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ORIGINAL ARTICLE

Identification of cell-associated and secreted serine-type peptidases in multidrug-resistant emergent pathogens belonging to the *Candida haemulonii* complex

Xênia M. Souto[1] · Lívia S. Ramos[1] · Marta H. Branquinha[1] · André L. S. Santos[1,2]

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Abstract

The *Candida haemulonii* complex (*Candida haemulonii*, *Candida haemulonii* var. *vulnera*, and *Candida duobushaemulonii*) comprises emerging opportunistic human fungal pathogens with recognized multidrug-resistance profiles. Little is known about the virulence markers produced by this fungal complex. However, it is recognized that *Candida* spp. express a large array of peptidases, which play multiple roles in different aspects of fungal-host interactions. In the present study, we have identified proteolytic enzymes in clinical isolates of the *C. haemulonii* complex using zymographic assays. Peptidases able to hydrolyze gelatin, casein, albumin, hemoglobin, and immunoglobulin G were detected in cell-free supernatants and cellular extracts taken from the three species forming the *C. haemulonii* complex. Overall, peptidases were preferentially evidenced at physiological pH and temperatures of 37–42 °C, with molar masses between 35 and 85 kDa. Peptidase profiles of *C. haemulonii* and *C. haemulonii* var. *vulnera* isolates were quite similar, contrasting to the peptidases produced by *C. duobushaemulonii*. Almost all peptidases were inhibited by phenylmethanesulfonyl fluoride (PMSF), thus classifying them as serine-type peptidases. Additionally, proteolytic cleavage of soluble azoalbumin was blocked by PMSF (65–95% inhibition depending on the fungal isolate). These unprecedented results have demonstrated the capability of the *C. haemulonii* complex to produce serine-type peptidases with an ability to cleave a broad spectrum of proteins, including key host components.

Introduction

Candidiasis represents the most important opportunistic mycosis in hospital settings and is one of the main causes of human infectious diseases worldwide. Although *Candida albicans* remains the most notorious species responsible for candidiasis, non-*albicans Candida* species, with reduced susceptibility to antifungal agents commonly used in the medical practice, have represented, in recent years, a substantial part of the clinical isolates collected in hospitals around the globe (Chen et al. 2009; Nucci et al. 2010; Pfaller et al. 2010; Papon et al. 2013; Sardi et al. 2013; Caggiano et al. 2015; Sehnaz et al. 2015).

Candida haemulonii was originally described in 1962 as *Torulopsis haemulonii* and was isolated from the gut of a fish (*Haemulon sciurus*) (van and Kolipinski 1962). Lavarde et al. (1984) reported the first clinical isolate of *C. haemulonii* which was obtained from the blood of a patient with renal failure. Since then, various kinds of infections associated with this yeast have been described, mainly in immunocompromised patients (Gargeya et al. 1991; Rodero et al. 2002; Khan et al. 2007; Ruan et al. 2010; Crouzet et al. 2011; Cendejas-Bueno et al. 2012; Ramos et al. 2015). Due to the similarities in the physiological characteristics, isoenzyme profiles, and DNA reassociation experiments, Lehmann et al. (1993) described, for the first time, the *C. haemulonii* complex. In that study, 25 isolates from different geographic origins and clinical sources were investigated, being described as two genetically distinct groups: *C. haemulonii* group I and *C. haemulonii* group II. Subsequently, Cendejas-Bueno et al. (2012) suggested the following new classification of this fungal complex, taking into

✉ André L. S. Santos
andre@micro.ufrj.br

Marta H. Branquinha
mbranquinha@micro.ufrj.br

[1] Laboratório de Estudos Avançados de Microrganismos Emergentes e Resistentes (LEAMER), Departamento de Microbiologia Geral, Instituto de Microbiologia Paulo de Góes (IMPG), Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil

[2] Programa de Pós-Graduação em Bioquímica, Instituto de Química (IQ), Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil

consideration both molecular and phenotypic approaches: *C. haemulonii* (formerly known as *C. haemulonii* group I), *C. duobushaemulonii* (formerly known as *C. haemulonii* group II), and *C. haemulonii* var. *vulnera* (a variety of *Candida haemulonii*). Undoubtedly, among the most striking features of the *C. haemulonii* species complex are its resistance to amphotericin B, its resistance/low susceptibility to azoles (e.g., fluconazole, itraconazole, and voriconazole), and its limited susceptibility to echinocandins (e.g., caspofungin). Indeed, it is this multidrug-resistance profile that has hindered the treatment of patients with deep infections and has increased the frequency of clinical failures followed by death (Rodero et al. 2002; Khan et al. 2007; Kim et al. 2009; Crouzet et al. 2011; Cendejas-Bueno et al. 2012; Li et al. 2015; Ramos et al. 2015).

Despite the growing importance of *C. haemulonii* complex infections in medicine, its interactions with the infected host are still poorly understood. However, it is well-known that fungi have the ability to digest polymeric compounds found in their environment into smaller components, which are easily absorbed by cells and utilized as carbon and nitrogen sources (Mahon et al. 2009). This characteristic is reflected in the high levels of activity of the hydrolytic enzymes found in cell extracts and conditioned culture supernatants of many of these microorganisms (Mahon et al. 2009). Recently, our research group described the production of different classes of hydrolytic enzymes/activities by species forming the *C. haemulonii* complex, which included aspartic peptidase, caseinolytic and hemolytic activities, phytase, esterase, and phospholipase (Ramos et al. 2017a). For instance, in that work, aspartic peptidase activity was detected (using agar plate methodology) in all of the clinical isolates of the *C. haemulonii* complex. In general, *C. duobushaemulonii* isolates were classified as "excellent" producers (*Pz* values between 0.399 and 0.100), while *C. haemulonii* and *C. haemulon*ii var. *vulnera* isolates were categorized as "good" producers (*Pz* values 0.699–0.400) (Ramos et al. 2017a).

