Candida parapsilosis, Candida orthopsilosis, and Candida metapsilosis virulence in the non-conventional host Galleria mellonella

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The incidence of fungal infections due to *C. parapsilosis* and closely related cryptic species (-*psilosis* complex) has increased in the last few years, but differences in virulence among these species have not been widely studied. Fifteen clinical isolates of *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*, including the type strains, were used to evaluate their virulence in *Galleria mellonella* larvae. Fluctuations in the hemocyte density and in the phagocytic activity were also tested. Differences in the median survival for these species were demonstrated at 37 °C (2.6 ± 1.02, 2.3 ± 0.92, and 4.53 ± 1.65 d for *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*, respectively). *Galleria mellonella* hemocytes phagocytosed *C. metapsilosis* strains more effectively than did for *C. orthopsilosis* and *C. parapsilosis* (*P* < 0.05). The phagocytosis rate was lower for *C. parapsilosis* than for *C. orthopsilosis* (*P* < 0.05). The hemocyte density was increased in larvae infected with *C. metapsilosis* compared with those infected with *C. parapsilosis* or *C. orthopsilosis* (*P* < 0.05). Moreover, in vitro studies of virulence factors such as pseudohyphae production and hydrolytic enzyme secretion showed that the capability of *C. metapsilosis* strains to produce those virulence factors was reduced. Infections due to -*psilosis* complex species produced tissue damage in *G. mellonella* and pseudohyphae could be also observed during infection with *C. parapsilosis*.

Introduction

Infections due to *Candida parapsilosis* are the second most common cause of candidemia in some European countries and Latin America.[1-3] However, in some invertebrate models, *C. parapsilosis* has shown reduced virulence compared with other *Candida* species.[4] In 2005, Tavanti et al. described two new species related to *C. parapsilosis* as a cause of *Candida* infections: *C. orthopsilosis* and *C. metapsilosis*.[5] The prevalence of these species is increasing, and *C. orthopsilosis* has been recently described as the fifth cause of fungemia in Spain over *C. krusei*.[6] Antifungal susceptibility patterns in these species showed reduced in vitro susceptibility to echinocandins although it has been reported that minimum inhibitory concentrations (MICs) to caspofungin for *C. orthopsilosis* and *C. metapsilosis* are lower than those for *C. parapsilosis* sensu stricto.[7]

Virulence of the *C. parapsilosis* complex has been studied using different models. An in vitro infection model using microglial cells pointed out *C. metapsilosis* as the least virulent member of the -*psilosis* complex.[8] Besides, virulence of *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* was also assessed in epithelial and epidermal tissue models. *Candida metapsilosis* was found to be the least pathogenic in that it produced fewer alterations

and yeasts could rarely be observed attached to the epithelium.[9] Despite known differences in virulence, it is unclear what virulence factors (biofilm development, lipases, or aspartyl proteinases) may play a role in the final outcome.[10-15]

Murine models are the gold standard for virulence studies; however, ethical considerations associated with mammalian models have made invertebrate models (*Caenorhabditis elegans*, *Drosophila melanogaster*, *Galleria mellonella*, *Dictyostelium discoideum*, etc.) an attractive alternative.[16] Several articles have shown the utility of *G. mellonella* as a model to study fungal infection.[17,18] *G. mellonella* provides important advantages such as larval maintenance at 25–37 °C. direct delivery of exact inoculum by injection, and mortality assessment without special equipment requirements.[17,19] Moreover, some of the fungal virulence factors described to be important in mammalian infections have also been found in *G. mellonella*.[17,19,20]

An additional advantage of *G. mellonella* is that its innate response is conserved in mammals. Hemocytes are important in the larva's cellular defense against bacteria and fungi since they act as phagocytic cells.[17,21,22] Fluctuations in the hemocyte density and in the phagocytosis rate have been related to the ability of the fungus to kill larvae, although several pathogens have developed strategies to avoid phagocytic killing.[23,24] Currently,

