection model because they can be easily kept and reproduced in large numbers, which improves statistical robustness (Curtis *et al*., 2022). They could be a good substitute for mammalian animal models when testing fungal pathogenicity because their immune response to infection is functionally comparable to the human innate immune response (Giammarino *et al*., 2024).

As in vertebrates, *G. mellonella* larvae have immune cells called hemocytes that may engulf other cells called macrophages. In addition, Bergin *et al*. (2003), Desalermos *et al*. (2012), and Mesa-Arango *et al*. (2013) found that fungal cells may use similar tactics to avoid destruction by hemocytes and macrophages. According to González-Santoyo and Córdoba-Aguilar (2012), the process of melanogenesis is an immunological response that depends on the enzyme polyphenol oxidase. This enzyme polymerizes melanin after converting phenols into quinones. Encircling the infection and stopping it from spreading and dying of starvation is one of many functions of this pigment in the immune response (Lu *et al*., 2014). Melanin intermediate pathways may also destroy the pathogen instantly. When testing for cytotoxicity and cell damage in both insect and human cells, melanin and phenol oxidase (PO) activity are often detected (Bergin *et al*., 2006).

In the present study, two fungal species, *A. fumigatus* and *C. albicans*, were compared for their pathogenicity using the *G. mellonella* larval model. The following variables were measured and compared in the test and the controls: (i) the ability of fungal cells to produce potentially virulent factors; (ii) the counts of fungal burden as the infection progresses; (iii) the hemolymph melanization, the density of hemocytes, the *in vivo* phagocytic potential, the concentration of hemolymph proteins and the activity of phenol oxidase throughout the infectious process; and (iv) the histopathological changes in larval tissues as a result of fungal infection. These immunological and cytological parameters may help detect fungal virulence at an early stage.

MATERIALS AND METHODS

All chemicals, media, and solvents used in this study were obtained from Sigma-Aldrich, UK.

The experimental insect

The greater wax moth, *G. mellonella* was obtained and cultured for several generations in constant darkness at 30 ±2°C and 60%-65% humidity, in the laboratory using a diet containing pollen and bee wax. Eggs hatched after about 7 days and the newly hatched larvae were maintained and fed a diet consisting of maize flour, 200g; wheat flour, wheat bran, and milk powder (100g each); glycerol (125 mL), honey (225g) and yeast (25g), until reaching 0.3-0.4g weight and 1.2-1.5 cm length, as previously described (Abo-Kersh and Barakat, 2023). The last instar larvae were obtained ~6 weeks after hatching and used in the subsequent experiments.

Fungal strains and growth conditions

This study used two clinical isolates: the filamentous fungus *A. fumigatus*, and the yeast, *C. albicans.* The selected fungi were identified and kept for one year at the Department of Microbiology, Faculty of Science, Ain Shams University, Cairo, Egypt. Fungal suspensions were prepared according to the National Committee for Clinical Laboratory Standards (NCCLS) - Document M38-A procedure (NCCLS, 2002) to verify the accuracy of treatment. Under moderate agitation (120 rpm) at 37°C for 48 h, fungal isolates were subcultured in Sabouraud-dextrose liquid medium (SAB), which were stored in 20% glycerol stocks. Before each experiment, the fungal cells were streaked over an agar plate containing 2% (w/v) agar in SAB broth at 37°C for 48 h. After that, the green colonies were transferred into the broth and continued growing for another 48 h at 37°C. To remove non-adherent cells, the fungal cells were washed twice with sterile phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM phosphate buffer, pH 7.2). These cells were then counted using a hemocytometer (Corning, New York, USA) and adjusted to a concentration of 10^8 cells/mL for injection. The inoculum injected was 10^6 cells/larva (Smith *et al*., 2022).

Production of extracellular hydrolytic enzymes

The conventional agar plate method was used for the qualitative evaluation of extracellular enzymes (lipase, chitinase, and protease) by growing fungi in specific growth media to detect the enzymes produced (Ramos *et al*., 2017). After 7 days of fungal culture on potato dextrose agar (PDA), the next 5–7 days were spent growing the fungus on a 5 mm disc in a solid medium that included substrate ingredients such as gelatin, olive oil, and colloidal chitin. The region of enzyme activity that surrounds the fungal colony was observed. The assays were performed in triplicate, and the control groups received media free of fungal cells. The specific media that were used to produce and detect enzymes were described as follows:

The protease

For determining protease activity, the fungi were cultured in a glucose-yeast extract-peptone agar (GYP) medium that included 8% gelatin. The gelatin was sterilized individually before being added to the GYP medium. The plates were then immersed in an ammonium sulfate solution until they were saturated; this created a see-through halo that encircled the fungal colony (Amirita *et al*., 2012).

The lipase

A PDA medium supplemented with 1% sterile olive oil was used to cultivate fungus to evaluate its lipase activity. After the incubation period, a sedimentation ring, formed by the precipitation of calcium salts, may be seen around the fungal colony (Amirita *et al*., 2012).