Microbial peptidases play physiological and pathological roles, for example, during nutrition, growth, differentiation, signaling, colonization, invasion, intracellular survival, evasion, dissemination, and immunomodulation of host defenses (Santos 2011). In fungi, several studies have indicated that the aspartic-, serine-, and metallo-type endopeptidases, as well as aminopeptidases, carboxypeptidases, and dipeptidylpep tidases, perform crucial biological roles and may also have important virulence attributes (Segal 2006; Yike 2011; Bochenska et al. 2013; Silva et al. 2014). From our present study, involving nine Brazilian clinical isolates, we herein report that secreted and cell-associated serine-type peptidases of the *C. haemulonii* species complex are able to cleave different key host proteins. This work is an important contribution to the understanding of the virulence factors linked to this fungal complex.

Materials and methods

Chemicals

Reagents used in electrophoresis, buffer components, peptidase inhibitors (*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), phenylmethanesulfonyl fluoride (PMSF), 1,10-phenanthroline, and pepstatin A), gelatin, casein, bovine serum albumin (BSA), human serum albumin (HSA), hemoglobin (Hgb), human immunoglobulin G (IgG), azoalbumin, 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT), and propidium iodide were obtained from Sigma-Aldrich (St Louis, USA). All other reagents were of analytical grade.

Microorganisms and growth conditions

Three Brazilian clinical isolates of each member forming the *C. haemulonii* complex were used in all of the experiments performed in the present study: *C. haemulonii* (LIP*Ch*2 from sole of the foot, LIP*Ch*7 from toe nail, and LIP*Ch*12 from blood), *C. duobushaemulonii* (LIP*Ch*6 from toe nail, LIP*Ch*8 from blood, and LIP*Ch*10 from bronchoalveolar lavage), and *C. haemulonii* var. *vulnera* (LIP*Ch*5 from toe nail, LIP*Ch*9 from urine, and LIP*Ch*11 from blood) (Ramos et al. 2015). The fungal isolates were previously identified by the automatized system VITEK® 2 and characterized by *ITS1–5.8S-ITS2* gene sequencing (Ramos et al. 2015). The yeasts (inoculum of 10^6 cells per mL) were cultured on Sabouraud broth at 37 °C for 48 h in an orbital incubator shaker (200 rpm) (Ramos et al. 2017b). All of the clinical isolates studied herein have reached their exponential phase at 48 h of in vitro growth on Sabouraud liquid medium as previously published by our research group (Ramos et al. 2017b).

Procurement of culture supernatants and cellular extracts

The fungal cultures (400 mL) were harvested by centrifugation (4000*g*, 10 min, 4 °C), and the supernatants were filtered through a 0.22-μm membrane (Millipore, São Paulo, Brazil). The cell-free culture supernatants were concentrated 100-fold in an Amicon ultrafiltration system (Amicon®, Beverly, USA) using a 10-kDa membrane (Santos and Soares 2005). The same volume of Sabouraud medium was also concentrated and used as a control to check for possible peptidase activity. In parallel, the number of fungal cells was counted using a Neubauer chamber, and 10^8 fungi were washed three times with phosphate buffered saline (PBS; 10 mmol/L NaH_2PO_4, 10 mmol/L Na_2HPO_4, 150 mmol/L NaCl; pH 7.2), suspended in 2.5% Triton X-100, and distributed in Eppendorf tubes containing glass beads (500 μm). Cellular lysis was performed in a cell homogenizer (FastPrep) by 5 cycles of 30 s,

alternating with an ice bath for 2 min. Then, the mixtures were centrifuged at 10,000*g* for 10 min at 4 °C, and the supernatants were collected. Protein concentration in both cellular and extracellular extracts was determined by the method described by Lowry et al. (1951) using BSA as the standard.

Viability tests

The ability of fungal cells to survive under growth conditions (37 °C for 48 h on Sabouraud medium) was assessed by measuring (i) mitochondrial dehydrogenase activity using a colorimetric assay that quantifies the metabolic reduction of XTT to a water-soluble, brown formazan product and (ii) propidium iodide uptake by damaged fungal cells due to a loss in cell membrane integrity. In these experiments, fungi were treated with either 0.4% paraformaldehyde or sodium azide (0.95 g/L) for 30 min in order to obtain non-viable cells to use as a negative control in the viability tests.

Zymographic assay

Peptidases were detected and characterized by electrophoresis on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1% gelatin, casein, BSA, HSA, Hgb, or IgG as proteinaceous substrates incorporated into the gels (Heussen and Dowdle 1980). The gels were loaded with 10–45 μL of cell-free supernatants (varying from 400 to 1400 μg of proteins) and cellular extracts (varying from 40 to 140 μg of proteins), following electrophoresis at a constant potential of 200 V at 4 °C for 2 h. Subsequently, the gels were incubated in 2.5% Triton X-100 for 1 h at room temperature under constant agitation in order to permit the enzymes' renaturation. The gels were incubated for 48 h under different conditions. The effect of pH on the peptidase activity was assessed by incubating the gels at 37 °C in 50 mmol/L sodium citrate (pH 5.0), 50 mmol/L sodium phosphate (pH 7.0), and 50 mmol/L glycine-NaOH (pH 9.0) buffers. The influence of temperature was assessed by incubating the gels at 28, 37, and 42 °C in 50 mmol/L sodium phosphate buffer (pH 7.0). The effect of peptidase inhibitors was also assessed by incubating the gels at 37 °C in 50 mmol/L sodium phosphate buffer (pH 7.0) in the absence (control) or in the presence of peptidase inhibitors able to block different peptidase classes: 10 μmol/L pepstatin A (aspartic peptidase inhibitor), 10 μmol/L E-64 (cysteine peptidase inhibitor), 10 mmol/L 1,10-phenanthroline (metallopeptidase inhibitor), and 10 mmol/L PMSF (serine peptidase inhibitor). The gels were then stained overnight with 0.2% Coomassie brilliant blue R-250 in methanol-acetic acid-water (50:10:40) and destained in methanol-acetic acid-water (5:10:85), to intensify the digestion halos. The peptidase activities in the gels were revealed by the presence of colorless zones indicative of protein digestion. The gels were dried, scanned, and digitally processed (Santos and Soares 2005). The molar mass of the peptidases was calculated by comparison with the mobility of Pierce™ Prestained Protein MW Marker (Thermo Fisher Scientific, São Paulo, Brazil).