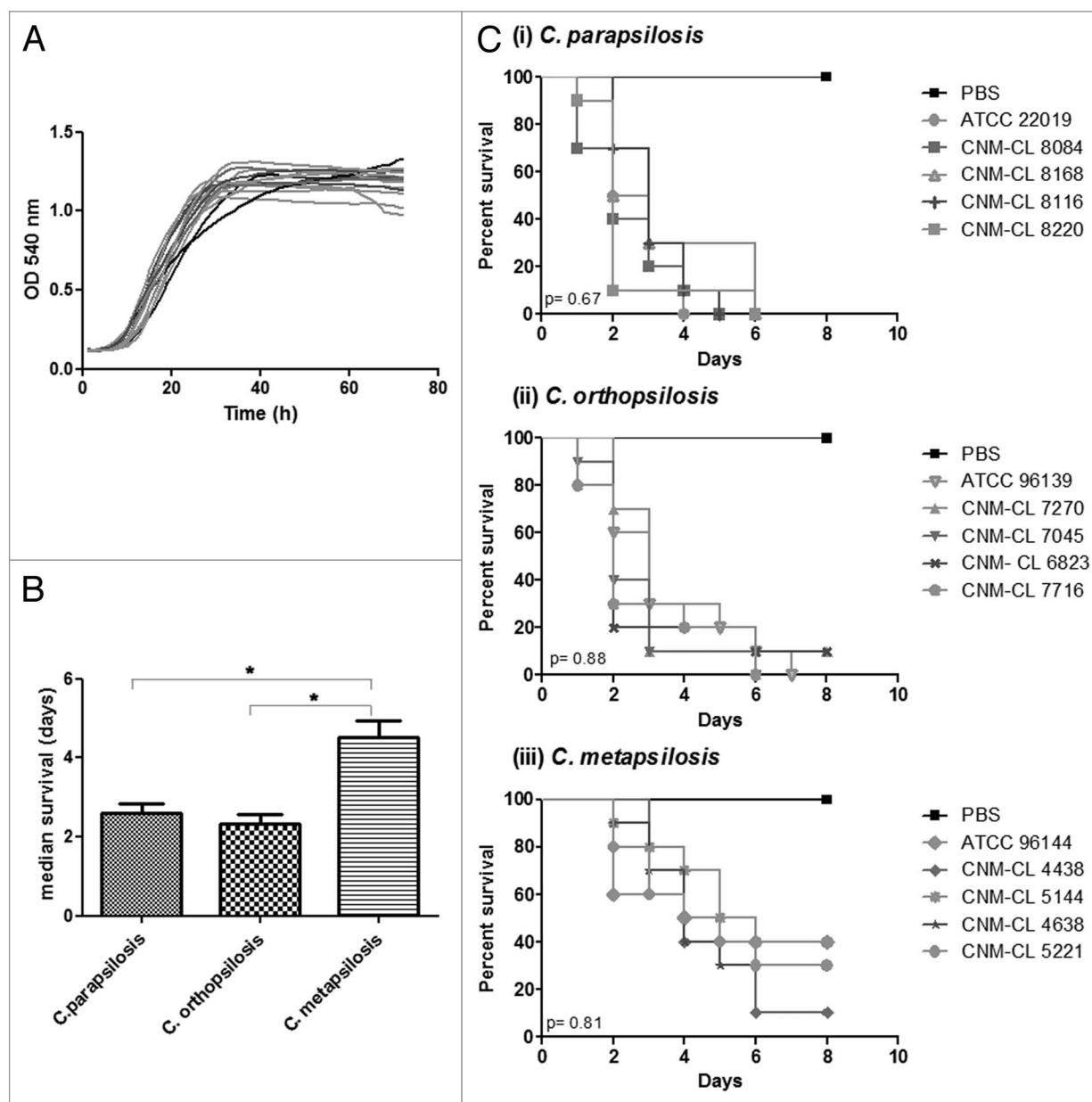**Figure 1.** (**A**) Growth curves of *C. parapsilosis* strains at 37 °C. (**B**) Differences in the median survival of *G. mellonella* larvae infected with *C. parapsilosis* species. (**C**. Survival curves of *G. mellonella* infected with five different strains of *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* at a final concentration of 2×10^6 yeasts/larva and incubated at 37 °C. *P* value below 0.05 is indicated with asterisk.

these two parameters, hemocyte density and phagocytosis rate, have been used to evaluate virulence of *Cryptococcus neoformans*, *Fusarium oxysporum*, *Aspergillus fumigatus*, *C. albicans*, *C. krusei*, *Histoplasma capsulatum*, *Paracoccidioides lutzii*, and *C. tropicalis*.[25-31]

In the present study, we demonstrate the ability of the species belonging to *C. parapsilosis* complex to produce lethal infection in *G. mellonella*. Additionally, hemocyte density, phagocytosis activity, pseudohyphae formation, and production of hydrolytic enzymes were also evaluated using five clinical strains of each of the species including their type strains.