The chitinase

The fungal growth on agar medium supplemented with 2% colloidal chitin allowed for the measurement of chitinase activity. A clear zone around active colonies is seen once the incubation time ends, which is a consequence of the monomeric N-acetylglucosamine liberated from colloidal chitin (Frandberg and Schnurer, 1998). To measure the enzyme activity, Bastos (2005) considered the halo diameter around the fungal colonies. To grade their output, we use the symbols +++ for high production, ++ for moderate, + for low, and — for none.

Mortality assay of *G. mellonella* larvae on fungal infection

According to Morio *et al*. (2019), the process of infecting *G. mellonella* larvae with fungal cells was carried out. Following the protocol laid forth by Walters and Ratcliffe (1983), a 10-µL syringe (Hamilton, Hamilton Company, Reno, NV, USA) was used to inject 10^6 fungal cells into the larval hemocoel via the last right proleg. The controls consisted of larvae that were not injected, and a sham group contained larvae that were injected with 10 µL of PBS. Ten infected larvae per group were cultured in the dark without food or water, at 37°C on Petri dishes lined with filter paper. Daily survival rates were recorded up to five days after treatment. Treated larvae were scored as dead when they met the following conditions: (1) not moving, (2) completely darkening bodies, (3) not reacting to touch, and (4) unable to turn themselves over while upside down. The assay was performed on three separate occasions; the average percentage of larval survival was calculated from data combined.

Quantification of larval melanization

Either 10^6 fungal cells or PBS were used to inoculate the larvae. The hemolymph of each larva was taken at 6, 12, 24, and 48 h and diluted 1:10 with the buffer. The insect physiological saline buffer was (IPS; 150 mM sodium chloride, 5 mM potassium chloride, 10 mM Tris-HCl, pH 6.9, 10 mM EDTA, and 30 mM sodium citrate). The samples were meticulously organized on 96-well microdilution plates. Measurements of optical density (OD_{405}) were taken to quantify melanin levels using a spectrophotometer (Spectramax M2e Molecular Devices, San Jose, California, USA), as per Scorzoni *et al*. (2013). In parallel, photographs were captured to observe the melanization process in the larval body.

Hemocyte density determination in the larval hemolymph

Ten larvae per group were incubated at 37°C for 6, 12, 24, and 48 h after being exposed to 10^6 fungal cells/larva or a solution of PBS (control). After each interval, 30µL of hemolymph was collected in IPS buffer according to Scorzoni *et al*. (2013). Centrifugation at 500 rpm for 5 min was used to collect the hemocytes; they were then washed twice in IPS to remove any remaining hemolymph proteins. A solution of PBS containing 5 mM glucose was then used to resuspend the cells. Hemocyte density was measured with a hemocytometer after a 1:10 dilution in IPS, and cell viability was assessed using the Trypan Blue stain. The units of display were hemocytes/mL.

***In vivo* phagocytosis assay**

The phagocytosis experiment was to determine whether the hemocytes of *G. mellonella* larvae phagocytosed fungal cells and to assess the phagocytic activity against the tested fungal species after different durations of infection. Ten microliters were administered to five-larvae groups after a solution containing 10^8 cells/mL had been first stained with 10 mg/L of Calcofluor white for 30 min at 37°C. Control larvae were injected with PBS. Hemolymph was collected in a 1:10 IPS buffer at 6, 12, 24, and 48 h, and centrifuged at 78 rpm for 4 min. After 30 min of pellet stabilization with 2% paraformaldehyde, the cells were placed on slides, covered, and immediately examined under an optical microscope (Zeiss, Jena, Germany). According to Scorzoni *et al*. (2013), 300 insect cells in triplicate from each treatment were counted and the percentage of hemocytes containing fungal cells was calculated.

Determination of phenoloxidase (PO) activity

After different time intervals, the hemolymph of fungus-injected larvae was spun at 20,000 rpm for 10 min, along with the control group. The protein content was measured in mg/mL in the collected supernatant and using the formula obtained from the standard calibration curve of bovine serum albumin (BSA) solution, as described by Bradford (1976), using a spectrophotometer. To measure the PO activity, the method developed by Ashida and Soederhaell (1984) was used to quantify the formation of dopachrome. 20 plasma and 780 µL of ice-cold PBS (0.01 M, pH 7.2) were mixed in an Eppendorf tube. The mixture was incubated at 25±2ºC after 800µL of 20 mM L-DOPA was added to each sample. After a 20-minute break, the absorbance was assessed at 490 nm. One PO unit is

equal to the amount of enzyme needed to increase absorbance by 0.001 min^{-1}, which is the standard unit for measuring PO activity in milliliters of plasma. For every decision, this test was repeated three times.

