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Candida metapsilosis as the least virulent member of the '*C. parapsilosis*' complex

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Results of recent molecular studies have provided evidence of three distinct species within the *Candida parapsilosis* complex, namely *Candida parapsilosis*, *Candida orthopsilosis* and *Candida metapsilosis*. While there are initial data pertaining to the virulence of these *Candida* species with respect to reconstituted epidermal and oral epithelial tissues, there have been no studies, as of yet, on their interaction with immune cells. Employing an *in vitro* infection model using microglial cells, we investigated the pathogenetic potential of different isolates of each of these three species. We show that *C. metapsilosis* isolates are more susceptible to microglia-mediated antifungal activity, as compared with those of *C. parapsilosis* and *C. orthopsilosis*. Interestingly, *C. metapsilosis* isolates are also phagocytosed to a lower extent, but the yeast-containing phagosomes exhibit the highest degree of acidification in comparison with the phagosomes containing *C. parapsilosis* or *C. orthopsilosis*. Furthermore, when assessing microglia secretory response to infection, comparable high levels of MIP-1α and little or no TNF-α production are observed with all of these *Candida* species. Finally, unlike *C. metapsilosis* infected cells, microglial cells infected with *C. parapsilosis* and *C. orthopsilosis* release high and time-dependent levels of lactate dehydrogenase (LDH). Overall, these findings point to *C. metapsilosis* as the least virulent member of the '*C. parapsilosis*' complex.

Keywords *Candida parapsilosis, Candida orthopsilosis, Candida metapsilosis,* microglia, phagosomal maturation

Introduction

Candida parapsilosis is the second most common yeast isolated from bloodstream infections around the world [1–4]. It is particularly associated with serious nosocomial infections and has long been considered a complex of three genetically distinct groups, namely I, II and III [5]. It was only in 2005 [6], that multigenic sequence analysis and internal transcribed spacer sequencing lead to the proposal to split the *C. parapsilosis* complex into three separate species, i.e., *C. parapsilosis, C. orthopsilosis* (formerly, *C. parapsilosis* group II), and *C. metapsilosis* (formerly, *C. parapsilosis* group III). Despite the clinical relevance of isolates of the *C. parapsilosis* complex as agents of opportunistic mycoses, little is known as to the virulence traits of each member of the complex and especially of the two rarely isolated species, *C. orthopsilosis* and *C. metapsilosis*. Recently, Gacser *et al*. [7] provided initial *in vitro* evidence on the differential ability of the species of the '*C. parapsilosis*' complex to invade reconstituted human epidermal and oral epithelial tissues. In particular, microscopic studies revealed that *C. parapsilosis* and *C. orthopsilosis* isolates caused major morphological changes, whereas *C. metapsilosis* exerted little or no effects on the human cells. Accordingly, cell cultures inoculated with *C. parapsilosis* and *C. orthopsilosis,* but not those exposed to *C. metapsilosis*, released lactate dehydrogenase (LDH). In further studies investigating *C. parapsilosis* specific virulence factors [8], lipase production has been associated with the capacity of the three species to produce

Received 16 November 2009; Received in final revised form 8 April 2010; Accepted 25 April 2010

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DOI: 10.3109/13693786.2010.489233

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biofilm and survive within macrophages. Moreover, Melo *et al*. [9] provide data on the ability of *C. orthopsilosis* and *C. metapsilosis* to produce biofilm.

It is well-established that the processes driving onset and outcome of microbial infections are regarded as a balance between the virulence of the microorganism and the ability of the host to prevent microbial colonization/invasion [10–12]. On the one hand, macrophages play an important indirect defence role by polarizing the specific immune response via antigen presentation to lymphocytes and directly by phagocytosis and killing of the ingested microorganisms [13]. On the other hand, there is increasing evidence of the ability of certain pathogens to develop strategies for intracellular survival, despite the potent antimicrobial environment generated by macrophages [14–18]. These mechanisms include pathogen escape from the phagosome or inhibition of phagosomal acidification and maturation [19]. In a recent study, we described the importance of the AFR1 encoding-gene on the virulence of another important fungal opportunistic pathogen, *C. neoformans* [18]. Known to code for fluconazole-resistance [20,21], AFR1 is also associated with the ability of the fungus to elude macrophage-mediated defences [18]. Unlike the knock-out counterpart, the AFR1 overexpressing yeast is capable of surviving inside brain macrophages. This is the result of significant delays in phagolysosome biogenesis and maturation which occurs when the macrophages are infected with the AFR1 +/+ yeast cells as compared with the AFR1 -/- infected counterpart [18].

The availability of immortalized cell lines which retain phenotypic and functional properties of the original tissue, facilitates the establishment of *in vitro* and *in vivo* infection models suitable for studies of the biomolecular mechanisms involved in host-pathogen interaction. As an example, the previously established *in vitro* microglial cell line BV2 [22] allowed the demonstration that *in vivo* microglial cells play a crucial role in preventing the outcome of experimental meningoencephalitis by *Candida albicans* [23]. Accordingly, when assessed *in vitro*, such cells exert phagocytic and anticandidal activity [24–26] and, once infected, they express a restricted pattern of secretory response [22,27,28]. This behaviour is likely interpretable as a tissue-specific reaction that tends to couple an efficacious antimicrobial host response with a concomitant maintenance of cerebral homeostasis.