Results

Differences among *C. parapsilosis* sensu lato (s.l.) virulence in *Galleria mellonella* are not due to differences in their growth rate

In the present study we compared different isolates of *C. parapsilosis*, *C. metapsilosis*, and *C. orthopsilosis* for their capability to kill *G. mellonella*. Five strains per species were evaluated. Inoculum standardization was assessed with the type strains and a 2×10^6 yeasts/larva inoculum was assessed to compare differences among species. No significant differences in growth rates at 37 °C were detected for any species ($P > 0.05$) (**Fig. 1A**).
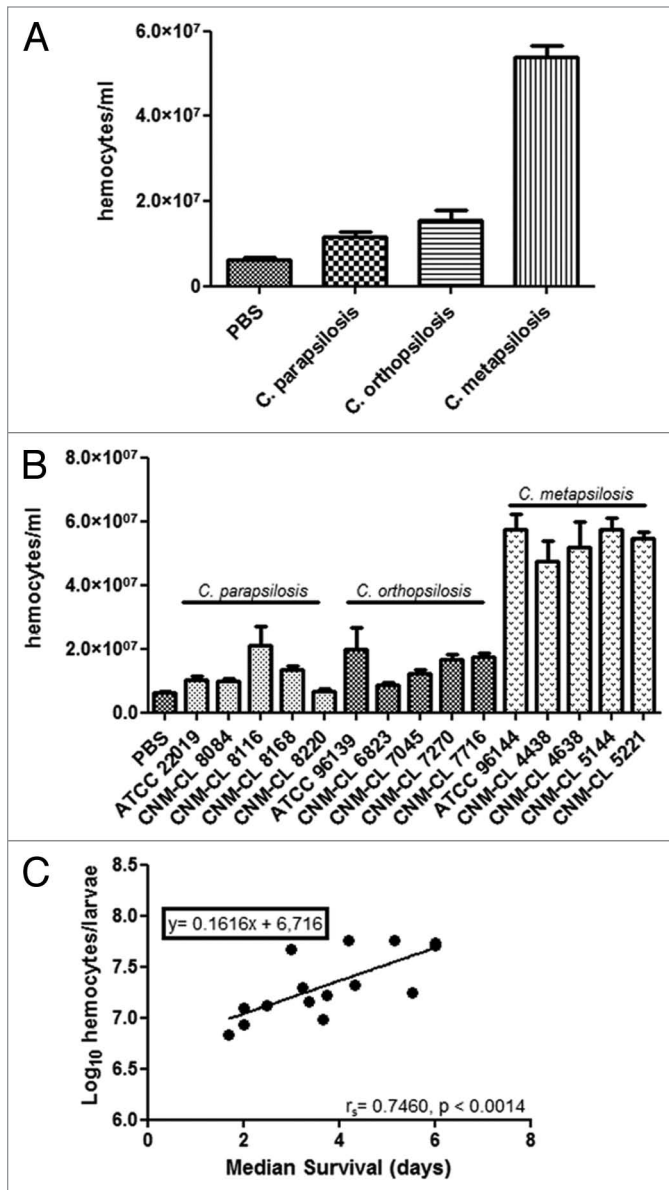
Figure 2. (**A**) Changes in the global hemocyte density during *C. parapsilosis* species infection. (**B**) Differences in the hemocyte density in *G. mellonella* due to infection with different *C. parapsilosis* strains. (**C**) Correlation between median survival and hemocyte density in *G. mellonella* larvae infected with *C. parapsilosis* strains. Significant differences (*P* < 0.05) in hemocyte density relative to that in the PBS treated larvae are indicated with an asterisk.

The median survival for larvae infected with *C. metapsilosis* strains was significantly higher than that of larvae infected with *C. orthopsilosis* and *C. parapsilosis* (*P* < 0.05). However, no differences in the median survival were detected when we compared *C. orthopsilosis* and *C. parapsilosis* infection (**Fig. 1B**, *P* > 0.05). No differences in the median survival were found within strains belonging to the same species (**Fig. 1C**, *P* > 0.05).

Hemocyte density in *Galleria mellonella* differs in *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*

Galleria mellonella larvae were infected with 10^6 yeasts/larva and hemocytes were enumerated after 2 h of infection at 37 °C (**Fig. 2A**). All the species induced an increase in the hemocytes present in the hemolymph of *G. mellonella* compared with controls (*P* < 0.05). Hemocyte population was significantly higher in larvae infected with *C. metapsilosis* than in those infected with *C. parapsilosis* or *C. orthopsilosis* (*P* < 0.05). Moreover, the number of hemocytes in the hemolymph of larvae infected with *C. orthopsilosis* was higher than that recorded in larvae infected with *C. parapsilosis* (*P* < 0.05). No differences in hemocyte density were observed when larvae were infected with different strains of the same species (**Fig. 2B**).