Histological study of larval tissues

To better understand how a fungal infection develops, experiments were carried out to look at the histological changes that occurred in the midgut and cuticle of *G. mellonella* larvae. After 24 h of infection, three larvae from each group were fixed in 10% buffered formalin with normal larvae. The larvae were then dehydrated using increasing dosages of ethanol. Following the xylene treatment, the specimens were embedded in paraffin. An optical microscope (Zeiss, Jena, Germany) was used for analysis after staining 3µm tissue slices with hematoxylin and eosin.

Statistics

The data were evaluated using the student's t-test in comparison to the untreated and PBS-treated samples and were shown as means ± standard deviation (SD). We used the terms "significant" ($P < 0.05$) and "very significant" ($P < 0.01$) to indicate statistical differences. Survivorship was conducted by averaging the percentage of larvae that survived all experiments. The fungal burden assay for *G. mellonella* was assessed using a two-way ANOVA. For multiple comparisons, the Bonferroni post-test was used to determine the significance level.

RESULTS

Screening of fungal extracellular enzymes

The results indicated that the studied fungi exhibited variability in the release of extracellular enzyme extents. The findings indicated that *C. albicans* had a superior capacity to produce chitinase and lipase compared to *A. fumigatus,* but both fungal species exhibited equivalent protease production (Table 1).

Virulence of fungal species in *G. mellonella* larvae

The ability of the tested fungi to infect and kill *G. mellonella* larvae was used to determine its virulence. On the first day after the fungal infection, there was a significant increase in larval mortality ($P<0.05$), which persisted until the experiment ended ($P<0.01$) or until pupation, whereas the control group showed little larval mortality. Complete larval mortality was recorded after the fourth-day post-inoculation in *C. albicans*-treated larvae (Figure 1A). The insect mortality showed significant differences among the tested fungi ($F = 12.68$, $P<0.05$) and post-injection times ($F = 180.42$, $P<0.01$). Injected larvae appeared darker in color than those of control larvae (Figure 2).

Table 1. Enzymatic activity of fungi isolated from solid medium

Tested fungi	Extracellular enzyme activity		
	Protease	Lipase	Chitinase
Candida albicans	+	+++	+++
Aspergillus fumigatus	+	+	++
Control	-	-	-

Weak (+), moderate (++), high (+++), unable to produce (−).

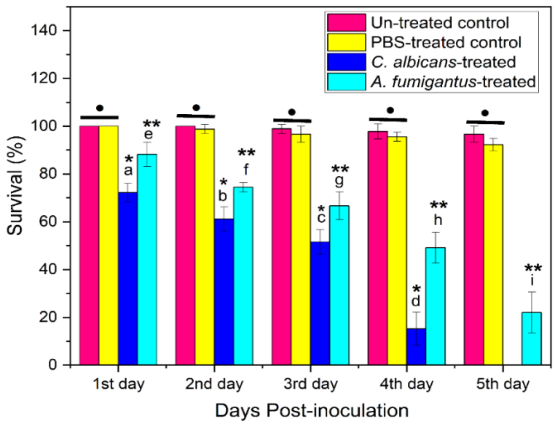


Figure 1. The percentage of *Galleria mellonella* larvae survived after being inoculated with 10^6 cells/larva of *Candida albicans* and *Aspergillus fumigatus* in the fourth proleg. The larvae were kept at 37°C for 4-5 days, with control groups also included. Based on three replicates with ten larvae per treatment, the values presented here are mean ± SD. (●P>0.05 in comparison between un-injected and PBS-injected at different post-injection times, *P<0.05 in comparison between PBS-injected and *Candida albicans*-injected at different post-injection times, and **P<0.05 in comparison between PBS-injected and *Aspergillus fumigatus*-injected at the different post-injection times). There is a definite difference between bars that do not share the same letter.

Darkness progression was marked by increased melanin pigmentation initially at the site of administration, which spreads throughout the entire larva before death. At all observation times, the larval cuticle remained intact and there was no hyphal penetration through the insect surface.

Hemolymph melanin of *G. mellonella* larvae after fungal infection

Fungal inoculation induced early melanization in the larvae within 6 h after injection, followed by increased pigmentation subsequently. Upon injection of larvae with 10^6 fungal cells, a notable buildup of melanin in the hemolymph was seen after 12 h, exceeding levels in non-injected larvae by at least twofold, with a steady rise reaching fivefold at 24 h post-infection. While there seemed to be an increase at 48 h post-inoculation, this was not statistically significant compared to the amount seen at 24 h (Figure 3). There was no significant difference ($F = 17.1$, $P>0.05$) between the tested fungi observed at all examination periods except at 12 h of injection ($F = 14.5$, $P< 0.001$).

(A) Normal (healthy) larvae

(B) *C. albicans*-infected larvae

(C) *A. fumigatus*-infected larvae

Figure 2. Impact of fungal infection on *Galleria mellonella* larvae, detected after 48 h of treatment, shown by varying degrees of melanization on the larvae. (A) larvae administered saline (serving as a control), (B) larvae infected with 10^6 cells of *Candida albicans*, and (C) larvae infected with 10^6 cells of *Aspergillus fumigatus*. Larvae exhibiting melanization were deemed dead, whereas those without melanization were regarded as alive.