In the present study, we conducted *in vitro* investigations of how and to what extent *C. parapsilosis, C. orthopsilosis* and *C. metapsilosis* isolates interact with BV2 microglial cells. In particular, eight different *Candida* isolates, belonging to the three species have been assessed for resistance/susceptibility to phagocytosis and anticandidal activity by microglial cells. Yeast-containing phagosome maturation, cytokine response and LDH release have also been evaluated. We show that, when compared to *C. parapsilosis* and *C. orthopsilosis, C. metapsilosis* is less susceptible to phagocytosis and more prone to intracellular acidification of the yeast-containing phagolysosomes. In addition, *C. metapsilosis* causes little or no damage to microglial cells, as opposed to what is observed with cells infected by *C. parapsilosis* and *C. orthopsilosis*. Cytokine response is comparable among the three species. The implications of such findings are discussed.

Materials and methods

Microglial cell line

The previously established murine microglial cell line BV2 [22] was maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% heat inactivated fetal bovine serum (Defined Hyclone, Logan, Utah, USA), 50 mg/ml gentamicin (EuroClone, Milan, Italy) and 2 mM L-glutamine (EuroClone, Milan, Italy), hereafter referred to as complete RPMI. Cells were detached by vigorous shaking and fresh cultures were started biweekly. Experimental cultures were set to a concentration of 5×10^5/ml in fresh medium the day before the experiment.

Candida *strains*

A selected panel of eight clinical isolates of members of the '*C. parapsilosis*' complex were kindly provided by Prof. Senesi. By multilocus sequence typing [6], the isolates had been identified as belonging to *C. parapsilosis* (CP37 and CP71 strain), *C. orthopsilosis* (CP47, CP331 and CP344 strain) and *C. metapsilosis* (CP86, CP87 and CP187 strain). Long-term storage was carried out in 20% glycerol at −80°C. Before testing, single colonies, obtained from Sabouraud dextrose agar (Oxoid, Hampshire, UK) cultures from each strain, were transferred to Sabouraud dextrose broth under shaking. When required, staining of yeast cells was achieved by incubating them with Oregon green 488 (Molecular Probes, Eugene, OR, USA) [18,29,30,34], 2 mg/ml in the dark at 37°C for 1 h. After labelling, yeast cells were washed twice with sterile phosphate-buffered saline (PBS, EuroClone, Whethereby, UK), counted and suspended in complete medium at the desired concentration.

Measurement of anticandidal activity

The assay was performed as detailed elsewhere [28]. Briefly, yeast cells (10^5/ml in complete RPMI, 100 µl per well) were plated in 96-well plates and BV2 cells were then added (10^6/ml in complete medium, 100 µl per well). After an additional 2 and 4 h of incubation, the plates were vigorously shaken and Triton X-100 (0.1% final

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concentration, Sigma St. Louis, MO, USA) was added to each well. Serial dilutions from each well were prepared in saline and plated (triplicate samples) on Sabouraud dextrose agar. The number of CFU was determined after 24–48 h of incubation. Control cultures consisted of yeast cells incubated in complete RPMI without effector cells. The results were expressed as percent of anticandidal activity according to the formula described elsewhere [28].

Phagocytosis assay

The assay was performed as detailed elsewhere [18,34]. In particular, Lab-Tek II chamber slides (Nalge Nunc International, Naperville, IL, USA) were treated with poly-L-lisine (Sigma, St. Louis, MO, USA), 10 μg per well, for 30 min. After two washes with PBS, BV2 cells (10^6/ml in complete medium, 100 μl per well) were transferred to chamber slides and inoculated with Oregon green 488 pre-labelled yeast cells (5×10^6/ml in complete medium, 100 μl per well). After 1 or 2 h incubation, Uvitex 2B (Polysciences Inc., Warrington, PA, USA) was added (20 μl/well) 15 min prior to each end point [18,29,30,34]. Then, cells were washed with PBS to remove the non-adherent yeast cells and fixed with 4% formaldehyde (freshly made from paraformaldehyde, obtained from Sigma, St Louis, MO, USA), for 30 min at 4°C, and immediately analyzed by epifluorescence microscopy. A minimum of 200 microglial cells per group were scored and any cell containing one or more particles was counted as phagocytic. The phagocytosis index was calculated as the total number of phagocytosed yeasts/total number of phagocytic cells.