A Spearman correlation coefficient (r_S) was determined by comparing the median survival and the hemocyte density data for each of the strains. From these r_S values, a Spearman correlation test was performed to determine whether those parameters varied regularly. A direct correlation between hemocytes and median survival was found (r_S = 0.7460) (**Fig. 2C**). *Candida metapsilosis* showed the highest median survival and the highest amount of hemocytes in the hemolymph in *G. mellonella*.

C. metapsilosis* is more effectively phagocytosed by *G. mellonella* hemocytes than *C. parapsilosis* and *C. orthopsilosis

To investigate whether the differences observed in virulence could be associated with differences in the efficiency of the innate immune system against these species, experiments of phagocytosis in vivo were performed. Larvae were infected with the type strains of each species and then incubated for 2 and 4 h. No differences in the phagocytosis efficiency were detected between 2 h and 4 h and thus phagocytosis at 2 h was determined for all of the strains. After 2 h of infection, all *C. metapsilosis* strains were more effectively phagocytosed by *G. mellonella* hemocytes than *C. parapsilosis* and *C. orthopsilosis*. Differences in the percentage of phagocytosis were statistically significant when compared among the species (*P* < 0.05) (**Fig. 3A**). No differences in the phagocytosis efficiency were detected intraspecies, except for *C. orthopsilosis* strain CNM-CL 7270 and *C. metapsilosis* strain CNM-CL 4438 that showed very low phagocytosis rates (**Fig. 3B**).

A positive correlation (r_S = 0.6714) was detected between percentage of phagocytosis and median survival (**Fig. 3C**). *Candida metapsilosis* showed the highest phagocytosis efficiency and the highest median survival (4.53 d). Percentage of phagocytosis and hemolymph density showed also a positive correlation (r_S = 0.4651) between *C. metapsilosis* strains with the highest phagocytosis efficiency and the highest values for hemocyte population after infection (**Fig. 3C**).

Pseudohyphae formation and secretion of hydrolytic enzymes

Secretion of hydrolytic enzymes and pseudohyphae production results are summarized in **Table 1**. All *C. parapsilosis* strains produced pseudohyphae after 24 h, while only 2 out of 5 strains of *C. orthopsilosis* were able to produce pseudohyphae after 48 h. In contrast, none of the *C. metapsilosis* strains produced hyphae or pseudohyphae after 48 h of incubation.

Table 1. Enzymatic profiles and pseudohyphae production of the strains included in this study

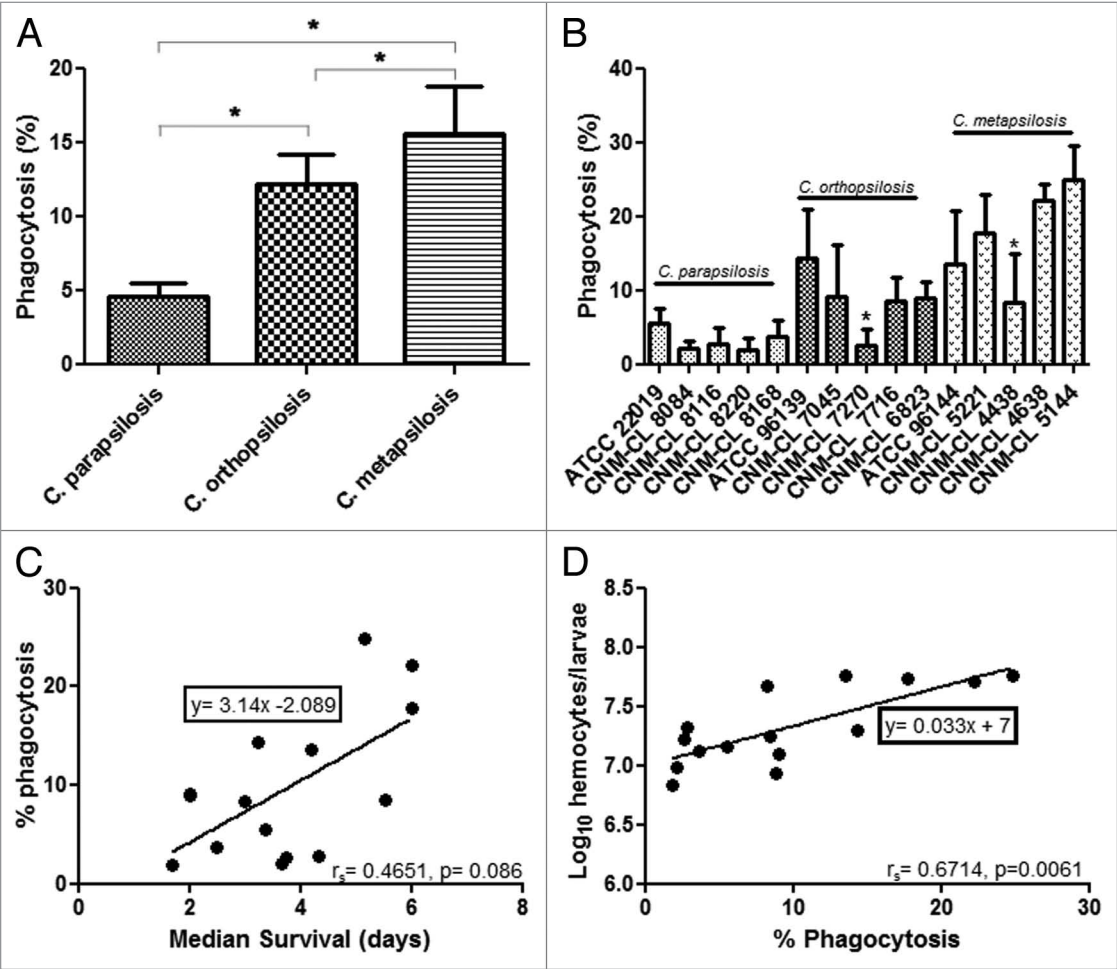| Species	Protease	Esterase	Urease	Hemolysin	24 h pseudohyphae	48 h pseudohyphae
C. parapsilosis	5/5	1/5	5/5	2/5	5/5	5/5
C. orthopsilosis	3/5	3/5	5/5	0/5	1/5	2/5
C. metapsilosis	1/5	4/5	5/5	2/5	0/5	0/5