Dendrogram analysis

Peptidase patterns observed in both cell-free culture supernatants and cellular extracts by zymographic assays with gelatin, casein, BSA, HSA, Hgb, and IgG were used to construct a dendrogram through the unweighted pair group method analysis (UPGMA). For these analyses, the PAST3 software package (Hammer et al. 2001) was applied. The results of peptidases' fingerprinting were collected into a matrix indicating the presence or absence (scored as 1 or 0, respectively) of specific peptidase bands in each zymographic analysis.

Quantitative peptidase activity assay

The peptidase activity was quantified using the method described by Plantner (1991). Briefly, the cell-free culture supernatants and cellular extracts (400 μg and 100 μg of protein, respectively) were incubated with azoalbumin (1.6 mg/mL) in 10 mmol/L sodium phosphate (pH 7.0), for 2 h at 37 °C, in the absence (control) or in the presence of 10 mmol/L PMSF. Following incubation, the reaction was terminated by adding 40 μL of 50% trichloroacetic acid, and samples were kept on ice for 1 h. The precipitate formed in each reaction was then removed by centrifugation at 500*g* for 15 min, and the fluid phase (200 μL) was added to 15 μL of sodium hydroxide (10 mmol/L) in a 96-well plate. For the background of the tests, only azoalbumin and sodium phosphate buffer were incubated at 37 °C for 2 h. In this case, the reaction was stopped with trichloroacetic acid prior to the addition of the fungal samples. The absorbance was measured at 440 nm in a microplate reader (SpectraMax M3; Molecular Devices, Sunnyvale, USA), and the specific peptidase activity was expressed in arbitrary units (U) per milligram of protein, where 1 unit of enzymatic activity is equivalent to the variation in optical density of 0.001 nm per min at 440 nm.

Statistical analysis

All the experiments were performed in triplicate, in three independent experimental sets, and data were expressed as mean ± standard deviation (SD). Data were analyzed by Student's *t* test using GraphPad Prism 5.0 software. *P* values less than or equal to 0.05 were considered statistically significant.

Results

Detection of peptidases in clinical isolates of the *C. haemulonii* complex

For all experimental sets, the fungal cells were obtained after 48 h of in vitro growth on Sabouraud medium, which correspond to cells at exponential growth phase. These cells were metabolically active as judged by the capability of their mitochondrial dehydrogenases in reducing the XTT salt to formazan and also by the lack of incorporation of a considerable amount of propidium iodide (< 10%) into the fungal cells (assessed using flow cytometry) (data not shown). Both of these viability assays attested that the fungi were viable along the experiments, suggesting that the extracellular contents were not arising from the lysis of damaged/dead cells.

Peptidase profiles of both cell-free culture supernatants (Fig. 1a; Table 1) and cellular extracts (Fig. 1b; Table 1) from nine clinical isolates forming the *C. haemulonii* complex were initially evaluated by SDS-PAGE containing different protein substrates (gelatin, casein, BSA, HSA, IgG, or Hgb) copolymerized into the gel matrix. Overall, peptidase activity was detected in all the studied fungal isolates, except for the supernatant obtained from LIP*Ch*11 in which no peptidase activity was detected regardless of the substrate evaluated. Two gelatinases, with apparent molar masses of 50 and 60 kDa, were detected in supernatants and cellular extracts of the isolates belonging to the *C. haemulonii* and *C. haemulonii* var. *vulnera* (Fig. 1a, b; Table 1). Furthermore, peptidases of 35 and 40 kDa, which were visualized in both extracellular and cellular samples, showed broad hydrolytic capabilities, degrading the remaining test proteinaceous substrates (casein, BSA, HSA, IgG, and Hgb). Besides that, a cell-associated 65-kDa peptidase, able to degrade only IgG, and a 70-kDa peptidase, able to degrade casein, BSA, and