Hemolymph protein concentration and phenoloxidase (PO) activity

As shown by Figure 4A, dramatic changes in the total hemolymph proteins (THPs) of *G. mellonella* larvae were observed between the uninjected and the PBS-injected controls, and between the controls and the fungus-injected insects. In PBS-injected insects. At a comparison level in Figure 4B, the PO activity was lower (P<0.05) at 6 h after the challenge with both fungal species as compared to the controls. After 12 h, till the end of the experiment, the PO activity was significantly higher than that of the controls in fungal-injected insects. –No statistical difference in this response was observed among the two fungal species (*P*>0.05).

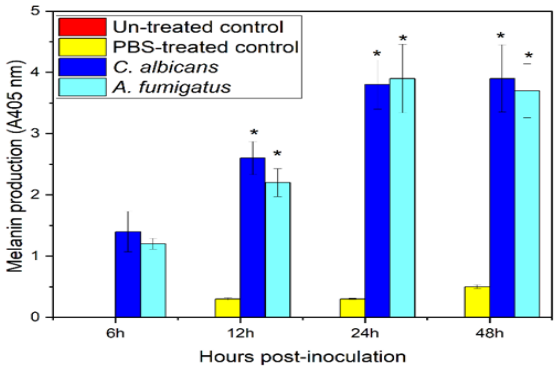


Figure 3. Hemolymph melanin content of *Galleria mellonella* larvae measured at different time points after injection with 10^6 *Candida albicans* and *Aspergillus fumigatus* fungi per larva. The data is shown as the average ± SD from three different replicates. **P*<0.05 in comparison between PBS-injected and fungus-injected at different post-injection times.

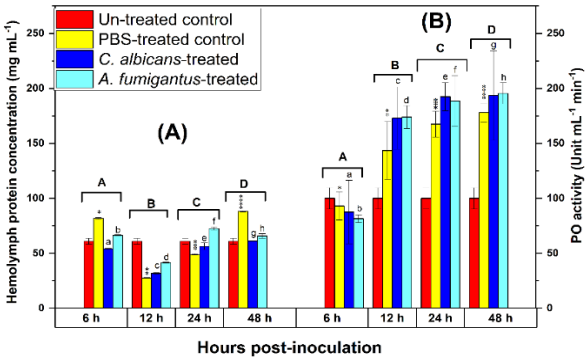


Figure 4. (A) Total hemolymph protein content (mg/mL) of *Galleria mellonella* larvae treated with PBS, *Candida albicans*, *Aspergillus fumigatus,* or control at different time intervals post-treatment. (B) Phenoloxidase (PO) activity (Units/mL/min). The data is shown as the average ± SD from three different studies. **P*<0.05 in comparison between uninjected and PBS-injected, PBS-injected and *Candida albicans*-injected, and between PBS-injected and *Aspergillus fumigatus*-injected at the different post-injection times). There is a definite difference between bars that do not share the same letter. Statistical differences in these responses were observed among the two fungal species (*P*>0.05).

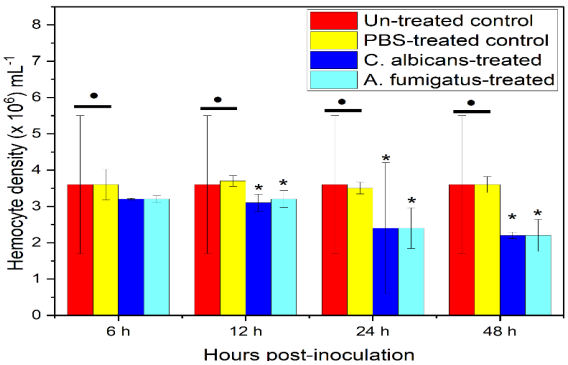


Figure 5. The density of hemocytes in *Galleria mellonella* larvae measured at different time points after injection of the larvae with 10^6 fungi cells of *Candida albicans* and *Aspergillus fumigatus* per larva. Data are plotted as means ± SD of three independent experiments. (●P>0.05 in comparison between un-injected and PBS-injected at different post-injection times, *P<0.05 in comparison between PBS-injected and *Candida albicans*-injected or *Aspergillus fumigatus*-injected larvae at different post-injection times.

Hemocyte density of *G. mellonella* larvae after fungal infection

Figure 5 illustrates that larval exposure to either fungal species led to a substantial reduction in hemocyte concentration, as seen at 12, 24, and 48 h post-infection. Either fungus insignificantly showed different hemocyte density across all post-infection intervals, however, they showed significant reductions when compared with the PBS controls. The number of hemocytes in non-infected larvae was like those inoculated with PBS (P>0.05).