Figure 3. (**A**) Percentage of phagocytosis at 2 h post infection with *C. parapsilosis*. (**B**) Average of phagocytosis percentages of *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*. (**C**) Correlation between median survival and percentage of phagocytosis during *C. parapsilosis* sensu lato infection. (**D**) Correlation between percentage of phagocytosis and hemocyte density during *C. parapsilosis* sensu lato infection. *P* value less than 0.05 is indicated with asterisk.

Regarding the secretion of hydrolytic enzymes, we found that all *C. parapsilosis* strains secreted proteases. Curiously, most of the *C. metapsilosis* strains showed esterase activity. However, in vitro production of hydrolytic enzymes varied among *C. orthopsilosis* strains and therefore no correlation with other virulence traits could be established for this group.

Candida parapsilosis sensu stricto induced pseudohyphae formation in *G. mellonella*

Histopathology studies of larvae infected with *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* type strains were performed at days 1, 2, and 3 post-infection (**Fig. 4**). At day 3 post-infection, *C. parapsilosis* was spread all over the fat body of

G. mellonella larvae and both yeasts and pseudohyphae forms could be found (white arrows, **Fig. 4B**). These pseudohyphae were formed by 2–3 blastoconidias and resemble those observed during *C. krusei* infection of *G. mellonella*.[28] In larvae infected with *C. orthopsilosis*, infection was spread all over the body fat of the larvae similar to the pattern observed in *C. parapsilosis* infection. However, only yeast forms were observed (red arrows, **Fig. 4D**). Finally, infection of larvae with *C. metapsilosis* resulted in focalized infection, and only yeast forms could be observed during the infection (black arrows, **Fig. 4F**). Yeasts were always found surrounded by tissue in a granuloma-like structure. Curiously, these structures varied in size during infection. At day 2
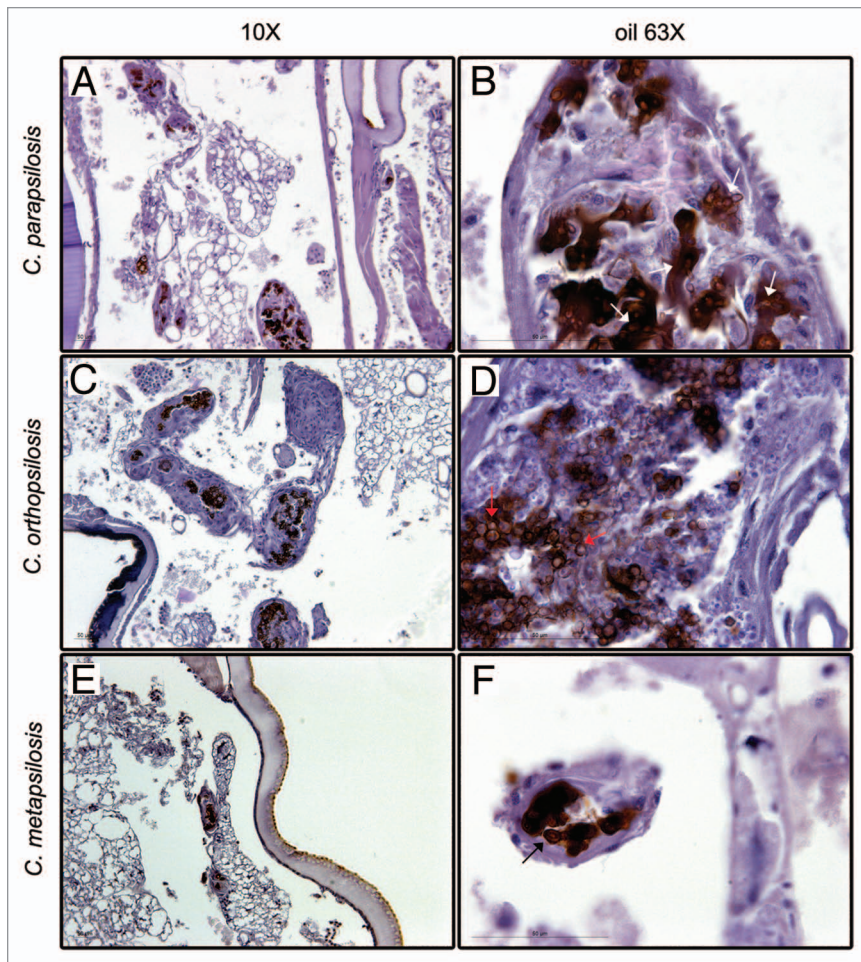
Figure 4. Histopathology *of G. mellonella* infection infected with 2×10^6 yeasts/larva of *C. parapsilosis* (**A and B**), *C. orthopsilosis* (**C and D**), and *C. metapsilosis* (**E and F**) at day 3 post infection. Panels show images taken with low magnification (**A, C, and E**) and high magnification (**B, D, and F**).