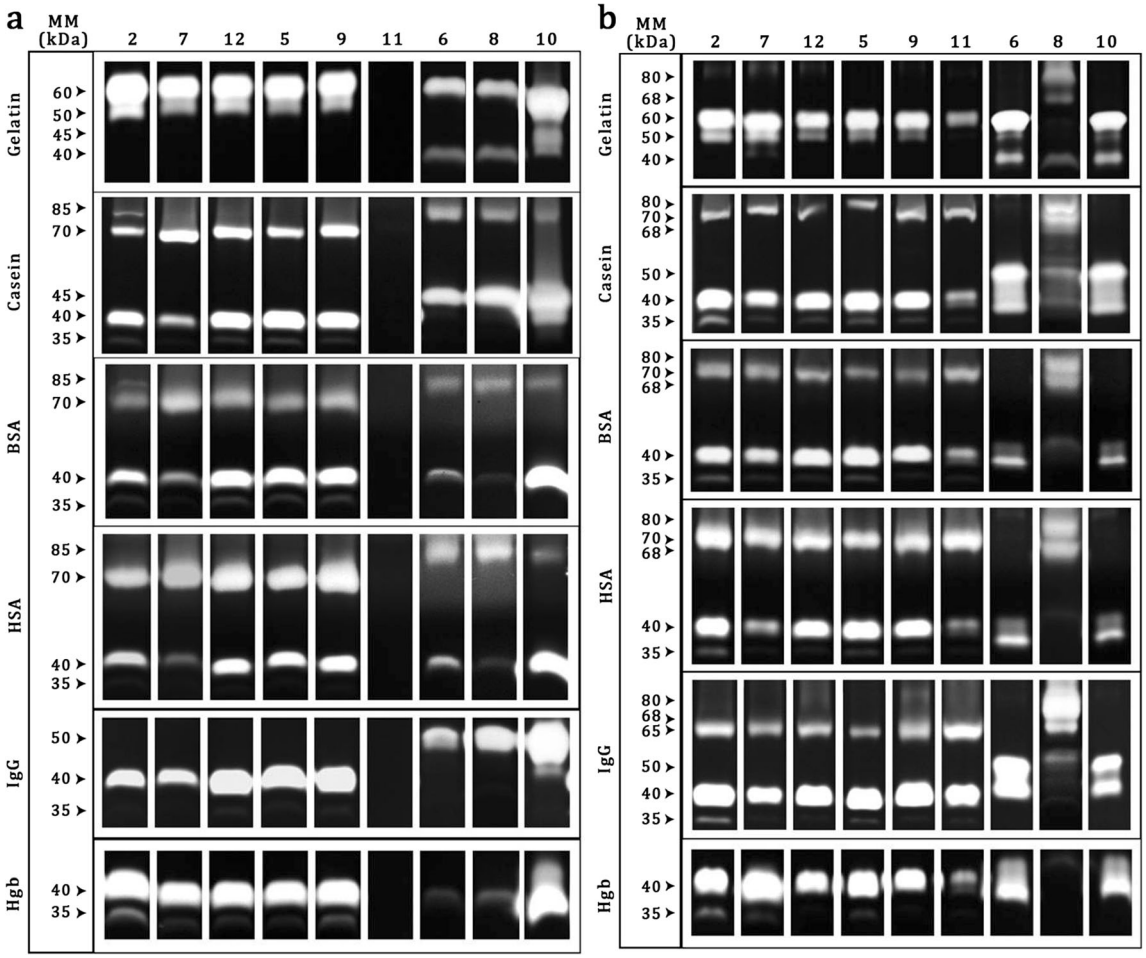

Fig. 1 Peptidase profiles detected in culture supernatants and cell extracts from clinical isolates of the *Candida haemulonii* complex. **a** Analysis of the peptidase activity profiles in culture supernatants and in **b** cell extracts of *C. haemulonii* (isolates LIP*Ch*2, LIP*Ch*7, and LIP*Ch*12), *C. haemulonii* var. *vulnera* (LIP*Ch*5, LIP*Ch*9, and LIP*Ch*11), and *C. duobushaemulonii* (LIP*Ch*6, LIP*Ch*8, and LIP*Ch*10) after electrophoresis on 12.5% SDS–PAGE with 0.1% gelatin, casein, BSA, HSA, Hgb, and IgG as substrates incorporated into the gel matrix. Gel strips containing the samples were incubated in 10 mmol/L sodium phosphate buffer, pH 7.0, for 48 h at 37 °C. The apparent molecular mass (MM) of the detected peptidases is expressed in kDa. The numbers on the top of each lane correspond to the code of the fungal isolates: 2, LIP*Ch*2; 7, LIP*Ch*7; 12, LIP*Ch*12; 5, LIP*Ch*5; 9, LIP*Ch*9; 11, LIP*Ch*11; 6, LIP*Ch*6; 8, LIP*Ch*8; and 10, LIP*Ch*10

Table 1 Peptidases detected in the *Candida haemulonii* species complex

Sample	Peptidases (kDa)					
	Substrate[a]					
	Gelatin	Casein	IgG	BSA	HSA	Hgb
Culture supernatant						
C. haemulonii						
LIP*Ch*2, LIP*Ch*7, and LIP*Ch*12	50, 60	35, 40, 70	35, 40	35, 40, 70	35, 40, 70	35, 40
C. haemulonii var. *vulnera*						
LIP*Ch*5 and LIP*Ch*9	50, 60	35, 40, 70	35, 40	35, 40, 70	35, 40, 70	35, 40
LIP*Ch*11	–	–	–	–	–	–
C. duobushaemulonii						
LIP*Ch*6 and LIP*Ch*8	40, 60	45, 85	50	40, 85	40, 85	40
LIP*Ch*10	40, 45, 50	40, 45, 85	50	40, 85	40, 85	40
Cell extract						
C. haemulonii						
LIP*Ch*2, LIP*Ch*7, and LIP*Ch*12	50, 60	35, 40, 70	35, 40, 65	35, 40, 70	35, 40, 70	35, 40
C. haemulonii var. *vulnera*						
LIP*Ch*5, LIP*Ch*9, and LIP*Ch*11	50, 60	35, 40, 70	35, 40, 65	35, 40, 70	35, 40, 70	35, 40
C. duobushaemulonii						
LIP*Ch*6 and LIP*Ch*10	40, 60	40, 50	40, 50	40	40	40
LIP*Ch*8	40, 68, 80	40, 50, 68, 80	50, 68, 80	40, 68, 80	40, 68, 80	–

[a] *IgG*, immunoglobulin G; *BSA*, bovine serum albumin; *HSA*, human serum albumin; *Hgb*, hemoglobin

HSA, evidenced in both supernatants and cellular extracts, were also detected in the *C. haemulonii* and *C. haemulonii* var. *vulnera* isolates (Fig. 1a, b; Table 1). In contrast, a great variability on the production of either cell-associated or secreted peptidases was clearly evident in the clinical isolates of *C. duobushaemulonii*, showing a typical isolate-dependent peptidase profile (Fig. 1a, b; Table 1). Interestingly, a good correlation between the peptidase profiles of *C. haemulonii* and *C. haemulonii* var. *vulnera* was observed in supernatants and in cellular extracts. However, these profiles differed significantly from those found for *C. duobushaemulonii* in both of the evaluated samples (Fig. 1a, b; Table 1).

Subsequently, a similarity analysis (based on UPGMA) was applied using all peptidase bands detected in both cell-free culture supernatants and cellular extracts (visualized by the zymographic assay) as markers (Fig. 2). At about 34% of similarity, two major groups were clearly separated for the *C. haemulonii* species complex: one formed by *C. haemulonii* and *C. haemulonii* var. *vulnera* isolates and the other one formed by *C. duobushaemulonii* isolates (Fig. 2).

Effects of pHs, temperatures, and inhibitors on the peptidases produced by the *C. haemulonii* complex

Based on the zymographic profiles (Fig. 1) and dendrogram analysis (Fig. 2), the supernatants and cellular extracts, with different proteolytic bands on each test substrate, were selected to further investigate their biochemical properties.

pH

In general, most of the peptidases detected in the fungal supernatants (Fig. 3a) and in the cellular extracts (Fig. 3b) showed activity at all of the evaluated pH values, presenting the highest hydrolytic capability at pH 7.0, when gels were incubated at 37 °C for 48 h. However, some peptidases detected in the cellular extract of LIP*Ch*8 with activity over BSA, HSA (40, 70, and 80 kDa), and casein (50 kDa) appeared to be most active at pH 5.0 (Fig. 3).