Phagocytic indices

Figure 6 illustrates that both fungal species were phagocytized in identical proportions by larval hemocytes. No changes were seen in the phagocytic capability of cells between the two fungal species examined in this study (P>0.05). Figure 7 illustrates that both granular cells (GRs) and plasmatocytes (PLs) exhibited phagocytic activity. Although the phagocytic activity of hemocytes that consumed *Aspergillus fumigatus* was superior to those that phagocytosed *Candida albicans* cells.

Histological study of *G. mellonella* larval tissue

Tissue sections, 24 h post-initiation of fungal infection, together with PBS controls and tissue sections from untreated larvae, are shown in Figure 8. The assessment of sagittal slices revealed the shape of the *G. mellonella* cuticle and internal organs dispersed underneath, the fat body and muscle fibers (Figure 8A, D). Two fungal species induced a fast advancement of infection, with the recruitment of spherical hemocytes seen in melanized nodules containing fungal cells (Figure 8B, C, E, F).

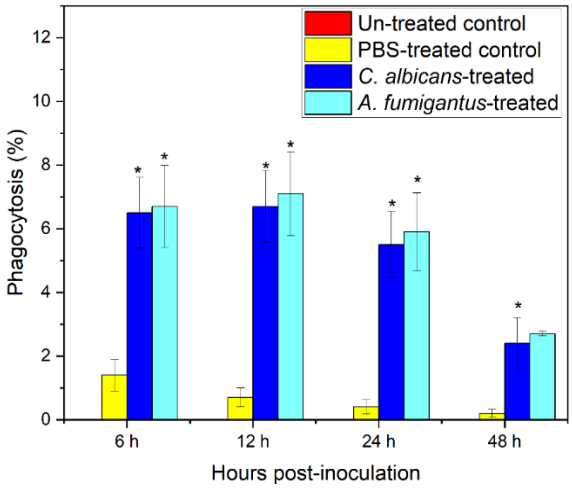
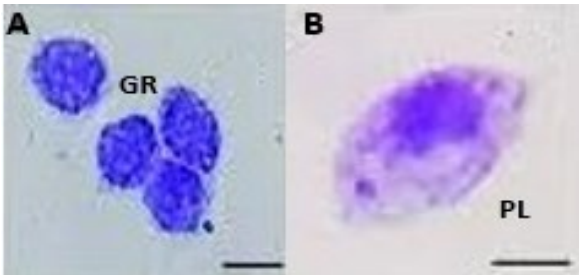
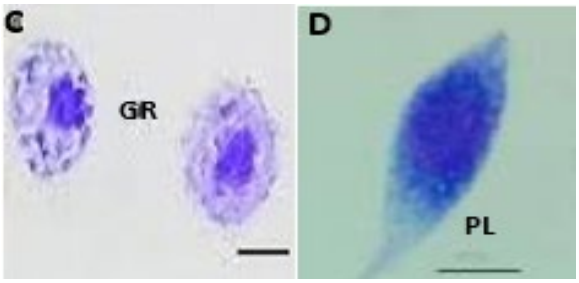


Figure 6. Percentage phagocytosis of *Galleria mellonella* larval hemocytes determined after different time intervals post-injection with 10^6 *Candida albicans* and *Aspergillus fumigatus* cells per larva injected and control larvae show statistically significant differences (*P≤0.05) and no changes were seen between the two fungal species examined (P>0.05).



Phagocytosis of *C. albicans*



Phagocytosis of *A. fumigatus*

Figure 7. Phagocytic activity of *G. Galleria mellonella* larval hemocytes determined at 12 h post-injection with 10^6 fungi per larva. (A) Granular cells (GRs) phagocytosed *Candida albicans* yeast cells, (B) Plasmatocytes (PLs) phagocytosed *Candida albicans* yeast cells, (C) Granular cells (GRs) phagocytosed *Aspergillus fumigatus* fungal cells, (D) Plasmatocytes (PLs) phagocytosed *Aspergillus fumigatus* fungal cells. Bar = 10 µm.

DISCUSSION

The fungi mostly linked to human illnesses include filamentous fungi such as *A. fumigatus* and the yeast *C. albicans* (Pfaller *et al*., 2007; Mukherjee *et al*., 2010). They are among the most dangerous molds, often exhibiting a fatality rate of up to 90% in people. Addressing these infections is very challenging owing to multidrug-resistant fungus strains. Moreover, the restricted supply of pharmaceuticals and their toxicity necessitates the continuous exploration of novel alternative compounds. This necessitates comprehensive research using cost-efficient and ethically uncomplicated host models.