differences could not be associated to differences in their growth rate.[35]

Macrophages play an important role in the immune response by phagocytosing and killing microorganisms.[36-38] The ability of macrophages to internalize medically important fungal species as *C. neoformans*, *C. krusei*, *Aspergillus* spp., *C. albicans*, and also species belonging to *C. parapsilosis* spp. complex has already been described.[28,36,39-43] The *Galleria mellonella* immune system consists mainly of hemocytes, which have phagocytic activity[44] and play an important role against pathogens.[45,46] Changes in the hemocyte density during infection can be used as a parameter of the response of the larvae to the infection.[44] We showed that these hemocytes can internalize *C. parapsilosis* species after 2 and 4 h of incubation as previously described for other fungal species.[26,28] Interestingly, although *C. metapsilosis* is the least phagocytosed of the *-psilosis* complex by microglial cells,[8] we have observed higher percentages of phagocytosis for *C. metapsilosis* strains compared with *C. parapsilosis* and *C. orthopsilosis* in our model. In fact, we observed the highest counts of hemocytes after 2 h of infection with *C. metapsilosis* compared with the other two species, suggesting a higher immune stimulation and therefore giving a possible explanation to the longer survival with this species.

A reduction in the number of phagocytic cells in larvae inoculated with *C. parapsilosis* and *C. orthopsilosis* species compared with *C. metapsilosis* infected larvae was observed. These two species reportedly develop pseudohyphae during the infection, as observed in histological preparations of the infected larvae. We demonstrated the capability of *C. parapsilosis* strains to develop pseudohyphae in vitro, while this characteristic was limited or absent in *C. orthopsilosis* and *C. metapsilosis* (**Table 1**). Pseudohyphae formation could lead to the death of the phagocytic population which could be the reason for the low numbers of hemocytes found in larvae infected with these species.[8,34,42]

The development of hyphae or pseudohyphae has been linked to virulence in some *Candida* species such as *C. albicans*, *C. krusei*, and *C. tropicalis*.[25,27,28] The reduced virulence of *C. parapsilosis* compared with *C. albicans* has been associated to the ability of the later to produce hyphal forms that are more adherent compared with the pseudohyphal forms produced by *C. parapsilosis*.[47,48] Other virulence traits, such as secretion of hydrolytic enzymes or pseudohyphae production, have been widely used to analyze differences in virulence among species belonging to the *-psilosis* complex.[42] No correlation between the production of hemolysins or urease and the development of pseudohyphae could be

post-infection, granuloma-like structures were bigger than at day 3 (data not shown).