Temperature

The vast majority of the peptidases detected in the fungal supernatants (Fig. 4b) and in the cellular extracts (Fig. 4b)

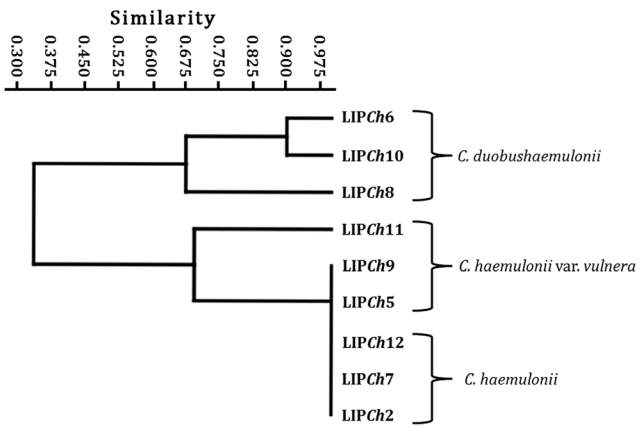

Fig. 2 Dendrogram (UPGMA) showing the relationships among nine clinical isolates belonging to the *Candida haemulonii* species complex (estimated by zymographic assays)

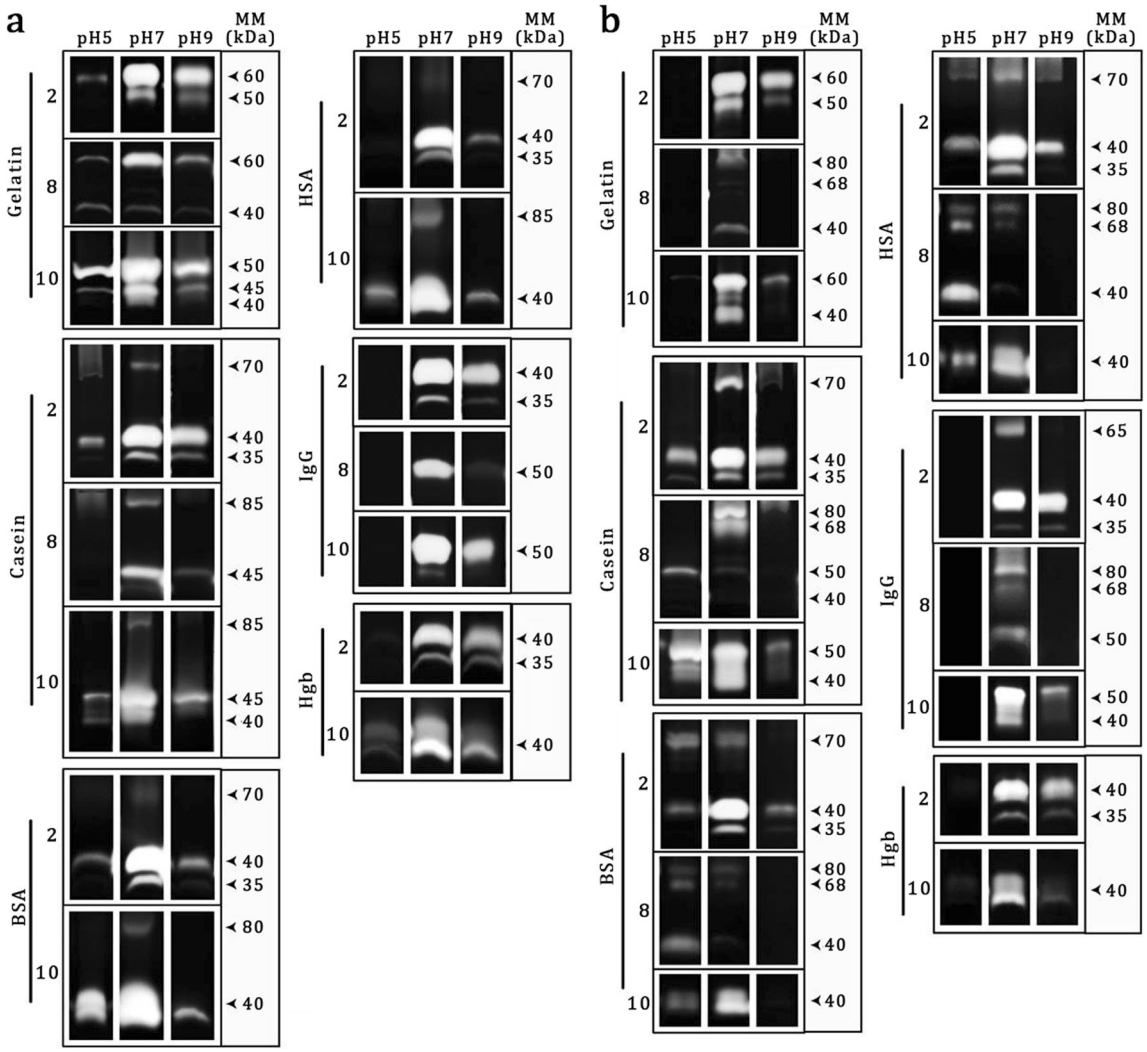

Fig. 3 Effects of pH on the peptidase activities detected in culture supernatants and cell extracts from clinical isolates of the *Candida haemulonii* complex. In this set of experiments, the clinical isolates presenting different peptidase profiles were selected. **a** Analysis of the peptidase activity profiles in culture supernatants and in **b** cell extracts of the *C. haemulonii* complex after electrophoresis on 12.5% SDS–PAGE with 0.1% gelatin, casein, BSA, HSA, Hgb, and IgG as substrates incorporated into the gels. Gel strips containing the samples were incubated in 50 mmol/L sodium citrate buffer, pH 5.0, 50 mmol/L sodium phosphate, pH 7.0, and 50 mmol/L glycine-NaOH, pH 9.0, for 48 h at 37 °C. The apparent molecular mass (MM) of the detected peptidases is expressed in kDa. The numbers on the left margins of the pictures correspond to the code of the fungal isolates. 2, LIP*Ch*2 (*C. haemulonii*); 8, LIP*Ch*8; and 10, LIP*Ch*10 (*C. duobushaemulonii*)

showed activity at all of the test temperatures, with the highest cleavages equally occurring at 37 and 42 °C, after 48 h at neutral pH (Fig. 4).