Here, we show that it is possible to use *G. mellonella* larvae to distinguish between different levels of pathogenicity among distinct groups of human pathogenic fungi. In contrast to other commonly used model systems, larvae provide a convincing and reproducible paradigm for studying the relative pathogenicity of fungal species. Among the several advantages of the *Galleria* model are its reduced costs and increased ethical acceptability. Furthermore, the similarity of *G. mellonella* immune system with the human innate immune system makes it an optimal experimental tool. However, despite its advantages, there are also some drawbacks to consider: the lack of guidelines regulating their use and nascence of adaptive immune response i.e., they do not produce antibodies, but are limited to the production of

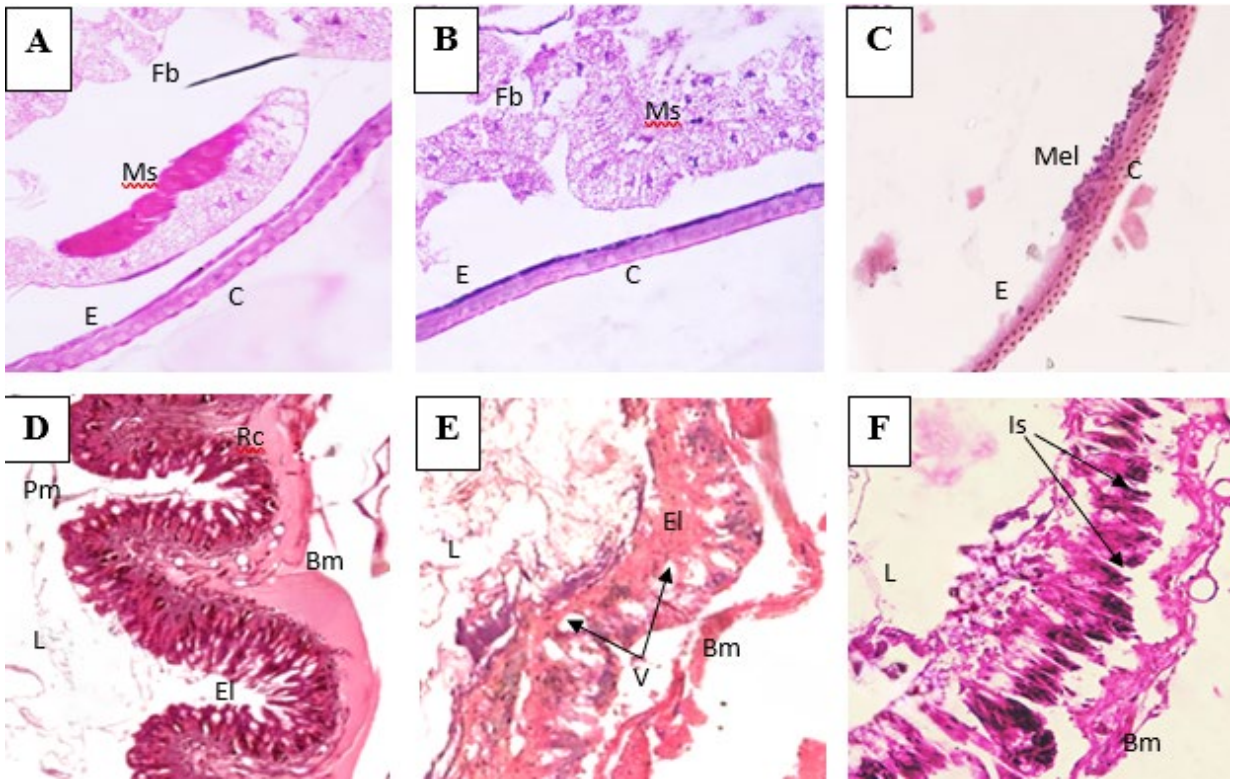


Figure 8. Light microscope photomicrographs of tissue sections from normal and fungus-treated *Galleria mellonella* larvae after 24 h of injection with 10^6 cells/larvae stained with hematoxylin and eosin. [A] Untreated larva showing normal cuticle (C) with its epidermis layer (E) and fat body (Fb), [B] *Candida albicans*-treated larva, and [C] *Aspergillus fumigatus*-treated larva showing melanin (Mel) formation beneath the cuticle layer (Note: the cells of the infected fat tissue became loose and were separated from each other). (D) Untreated larva showing normal midgut with its epithelium layer (El), gut lumen (L), peritrophic membrane (Pm), basement membrane (Bm) and regenerative cells (Rcs). (E) *Candida albicans*-treated larva, and (F) *Aspergillus fumigatus*-treated larva showing the formation of some vesicles (V) which are shed into the gut lumen of the epithelial cells (Note: (1) elongation of midgut epithelium leaving intracellular spaces (Sp) in between, (2) the columnar cells are destroyed and extrude their contents into the gut lumen until the nucleus itself with early stage of lyses, (3) complete separation of columnar epithelium from basement membrane and replication of regenerative cells beneath it, (4) complete damages to the basement membrane, and (5) all that remains of the anterior midgut epithelium is a disorganized matrix of cellular debris of necrotic epithelial layer and fungal pathogen and its germination hypha (original magnification × 200).