Phagolysosome acidification assay

The assay was performed as detailed elsewhere [18,34]. Briefly, BV2 cells were dispensed in Lab-Tek II chamber slides as in the 'Phagocytosis assay'. The acidotropic dye LysoTracker DND-99 (75 nM, obtained from Molecular Probes, Eugene, OR, USA) [18,33,34] was added and the BV2 cells were infected for 2 h, as described above. Samples were then fixed with 4% formaldehyde and immediately examined by fluorescence microscopy. Control groups consisted of uninfected cells. A minimum of 200 yeast-containing phagosomes were scored; the percent of acidic phagolysosomes was evaluated as the ratio between the number of Lyso-Tracker-labelled phagosomes and the total number of yeast-containing phagosomes.

Cytokine measurement

The assay was performed as detailed elsewhere [26]. Briefly, BV2 cells (10^6/ml) were inoculated with yeast cells (10^7/ml) or exposed to LPS (1 μg/ml, Sigma, St Louis, MO, USA) for 6 h in 24-well plates. Then, the supernatants were collected and frozen at −80°C. MIP-1α and TNF-α levels were measured by sandwich ELISA according to the manufacturer's protocol (R&D Systems, MN, USA). The reaction was read as OD using a Microplate Reader (Sunrise). Experiments were repeated three to five times and each sample was run in triplicate. Results were expressed as pg/ml.

Lactate dehydrogenase (LDH) measurement

BV2 cells (10^6/ml) were infected with yeast cells (10^7/ml) for 24, 48 and 72 h in 24-well plates. In parallel groups BV2 cells were either treated with LPS (1 μg/ml) or incubated in complete RPMI and then exposed to Tryton X-100 (0.01%), at the end of the incubation time as positive controls. Yeast cells alone, incubated under identical conditions, were included as negative control. At the different time-points, the supernatants were harvested and the levels of LDH activity was immediately measured and expressed as U/L.

Epifluorescence microscopy

Images were generated and captured with a Nikon Eclipse 90i system, equipped with Nomarski differential interference contrast (DIC) optics. The overlapping signals of Oregon green-labelled yeasts (green fluorescence) with either the Uvitex 2B (blue fluorescence) or the red fluorescence of the LysoTracker were interpreted as colocalization. At each time point, samples were photographed with a DS-5Mc Nikon digital camera and the photographs were then analysed by the NIKON software program.

Statistical analysis

Statistical analysis was performed by one-way Anova with a Bonferroni correction post-test. The results in the figures and in the table are the mean ± standard deviation (SD) of three to five independent experiments, if not specified otherwise.

Results

Comparison among C. parapsilosis, C. orthopsilosis *and* C. metapsilosis *isolates in terms of resistance/susceptibility to microglia-mediated antifungal activity*

In the present study, we compared different isolates of *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*, for their resistance/susceptibility to microglia-mediated

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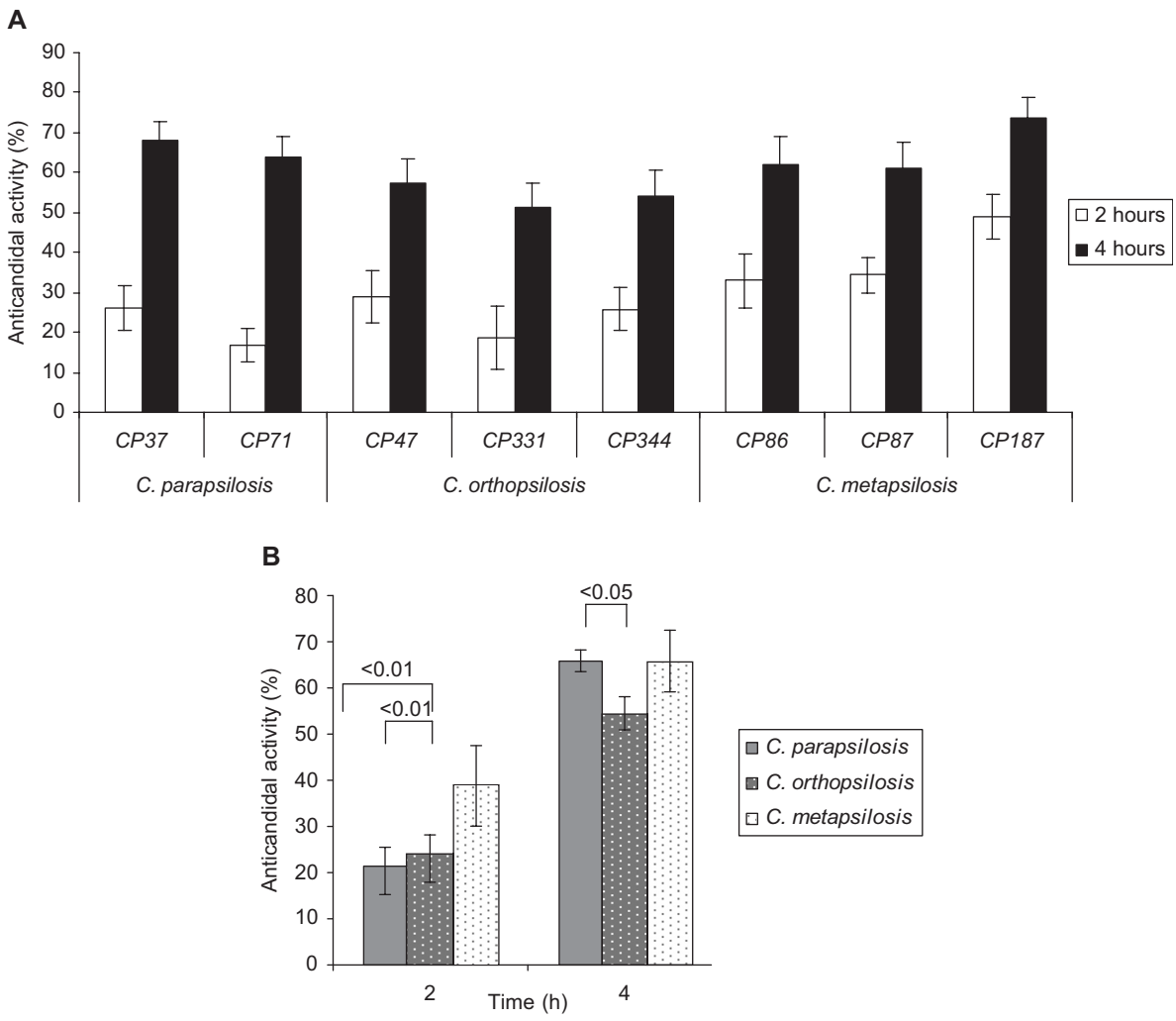