Peptidase inhibitors

Overall, PMSF (at 10 mmol/L) partially or fully inhibited the enzymatic activities of all the peptidases detected in the fungal supernatants (Fig. 5a) and in the cellular extracts (Fig. 5b) of the *C. haemulonii* species complex when incubated for 48 h at pH 7.0 and 37 °C. Additionally, 1,10-phenanthroline (10 mmol/L) was also able to inhibit the enzymatic activity of some secreted and cell-associated peptidases with activity over BSA and IgG (Fig. 5a, b). Both pepstatin A (10 μmol/L) and E-64 (10 μmol/L) did not interfere with the peptidase activities observed in all the evaluated systems (Fig. 5).

Quantification of peptidase activity

To varying extents, peptidase activity was quantified in all of the analyzed samples (supernatants and cellular extracts) using azoalbumin as soluble substrate (Fig. 6). Regarding the clinical isolates of each member of this fungal complex, differences in peptidase activities were detected in supernatants (≈ 4–9 U/mg for *C. haemulonii*; 3–6 U/mg for *C. haemulonii* var. *vulnera*; and 6–9 U/mg for *C. duobushaemulonii*) (Fig. 6a) and in cellular extracts (≈ 7–

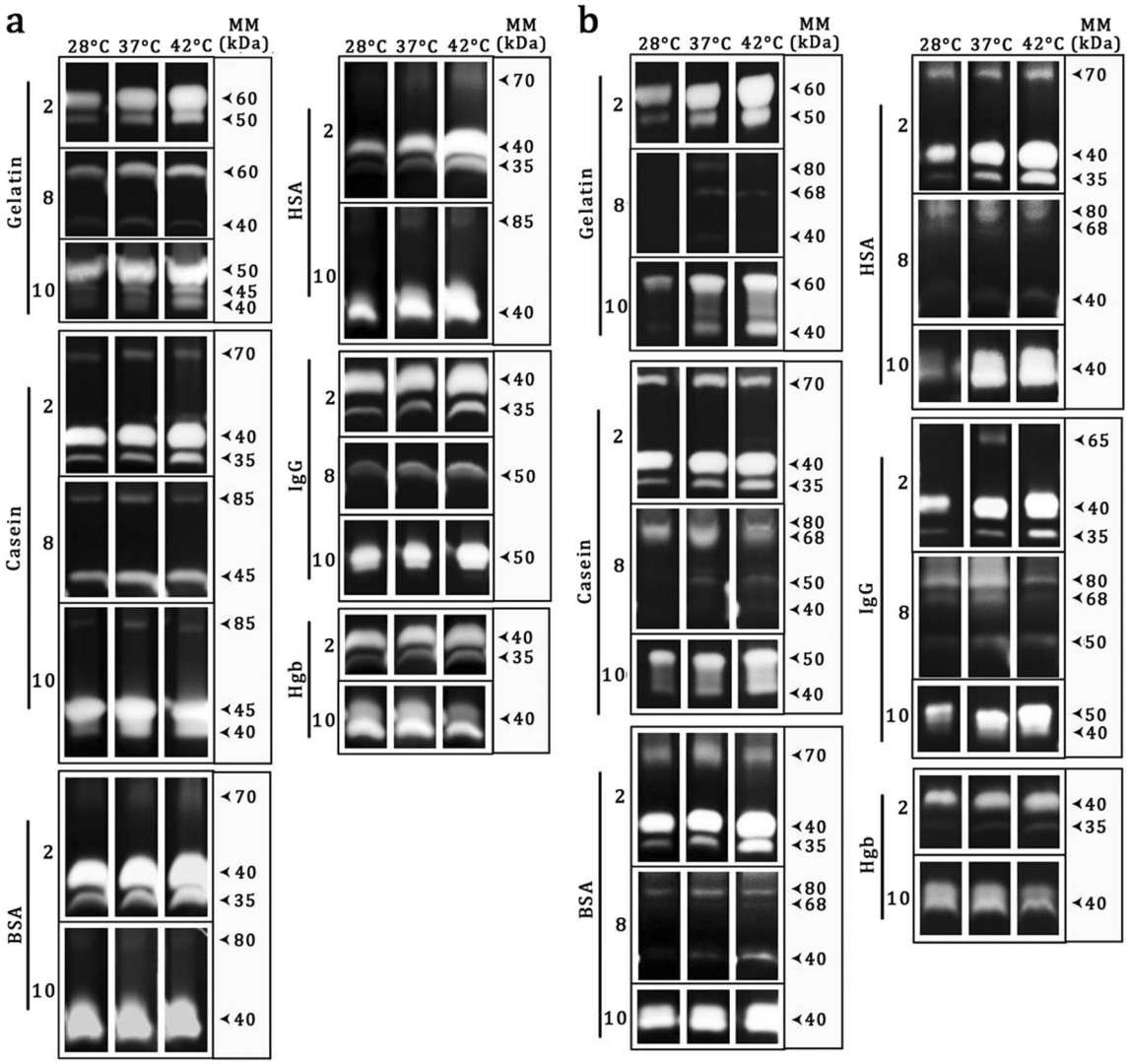

Fig. 4 Effects of temperature on the peptidase activities in culture supernatants and in cell extracts from clinical isolates of the *Candida haemulonii* complex. In this set of experiments, the clinical isolates presenting different peptidase profiles were selected. **a** Analysis of the peptidase activity profiles in culture supernatants and in **b** cell extracts of the *C. haemulonii* complex after electrophoresis on 12.5% SDS–PAGE with 0.1% gelatin, casein, BSA, HSA, Hgb, and IgG as substrates incorporated into the gels. Gel strips containing the samples were incubated in 10 mmol/L sodium phosphate buffer, pH 7.0, for 48 h, at 28, 37, or 42 °C. The apparent molecular mass (MM) of the detected peptidases is expressed in kDa. The numbers on the left margins of the pictures correspond to the code of the fungal isolates. 2, LIP*Ch*2 (*C. haemulonii*); 8, LIP*Ch*8; and 10, LIP*Ch*10 (*C. duobushaemulonii*)