proteins that confer only non-specific immunity (Kavanagh and Reeves, 2004; Browne *et al.,* 2013; Jacobsen, 2014; Kavanagh and Sheehan, 2018; Silva *et al*., 2018; Serrano *et al*., 2023, Giammarino *et al.,* 2024). Given the specific interest in developing non-mammalian host models to investigate microbial virulence and mitigate the ethical implications of animal testing, we previously elucidated the usefulness of the *Galleria* model host for evaluating antibiotic activity against *Escherichia coli* (Abo Kersh and Barakat, 2023). The current work examined the efficacy of this model against prevalent opportunistic fungal infections, *C. albicans* and *A. fumigatus*, along with their pathogenic pathways to evaluate host-pathogen interactions. Furthermore, the insect immune system exhibits several similarities to the human innate defense. Thus, *Galleria* could be used in research concerning the pathogenicity of human diseases (Kavanagh and Sheehan, 2018).

The pathogenicity of these fungal species is widely known. Nevertheless, *G. mellonella* larvae exhibited greater susceptibility to *C. albicans* than *A. fumigatus*. Consequently, these findings corroborated the virulent difference of the two examined fungal species. The disparities in relative pathogenicity may be attributed to the broader spectrum of virulence factors (phospholipase activity, and cellular adherence to colonize the host epithelial surfaces) shown by *C. albicans* (Barrett-Bee *et al*., 1985; Odds, 1994; *Cotter et al*., 2000), or maybe to the upregulation of these virulence factors. The mortality of larvae may be attributable to the abundance of fungal cells in the insect's hemolymph as reported by (Dunphy and Thurston, 1990). Disparities in fungal growth rates and the production of tissue-damaging hydrolytic enzymes may explain the differences in virulence across species. The low-virulent *A. fumigatus* species showed diminished extracellular enzymatic activities. This supports the original finding of Rossoni *et al*. (2013), who first linked *in vitro* enzyme production with *in vivo* pathogenicity.

Further studies were conducted in the fields of immunology and cytology to investigate the host-pathogen interactions that have a role in the development of virulence factors in *G. mellonella* larvae. As the first line of defense against a wide variety of pathogens, the cuticle - a component of the *Galleria* innate immune system - contains chitin, fatty acids, lipids, and sterols (Vincent and Wegst, 2004). The detection and defense against invader microbes, as well as the transportation and storage of nutrients, are all essential functions of the hemolymph, which includes hemocytes, a variety of soluble proteins, and immunological effectors (Barakat, 2006).

The dark color of larvae that have been injected with both fungal species under study is associated with the production of the melanin pigment, an immune response by the insect to non-self-matter such as fungi. After larval injection, a gradual darkening of the skin began, and the degree of darkening increased with time and correlated with the type of inoculum. As the infection worsens, the melanin begins to spread from little black spots on the larval cuticle surface to the whole surface. A complete darkening of the larvae's skin might be the outcome of this, signaling a deadly infection that eventually kills the larvae. Based on their investigations into the virulence factors of *C. albicans* and *A. fumigatus*, respectively, Durieux *et al*. (2021) and García-Carnero *et al*. (2020) have reached similar conclusions. Melanization begins when certain chemical patterns are detected by receptors on the surface of cells, such as β-1,3-glucan, lipopolysaccharides (LPS), and peptidoglycans (Whitten *et al*., 2004). According to Kavanagh and Reeves (2004), C-reactive protein is produced and then moved to the cuticle, the damaged area, or the hemolymph until melanin polymerization takes place. This protein is like Toll-like receptors (TLRs) in mammals.

Melanization helps eliminate pathogens by activating the prophenoloxidase cascade. This cascade produces molecules that are extremely toxic to microbes, which the insect uses for phagocytosis (Binggeli *et al*., 2014) and encapsulation (Jiravanichpaisal *et al*., 2006), and eventually to form a cellular barrier that regulates invasion. Melanin is made possible by this enzyme that helps oxidize phenols to quinones. The quinones are subsequently formed via polymerization, which does not need enzymes (Trevijano-Contador and Zaragoza, 2014). Our results on PO activity agree with those of García-Carnero *et al*. (2020), who found that inoculating *G. mellonella* larvae with *C. albicans* increased enzyme activity. On the other hand, our results contradict those of Vertyporokh and Wojda (2020), who found that the same organisms (*G. mellonella* larvae) had a significant decrease in PO activity when given yeast cells. This contradiction might be due, in part, to differences in the *C. albicans* strains tested or the dose utilized.

Regarding the impact of PO activity and melanin production on insect defense mechanisms, it seems that larvae with low survival periods increased the levels of both. Therefore, it is reasonable to assume that the strong activation of these humoral effectors causes the insect to redirect its resources towards this reaction, which might harm its fitness. Also, different fungal species have cell wall glucan at different rates, which might induce PO activity and, therefore, melanization (Pereira *et al*., 2018; Pérez-García *et al*., 2016). In addition, it has been suggested by Pereira *et al*. (2018) that the PO activity and melanin could be activated by the cell wall protein (glucan), and exposure to this molecule can vary according to the fungal species (Pérez-García *et al*., 2016).