Fig. 1 Susceptibility of different *Candida parapsilosis*, *Candida orthopsilosis* and *Candida metapsilosis* isolates to antifungal activity by microglia. Yeast cells were exposed to BV2 cells at E:T = 10:1. After 2 and 4 h, the percent of anticandidal activity was determined as detailed in Materials and methods. Panel A shows the anticandidal activity against each isolate (mean value +/– SD of 3 independent experiments). Panel B shows the mean +/– SD of the values depicted in Panel A.

defences. In particular, BV2 cells were infected with the eight different *Candida* isolates, for different times, at an E:T ratio of 10:1 after which the anticandidal activity of the cells was measured, as detailed in Materials and methods. Significant levels of antifungal activity were observed against all the *Candida* isolates within 2 h and it appeared that this activity was time-dependent, since a further increase in anticandidal action was noted after 4 h in all cases (Fig. 1A). When comparing the mean anticandidal activity observed among isolates belonging to each species, *C. metapsilosis* appeared significantly more susceptible, especially in the 2 h assay, than *C. parapsilosis* and *C. orthopsilosis* to BV2 cell activity, (Fig. 1B).

Comparison among C. parapsilosis, C. orthopsilosis *and* C. metapsilosis *isolates in terms of resistance/susceptibility to phagocytosis by microglia*

With the purpose of detailing the events involved in *Candida* microglia interaction, fluorescence studies were performed, as detailed elsewhere [18,34]. Using this methodology, we performed a time-dependent phagocytosis assay comparing all the isolates. As shown in Fig. 2A, the percent of phagocytic cells varied to some extent and no time-dependency was observed with the exception of *C. metapsilosis*. The trend was better defined when comparing the mean phagocytosis observed among isolates belonging to each species. As shown in Fig. 2B, *C. metapsilosis* was significantly less phagocytosed than *C. orthopsilosis* or