16 U/mg for *C. haemulonii*; 11–15 U/mg for *C. haemulonii* var. *vulnera*; and 7–10 U/mg for *C. duobushaemulonii*) (Fig. 6b). However, no significant differences ($P > 0.05$) were detected considering the mean of peptidase activity among the species forming the *C. haemulonii* complex (Fig. 6a, b, *insert*). In parallel, PMSF (at 10 mmol/L) significantly inhibited the peptidase activity in all of the supernatants (≈ 79–87% for *C. haemulonii*; 75–84% for *C. haemulonii* var. *vulnera*; and 63–72% for *C. duobushae mulonii*) and in the cellular extracts (≈ 64–69% for *C. haemulonii*; 77–96% for *C. haemulonii* var. *vulnera*; and 75–80% for *C. duobushaemulonii*) from the *C. haemulonii* complex (Fig. 6a, b).

Discussion

The difficulties encountered in the treatment of infections caused by the *C. haemulonii* species complex have driven the search for new drugs and novel therapeutic targets. In this context, peptidases are involved in different interactive stages between fungi and hosts, being considered as potential virulence attributes (Hube 2000), and it is the fact which has prompted us to investigate the potential peptidases' arsenal produced by the *C. haemulonii* complex.

Candida haemulonii and *C. haemulonii* var. *vulnera* isolates produced two secreted/cell-associated peptidases (50 and 60 kDa) that hydrolyze gelatin. Zymography also revealed

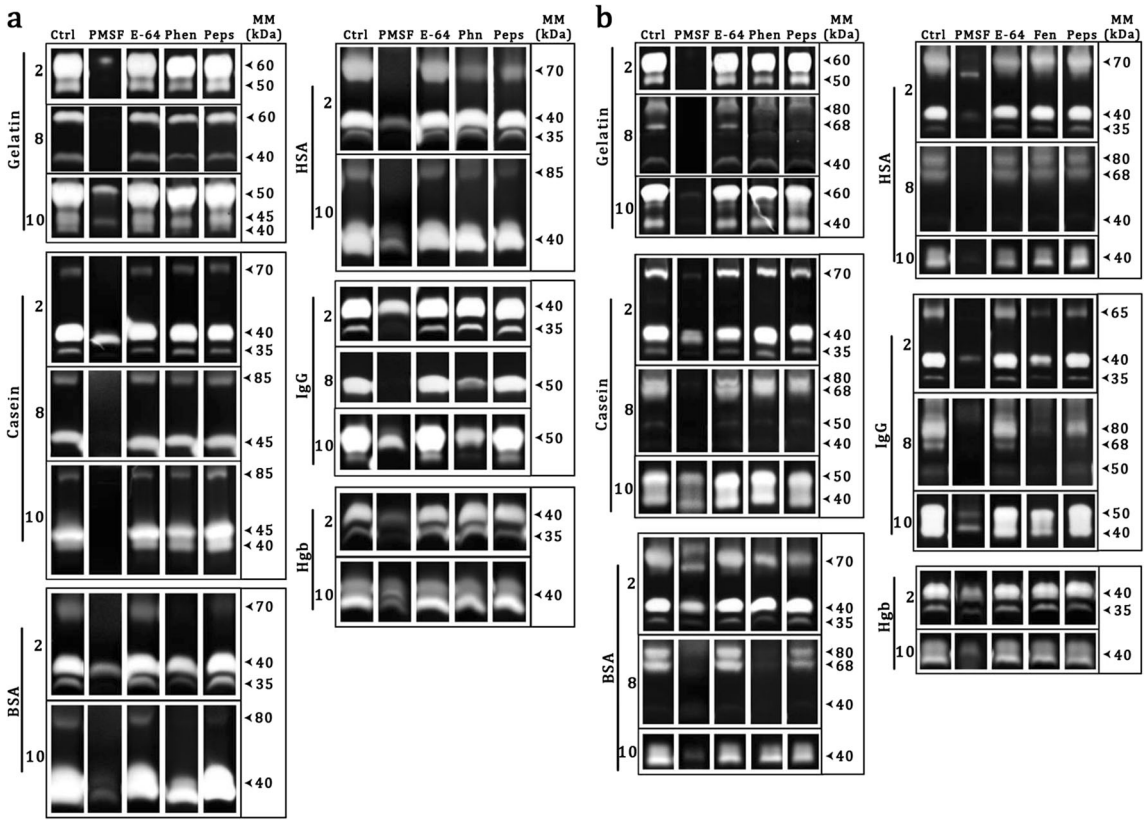

Fig. 5 Effect of inhibitors on the peptidase activities in culture supernatants and cell extracts from clinical isolates of the *Candida haemulonii* complex. In this set of experiments, the clinical isolates presenting different peptidase profiles were selected. **a** Analysis of the peptidase activity profiles in culture supernatants and in **b** cell extracts of the *C. haemulonii* complex after electrophoresis on 12.5% SDS–PAGE with 0.1% gelatin, casein, BSA, HSA, Hgb, and IgG as substrates incorporated into the gels. Gel strips containing the samples were incubated in 50 mmol/L sodium phosphate buffer, pH 7.0, for 48 h at 37 °C, in the absence (ctrl) or in the presence of 10 mmol/L PMSF, 10 mmol/L 1,10-phenanthroline (Phen), 10 μmol/L E-64, or 10 μmol/L pepstatin (Peps). The apparent molecular mass (MM) of the detected peptidases is expressed in kDa. The numbers on the left margins of the pictures correspond to the code of the fungal isolates. 2, LIP*Ch*2 (*C. haemulonii*); 8, LIP*Ch*8; and 10, LIP*Ch*10 (*C. duobushaemulonii*)