Discussion

Since the description of cryptic species of *C. parapsilosis*, several molecular tools to differentiate them have been described, but virulence studies remain scarce. In the present work, we compared *C. metapsilosis*, *C. parapsilosis*, and *C. orthopsilosis* pathogenicity using *Galleria mellonella* as a model host. *Galleria mellonella* has been used to study virulence of some *Candida* spp., *Cryptococcus* spp., *Fusarium* spp., and *Aspergillus* spp, showing strong correlation with mammal models.[17,20,25-33]

In agreement with previous studies in other models, differences in virulence for *C. parapsilosis* complex were detected in *G. mellonella*. *Candida metapsilosis* was the least virulent of the *-psilosis* complex[7-9,34] with a median survival longer than that one observed with the other two species (4.53 d vs. 2.6 and 2.3 d for *C. parapsilosis* and *C. orthopsilosis* respectively). However, these

established for any of the -psilosis species. However, all *C. parapsilosis* strains showed proteinase activity and were able to develop pseudohyphae, which could lead to the evasion of the phagocytosis observed in vivo. In contrast, no pseudohyphae formation was observed in any of the *C. metapsilosis* and only one strain showed proteinase activity. In addition, most *C. metapsilosis* produced esterase as described in Ge et al.[49] while only one *C. parapsilosis* was able to produce this enzyme. Finally, *C. orthopsilosis* strains were the most heterogeneous group regarding the production of both hydrolytic enzymes and pseudohyphae, and therefore no correlation could be established.[42] Other unknown virulence factors might be involved in the pathogenesis of this last species.

We were able to correlate the degree of damage on the histological preparations of *G. mellonella* to the virulence showed for each species, as described for *C. tropicalis* infections.[27] *C. metapsilosis* induced minor alterations in *G. mellonella* compared with both *C. parapsilosis* and *C. orthopsilosis*. *Candida parapsilosis* produced pseudohyphae and severe tissue damage in the larvae with numerous areas of infection. Occasional pseudohyphal structures were also detected in *G. mellonella* infected with *C. orthopsilosis*, although the main structures observed were clustered yeast cells surrounded by tissue structures. Several studies have also shown that pseudohyphae production is not common in *C. orthopsilosis*.[13,42] Differences in virulence have been described between *C. orthopsilosis* and *C. parapsilosis* although the incidence varies between studies.[1,2,6,8,12,50] Our results suggest that initial phagocytosis does not predict the final outcome of *C. orthopsilosis* infection since despite being easily phagocytized by hemocytes, *C. orthopsilosis* could cause disseminated infection.

Our work demonstrates that *G. mellonella* is a good alternative model to study virulence in species belonging to the -psilosis complex. The results are in agreement with previous work that pointed out *C. metapsilosis* as the least virulent of the *C. parapsilosis* species complex.[8] Differences in the production of virulence factors in vitro, such as pseudohyphae production or proteinase and esterase activity, could be linked to the differences observed in phagocytosis in vivo. Moreover, for the first time, virulence has been associated with differences in hemocyte influx in response to the infection, phagocytosis efficiency and the capability of undergoing morphological changes that can lead to tissue damage.

Materials and Methods

Yeast strains and culture conditions

Fifteen strains of *C. parapsilosis* complex isolated from blood cultures were used in this study. *Candida parapsilosis* ATC. 22019 (T), *C. orthopsilosis* ATC. 96139 (T), and *C. metapsilosis* ATC. 96144 (T) were used as controls. Yeast isolates were identified to species level by sequencing the ITS (Internal Transcribed Spacers) region of the rDNA. *Candida parapsilosis* cultures were grown in liquid Sabouraud (SAB) overnight at 30 °C with shaking (150 rpm).

Growth curves

Growth curves in SAB were determined for all the strains of *C. parapsilosis* complex. Suspensions of *C. parapsilosis* at 10^5 yeasts/ml were prepared in SAB and 200 μl were inoculated in a 96-well plate and incubated for 72 h with moderate shaking. Optical density at 540 nm was measured every 30 min in an iEMS Spectrophotometer (Thermofisher) and plotted using Graph Pad Prism 5 software. Growth curves were performed in triplicate at 37 °C. Differences in the curves were calculated by non-parametric Kruskal–Wallis test using Graph Pad Prism 5 software.

Galleria mellonella survival experiments

Galleria mellonella caterpillars in the final instar larval stage were obtained by R.J. Mous Livebait. For experiments, larvae weighing between 0.3 and 0.5 g were selected. Prior to experiments larvae were incubated in the dark at 37 °C overnight.