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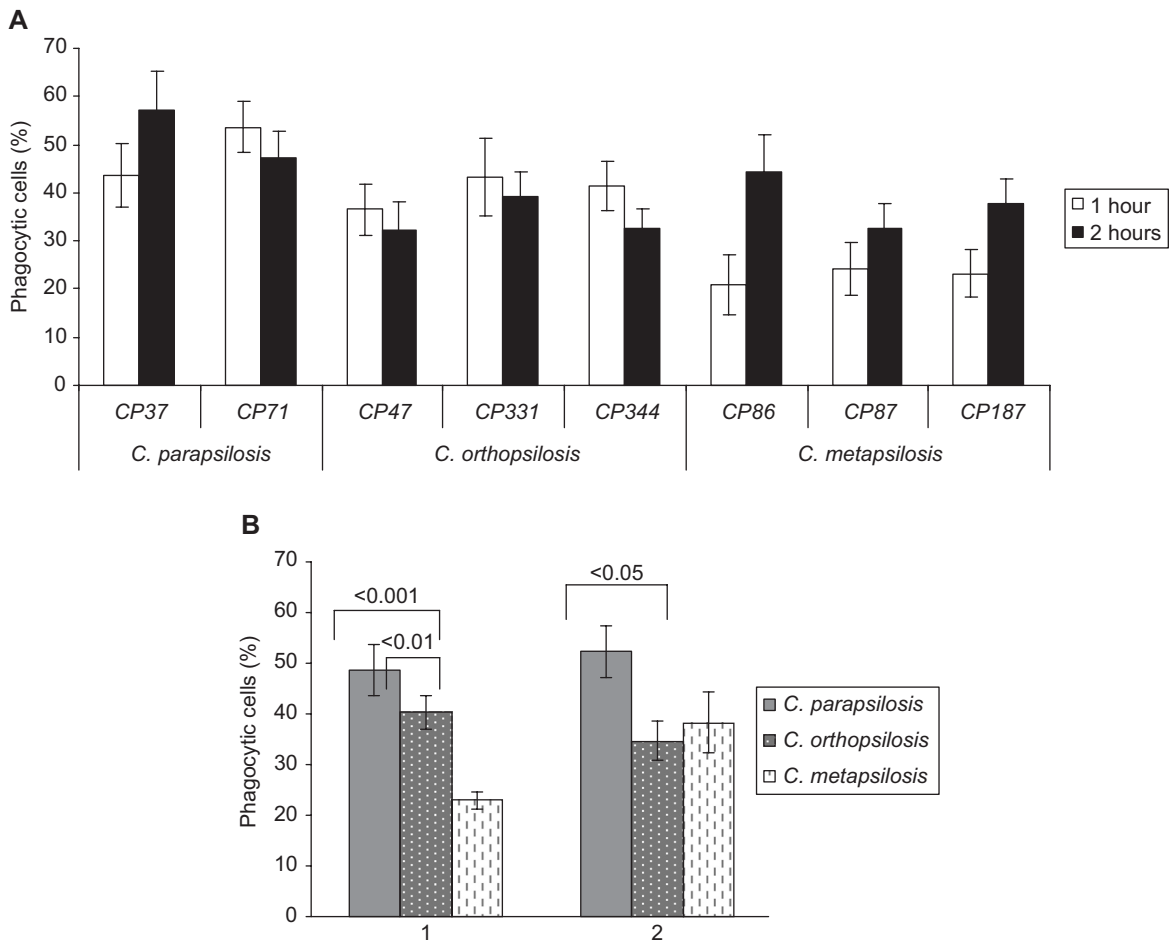

Fig. 2 Susceptibility of different *Candida parapsilosis*, *Candida orthopsilosis* and *Candida metapsilosis* isolates to phagocytosis by microglia. Oregon green 488 pre-labelled yeast cells were exposed to BV2 cells (E:T = 1:5) for 1 and 2 h. At each end point, Uvitex 2B was added for 15 min; the cultures were then washed, fixed and analysed by epifluorescence microscopy (panel A). Panel B shows the mean +/– SD of the values depicted in Panel A phagocytosis values.

C. parapsilosis. Furthermore, the difference was highly significant after 1 h of infection ($p < 0.001$: *C. parapsilosis* vs *C. metapsilosis*; $p < 0.01$: *C. orthopsilosis* vs *C. metapsilosis*). When the phagocytosis efficiency was determined by evaluating the phagocytosis index, we observed values ranging from 5–7 and no statistically significant differences occurred between groups (data not shown).

Comparison among C. parapsilosis, C. orthopsilosis *and* C. metapsilosis *isolates in terms of phagolysosomal maturation within infected microglia*

To investigate the events following the yeast internalization by microglia, the fate of the yeast-containing phagosomes was evaluated in terms of acidification. In particular, the acidification of the vacuoles were assessed 2 h post infection, by adding the LysoTracker red DND-99 dye in the phagocytosis assay, as detailed elsewhere [18,34]. Figure 3A shows fields representative of the following conditions; (1) BV2 cells and the yeast cells were observed by DIC (a, f and k), (2) the extracellularly localized yeast cells were evidenced by Uvitex 2B (b, g and l), (3) the total yeast cells were detected by Oregon green staining (c, h and m), (4) the LysoTracker staining identified acidic intracellular compartments (d, i and n), (5) by merge, the intracellular yeasts within acidic vacuoles were depicted. As shown in Fig. 3, LysoTacker staining was not evident in the *C. parapsilosis*-infected cells (d) and one or numerous acidic vacuoles were observed when assaying phagosomes (n) containing *C. orthopsilosis* (i) or *C. metapsilosis*. These differences were confirmed by the merge images (e, j and o). Fig. 3B shows the percent of acidic phagolysosomes in each experimental group. The extent of the phenomenon was related to the isolate employed, with the three isolates of *C. metapsilosis* showing consistently the highest percent of acidic phagolysosomes. As detailed in Fig. 3C, when