Phagocytosis, encapsulation, and nodulation of pathogens into the body are all functions of hemocytes, which are responsible for cellular immune responses. According to Wu *et al*. (2016), hemolymph may include freely circulating hemocytes or they can be attached to certain internal organs like digestive and reproductive systems. To find out how fungal infection affects insects' immune responses, we looked at *G. mellonella* larvae to see whether different kinds of fungi affected the density of their hemocytes. Like one another, both fungal species significantly reduced hemocyte content when injected into larvae. When *G. mellonella* is infected with microbes, the density of its hemocytes changes (Bergin *et al*., 2003). Several fungal isolates are more harmful during infection in *G. mellonella* where there is a drop in hemocyte levels (Bergin *et al*., 2003; Sheehan and Kavanagh, 2018; Sheehan *et al*., 2018; Stączek *et al*., 2020). Matha and Acek (1984) and Mesa-Arango *et al*. (2013) suggested that the reduction of hemocytes after fungal infection is due to their aggregation, and not to fungal virulence, however, the exact mechanism underlying this observation is still not well understood. According to Kumar and Khan (2018), phagocytosis plays a pivotal role in the innate immune response of *G. mellonella*. Because phagocytosis is crucial for clearing fungal infections, we looked at the functional viability of larval hemocytes. Therefore, we compared the phagocytosis of both fungal cells (*A. fumigatus* and *C. albicans*). Larval hemocytes phagocytized both types of fungi (i.e., the two fungal species did not differ in their phagocytic capabilities). In phagocytosis, fungal cells are taken in by the larva

and stuffed within its hemocoel. This happens when small infectious agents, such as bacteria, fungi spores, or other foreign bodies are recognized at the surface of hemocytes (Bergin *et al*., 2003; Desalermos *et al*., 2012; Mesa-Arango *et al*., 2013). While this finding supports the hypothesis put out by Mesa-Arango *et al*. (2013) regarding fungal species facilitating larval death as a means of phagocytosis avoidance, it does not provide any clarity on the species-specific differences in virulence. Since the two fungal species studied were able to be recognized by the phagocytes, there were no significant differences in the phagocytosis percentage of hemocytes.

The adherent properties of the hemocytes (plasmatocytes and granulocytes) of *G. mellonella* larvae were further shown by our previous study (Barakat *et al*., 2024) to indicate that these cells are phagocytic. According to Wu *et al*. (2016), these cells are comparable to human neutrophils and make up most of the blood cells. What exactly in insects is mostly responsible for phagocytosis is a matter of debate. After 24 h of treatment with 10^6 cells, larvae of *G. mellonella* had destroyed the non-sclerotized cuticle, epidermal layer, muscles, fat bodies, and midgut epithelium. The occurrence of integument and midgut disturbance is dependent on the promotion of protein degradation in these layers (Ibrahim *et al*., 2019). The fungal effect as destructive in the cuticle layer is possibly related to fungal hydrolytic enzymes. The damaged tissues stimulated the insect defense mechanisms through the signaling pathways which regulate the expression of immune effector molecules to be liberated in hemolymph (Wojda *et al*., 2020). The treated larvae observed large vacuoles, dimensions of the fat bodies, and complete separation of epidermal cells. These results are supported by Perdoni *et al*. (2014). Fungi also produce pathological changes in the columnar cells of the midgut by extruding their contents into the gut lumen. These results are consistent with Curtis *et al*. (2022) who indicated that the death of larvae by fungal species was due to toxic proteins extracted by fungi leading to a progressive bleeding of the midgut epithelium into the gut lumen with lyses of the epithelium layer.

The presence of melanin deposition, as shown by histological investigation, reflects the immune response of *G. mellonella*. In line with previous findings in numerous studies on fungal infections, melanin is primarily found near the cuticle, along with the epidermis and fat body (Bergin *et al*., 2003; Eisenman *et al*., 2014; Perdoni *et al*., 2014; Wuensch *et al*., 2018; Sheehan and Kavanagh, 2019; Torres *et al*., 2020).

CONCLUSION

Based on the pre-mentioned characteristics, *G. mellonella* larvae are considered an ideal model for studying fungal virulence. Larval burden as well as some immunological and cytological parameters, were shown to be associated with shorter larval survival after fungal inoculation, indicating that they might be used as early predictors of virulence. Evidence suggests that *C. albicans* is a more pathogenic yeast than *A. fumigatus*.

CONFLICT OF INTEREST

No conflict of interest to declare.

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AUTHOR CONTRIBUTIONS

Conceived and designed experiments: E.M.S.B. and A.S.B. - Performed the experiments, analyzed the data, and wrote the manuscript (original draft): M.O.A., H.A.A. and Z.A.S. - Supervision, and writing (review and editing): E.M.S.B. and A.S.B. All authors read and approved of the final manuscript.

DATA AVAILABILITY

All data is presented within the article.

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