Galleria mellonella larvae were cleaned with ethanol 70% and Hamilton syringes were disinfected with diluted bleach and ethanol 70%, and washed several times with PBS. All inoculum were prepared in PBS with ampicillin at final concentration of 2 μg/ml. Inoculum standardization was conducted with the three type strains and an inoculum of 2×10^8 yeasts/ml was selected as the most appropriate to monitor virulence. Larvae were inoculated with 10 μl of a 2×10^8 yeasts/ml (2×10^6 yeasts per larva). Untouched larvae and larvae inoculated with PBS-ampicillin were used as uninfected controls. *Galleria mellonella* larvae were incubated at 37 °C and experiments were performed at least three times independently for all strains. Larval death was determined by visual inspection daily. Killing curves were analyzed by Kaplan–Meier method using Graph Pad Prism 5 software. Differences in the median survival among species were evaluated by Mann–Whitney U test.

Determination of hemocyte density in *G. mellonella* larvae

Five *G. mellonella* larvae were inoculated with 10^6 yeasts/larva of each of the 15 strains tested in the virulence experiments. Larvae inoculated with 10 μl of PBS served as controls. After inoculation, larvae were incubated in dark at 37 °C for 2 h. Then, hemolymph was obtained by insertion of a lancet into the hemocell and diluted 1:1 in PBS to prevent melanization. Density was assessed by counting the hemocytes using a hemocytometer. Statistical analysis was performed by one-way ANOVA to test differences among strains and *t* test was performed to see differences among species by using Graph Pad Prism 5 software.

Phagocytosis assay

Strains were grown on SAB overnight at 30 °C with shaking (150 rpm). Cells were stained with Calcofluor white at 10 mg/ml for 30 min at 37 °C and washed twice in PBS. Three caterpillars were inoculated with 10^6 yeasts/larvae for each of the strains and incubated for 2 h at 37 °C. After incubation, hemolymph was recovered and washed with PBS to remove the non-internalized yeasts. The hemocytes were placed on coverslips and then allowed to adhere for 30 min. Samples were mounted in Fluoromount G and images were taken to quantify the phagocytosis with a Leica DMI 3000B fluorescent microscope. A minimum of 100 hemocytes per group were scored. Experiments were performed in triplicate on different days. Statistical differences among strains and species were calculated using one-way ANOVA and *t* test respectively using Graph Pad Prism 5 software.

Statistical correlations between median survival, percentage of phagocytosis and hemocyte density data were assessed by determining nonparametric Spearman correlations and linear regression analysis using Graph Pad Prism 5 software.

Filamentation assay

Cell suspensions were prepared at 10^8 yeasts/ml in PBS. Two hundred microliters were inoculated into 10 ml of YPD and incubated at 37 °C up to 48 h. Cultures were observed under a Leica 300B microscope at both 24 and 48 h.

Production of extracellular hydrolytic enzymes

The production of secreted aspartyl proteinases (sap), esterases, hemolysins, and urease were determined at 37 °C as previously described.[51,52]

Plate assays containing each specific test medium were used for each of the determinations. An aliquot of 5 μl of yeast suspension (10^7 cells/ml) or 10 μl for hemolysis assay was spotted on each medium. The plates were incubated for 10 d at 37 °C.

For aspartil proteinase activity determination, the media was prepared with 1% bactoagar (Difco, DF214030) 0.1% KH_2PO_4, 0.5% $MgSO_4$, 1% glucose (pH 5.0), and 0.16% of bovine serum albumin. Aspartil proteinase production was detected using 1.25% w/v amidoblack (90% metanol and 10% acetic acid).

Esterase activity was determined using Tween 80 opacity test medium containing 10 g Bacto peptone (Difco, DF211677), 5 g $NaCl_2$, 0.1 g $CaCl_2$, 15 g bacto agar (Difco, DF214030) in 1 l distilled water; pH was adjusted at 6.8 and then the media was autoclaved. When the medium was cooled, 5 ml of Tween 80 (Sigma, P8074) were added. The production of lipolytic enzymes was detected as a precipitation halo around the colonies.

Hemolysin and urease activities were determined by using blood sheep agar (Difco, DF240920) and Christensen medium (BD, 221097) respectively according to manufacturer's instruction.

Histological examination

Histopathology studies were assessed to determine the course of the infection in *G. mellonella*. Tissue sections were obtained on the first, second, and third day after infection with *C. parapsilosis s.l.* type strains. Larvae inoculated with PBS were included as a control. Samples were processed according to a protocol previously described.[27] Periodic acid Schiff (PAS) staining was performed and sections were microscopically examined. Histological sections were observed under a Leica 3000B microscope with 10× and 63× objectives. Pictures were taken with a Leica DFC 300FX camera using Leica Microsystems Software.

Disclosure of Potential Conflict Of Interest

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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