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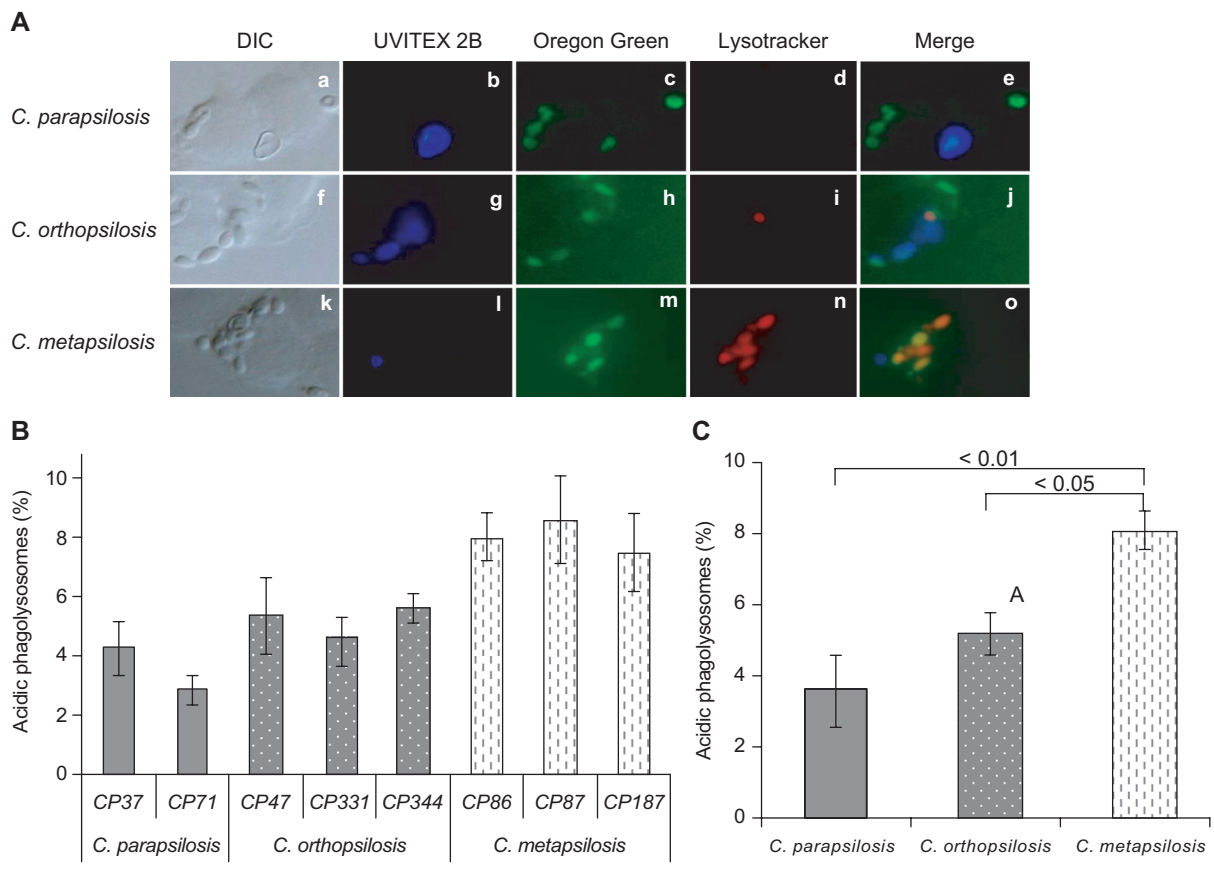

Fig. 3 Acidification of phagolysosomes containing yeast cells. Oregon green 488 pre-labelled yeast cells were exposed to BV2 cells (E:T = 1:5), for 2 h; then LysoTraker dye was added. After counterstaining with Uvitex 2B, samples were fixed and then visualized by epifluorescence microscopy (Panel A). (a), (f) and (k) DIC images, (b), (g) and (l) the Uvitex 2B image showing the blue-stained extracellularly localized yeast cells, (c), (h) and (m) the Oregon green image showing total yeast cells, (d), (i) and (n) LysoTracker staining, (e), (j) and (o) the merge image showing the LysoTracker-positive compartment. Panel B shows the results +/– SD, expressed as percent of acid phagolysosomes. Panel C shows the mean values +/– SD of the values depicted in Panel B.

considering the mean values within each species, cells infected with *C. metapsilosis* showed values significantly higher than the other two groups.

Microglia secretory response to infection by C. parapsilosis, C. orthopsilosis *and* C. metapsilosis

In order to investigate the impact of different *Candida* species on microglial cell response to infection, BV2 cells were exposed or not to the eight yeast isolates and then assessed for cytokine response. In particular, BV2 cells were infected for 6 h with *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* yeast cells (E:T = 1:10) or exposed to LPS (1 μg/ml), after which the production of MIP-1α and TNF-α was measured. As depicted in Fig. 4A, LPS greatly enhanced MIP-1α production, with high levels of MIP-1α also found in BV2 infected cells in comparison to uninfected controls. However, no statistically significant differences were detected when comparing BV2 cells exposed to each of the three species. Furthermore, little or no response was observed in terms of TNF-α production (data not shown).

LDH release by C. parapsilosis, C. orthopsilosis *and* C. metapsilosis-*infected microglial cells*

To assess the impact of infection with the three studied *Candida* species on the status of BV2 cells, we compared the release of LDH by microglial cells exposed to *C. parapsilosis*, *C. orthopsilosis* or *C. metapsilosis* yeast cells for 24, 48 and 72 h, at E:T = 1:1 (Table 1). In parallel groups, BV2 cells were incubated in medium alone, in the presence of LPS or the cells were simply treated with Tryton X-100. As predicted, the highest LDH activity was found in the Tryton X-100 lysed cells. Appreciable and time-dependent LDH activity was observed in BV2 cells

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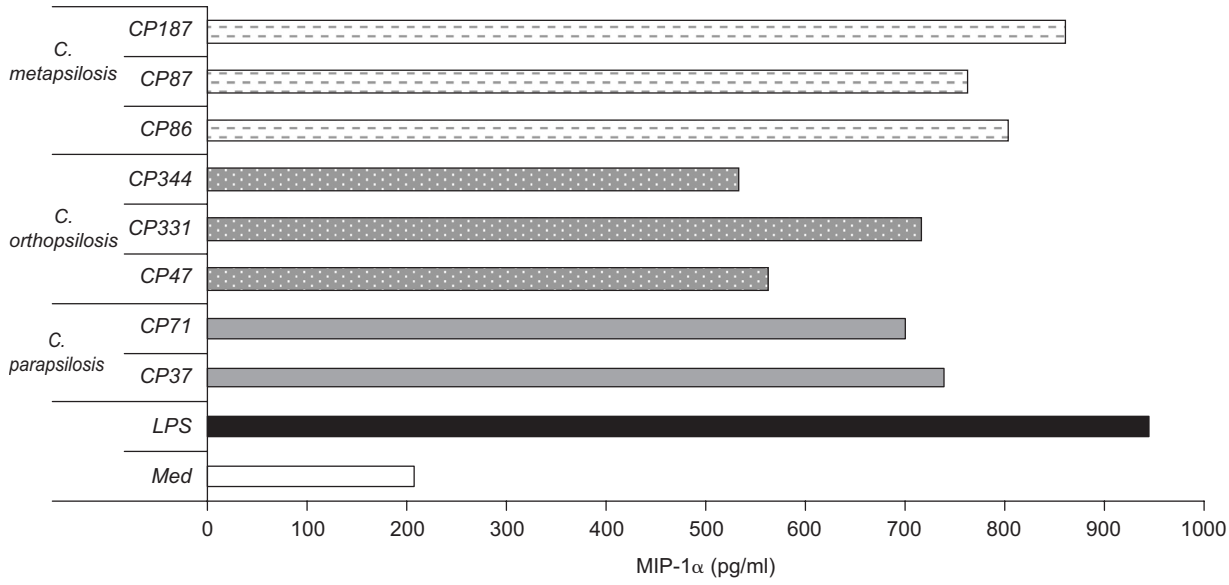

Fig. 4 Levels of MIP-1α in BV2 cells infected with the different isolates of *Candida parapsilosis*, *Candida orthopsilosis* and *Candida metapsilosis*. BV2 cells were infected with *Candida* at E:T = 10:1 for 6 h. In parallel, lipopolysaccharide treatment (LPS, 1 μg/ml) was included as positive control. Then, the supernatants were harvested and MIP-1α levels were evaluated, as detailed in Materials and methods. Values are expressed as pg/ml.

exposed to LPS, even though some toxic effects occurred at the time, as confirmed by the fact that the LDH levels in LPS-treated cells was below those of the controls at the latest time 72 h (470 vs 553). When microglial cells were infected with the three studied *Candida* species, we found no variations in LDH activity with respect to the control baseline at 24 h. Later, a time-dependent increase in LDH was detected in all the infected cells; in particular, *C. orthopsilosis* and *C. parapsilosis* induced the highest and intermediate levels of LDH, respectively, while *C. metapsilosis* infected cells released the lowest levels of LDH, at all the time-points considered.

Discussion

While three distinct serotypes have long been recognized within *C. parapsilosis*, it was only recently on the basis of molecular studies that a proposal was made to divide it into three distinct species [6]. In the present work, we compared *C. parapsilosis, C. orthopsilosis* and *C. metapsilosis* with respect to their pathogenic potentials by means of an *in vitro* infection model employing microglial cells.

As recently summarized in a review [35], the initial interaction between host immune cells and *C. albicans* leads to a set of standard, and possibly redundant, pathways that stimulate phagocytosis and the subsequent killing of the fungal cells. In this study, we showed that, upon interaction with brain macrophages, the growth of *C. parapsilosis, C. orthopsilosis* and *C. metapsilosis* were inhibited in a time-dependent manner. Interestingly, *C. metapsilosis* isolates were more inhibited than *C. parapsilosis* or *C. orthopsilosis*, especially in the 2 h assay. Unexpectedly, epifluorescence microscopy analysis revealed that isolates belonging to *C. metapsilosis* are phagocytosed to a lower extent than the other two species. Furthermore, when evaluating the phagosome maturation within microglia cells infected with the three species, the vacuoles containing *C. metapsilosis* cells undergo the highest degree of acidification. Taken together, the present findings indicate that *C. metapsilosis* is less susceptible to phagocytosis but, once internalized, it is also the least effective in counteracting host intracellular antimicrobial defences. From the pathogen point of view, the fact that only about half of the phagocytic cells are infected with *C. metapsilosis* may be interpreted as a better ability of those species to resist macrophage-mediated ingestion. Moreover, the results concerning phagolysosome acidification imply that, once internalized, *C. metapsilosis* has a reduced chance of surviving within the phagocyte. Since intracellular localization and subsequent persistence have been established as virulence traits for fungi [14,15], we may conclude that *C. metapsilosis* is defective in both respects. Thus, it may be considered as the least virulent species among the '*C. parapsilosis*' complex. In line with this conclusion, recent population-based epidemiological studies [7,36–38] showed that *C. metapsilosis* is indeed rarely isolated from patients. In particular, Lockhart *et al*. [37] demonstrated that

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Table 1 Levels of LDH activity in BV2 cells infected with different isolates belonging to *Candida parapsilosis*, *Candida orthopsilosis* and *Candida metapsilosis* species.

Strains	Treatment	LDH (U/L)		
		24 h	48 h	72 h
None	Medium	32	183	553
None	Tryton X-100	1585	1655	1484
None	LPS	80	348	470
C. parapsilosis	CP37	36	774	1163
	CP71	37	607	1160
	Mean value ± SD	36.5 ± 0.7	690.5 ± 118.1	1161.5 ± 2.1
C. orthopsilosis	CP344	41	1130	1323
	CP331	38	911	1325
	CP47	35	1097	1270
	Mean value ± SD	38.0 ± 3.0	1046.0 ± 118.1	1306.0 ± 31.2
C. metapsilosis	CP187	38	259	601
	CP86	37	255	686
	CP87	35	381	702
	Mean value ± SD	36.7 ± 1.5	298.3 ± 71.6*	663.0 ± 54.3*

*$P < 0.01$ (*C. parapsilosis* vs *C. orthopsilosis*) or (*C. parapsilosis* vs *C. metapsilosis*).

C. metapsilosis is the least represented species of the '*C. parapsilosis*' complex (1.8%) in the worldwide collection of clinical isolates.

It is known that *C. albicans* infection induces the production of important cytokines, which play a crucial regulatory role in initiating host immune response [39–41]. Similarly, BV2 cells infected with *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* produce high levels of MIP-1, but no significant differences are observed with respect to these three *Candida* species. In contrast, TNF-α production is totally unaffected by infection. Thus, these data ascribe to the members of the '*C. parapsilosis*' complex an immunomodulating property, consisting of selective induction of MIP-1α but not TNF-α in microglia, irrespective of the fungal species. A similar trend of secretory response to infection has been previously described in a study employing *C. albicans* as the microbial insult [26]. We may interpret such a subtle control of secretory response by microglia as an effort to minimize any potential toxic effects, by a cytokine such as TNF-α, within brain.

Finally, significant differences in term of microglial cell injury after infection with isolates of the complex have been revealed by evaluating the release of LDH. In particular, little LDH release was observed in BV2 cells infected with *C. metapsilosis* strains with respect to uninfected controls. In contrast, infections by *C. parapsilosis* and *C. orthopsilosis* induced high levels of LDH release. This phenomenon further increased by time, implying gradual deleterious effects due to both infectious agents. This finding is in line with a recent study of Gacser *et al*. [7] that showed that upon infection of reconstituted human tissue with clinical and laboratory isolates belonging to the '*C. parapsilosis*' complex, *C. metapsilosis* induces minimal damage when compared with *C. parapsilosis* and *C. orthopsilosis* isolates [7].

Overall, the present report describes differences among *C. parapsilosis, C. orthopsilosis* and *C. metapsilosis* in terms of fungus-host cell interaction. Although the numbers employed in this study was low, it does point to the importance of achieving species identification among the '*C. parapsilosis*' complex in clinical microbiology laboratories to better tailor patient treatment. In addition, this would provide precious epidemiological data and contribute to the elucidation of the etiological role of the three distinct species, *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*, in clinical settings.

Acknowledgements

We wish to acknowledge Prof. A. Todaro (Università di Modena e Reggio Emilia, Modena) for sharing his experience and providing access to the epifluorescence Eclipse 90i Nikon microscope. Special thanks are also forwarded to Prof. S. Senesi (Dipartimento di Biologia, Pisa) who provided us with the eight isolates, already identified by multilocus sequence typing procedures as belonging to '*C. parapsilosis*' complex. Finally, we acknowledge the Laboratorio Analisi Chimico-Cliniche Azienda Ospedaliero-Universitaria di Modena for measuring the lactate dehydrogenase activity.

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This study was partially supported by the Ministero Italiano Università e Ricerca - Cofin 2007 (n. 2007BXRBFR-004 to EB).

Declaration of interest: None.

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This paper was first published online on Early Online on xx xx xxxx.

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