peptidases in the supernatants and in the cellular extracts of *C. haemulonii* and *C. haemulonii* var. *vulnera* having different hydrolytic capacities, being able to cleave casein, BSA, HSA, IgG, and Hgb (35 and 40 kDa), casein, BSA, and HSA (70 kDa) and only IgG (65 kDa). Conversely, a wide variety of secreted and cell-associated peptidases with the ability to degrade all of the abovementioned substrates were detected in *C. duobushaemulonii* isolates. Gelatin and casein were used as substrates because they are easily hydrolyzed by various peptidases (Rao et al. 1998). Besides that, gelatin is a denatured form of collagen, itself a known constituent of the extracellular matrix of the connective tissue of animals. In *Candida* spp., it has been suggested that the ability of peptidases to degrade extracellular matrix proteins may be related to their role in removing host barriers during the in vivo infection process, providing nutrients for growth and favoring the penetration/colonization of host tissue (Hube 2000). The hydrolysis of Hgb (main protein in red blood cells) and albumin (a carrier protein found in high amounts in serum) by *Candida* spp. has been associated with the uptake of nutrients (iron and amino acids) during mammalian infection (Moors et al. 1992; Chaffin et al. 1998; Hube 2000; Santos and Soares 2005; de Melo et al. 2007; Noble 2013; Ramachandra et al. 2014). The IgG molecules are known to play important roles in humoral host defense mechanisms against infections caused by *Candida* spp.; however, their degradation by fungal peptidases could result in its immunological escape (Casadevall 1995; Santos et al. 2006).

The peptidase profiles observed in *C. haemulonii* and *C. haemulonii* var. *vulnera* were identical for either supernatants or cellular extracts. Contrarily, differences in the proteolytic profiles were clearly observed among the isolates of *C. duobushaemulonii*, as well as between them and the isolates of *C. haemulonii* and *C. haemulonii* var. *vulnera*. These data reflect the genetic similarity existing between the microbial members of the complex, being most striking between *C. haemulonii* and *C. haemulonii* var. *vulnera* (Cendejas-Bueno et al. 2012). Additionally, the quantitative evaluation of peptidase activities indicated differences between the isolates of each member of the *C. haemulonii* complex; however, the mean proteolytic activities were not different among the species forming this fungal complex.

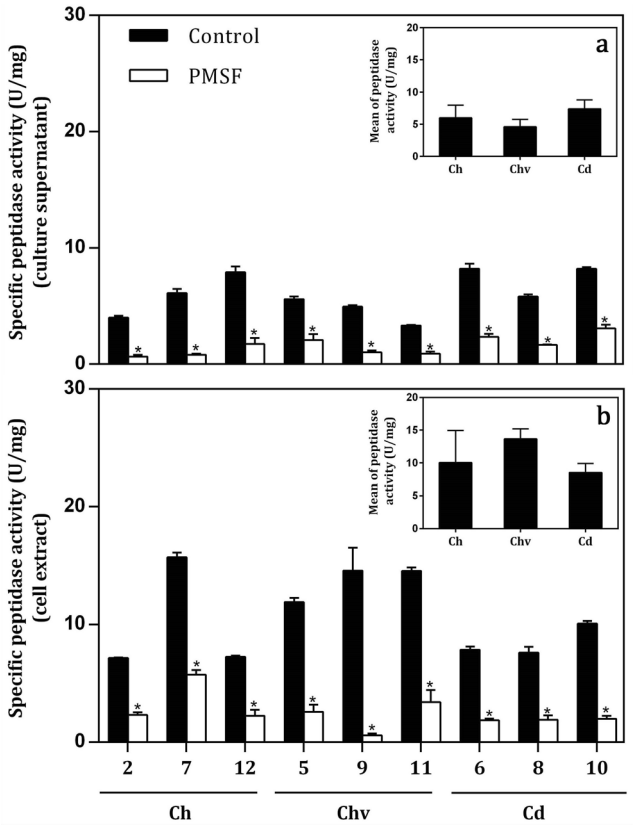

Fig. 6 Peptidase activity measured in the culture supernatants and in cell extracts from clinical isolates of the *Candida haemulonii* complex. **a** Measurement of peptidase activity in culture supernatants and in **b** cell extracts of *C. haemulonii* (*Ch*; isolates LIP*Ch*2, LIP*Ch*7, and LIP*Ch*12), *C. haemulonii* var. *vulnera* (*Chv*; LIP*Ch*5, LIP*Ch*9, and LIP*Ch*11), and *C. duobushaemulonii* (*Cd*; LIP*Ch*6, LIP*Ch*8, and LIP*Ch*10) using azoalbumin (1.6 mg/mL) as the protein substrate in 50 mmol/L sodium phosphate (pH 7.0), for 2 h at 37 °C, in the absence or in the presence of 10 mmol/L PMSF. The peptidase activity is expressed in arbitrary units (U) per milligram of protein, where one unit of activity is equivalent to the variation in optical density of 0.001 nm per min at 440 nm. The bars represent the mean ± SD from at least three independent experiments. The asterisks indicate significant difference in the peptidase activity considering the isolates treated (white bars) or not (black bars) with PMSF ($P < 0.05$). Inserts represent the mean of peptidase activity (in the absence of PMSF) among the isolates of each member of the *C. haemulonii* complex. The numbers on the bottom of each bar correspond to the code of the fungal isolates: 2, LIP*Ch*2; 7, LIP*Ch*7; 12, LIP*Ch*12; 5, LIP*Ch*5; 9, LIP*Ch*9; 11, LIP*Ch*11; 6, LIP*Ch*6; 8, LIP*Ch*8; and 10, LIP*Ch*10

PMSF blocked the activity of almost all of the peptidases produced by *C. haemulonii* complex, thus classifying them as serine-type peptidases. Fungal serine peptidases constitute an important group of intra- and extracellular peptidases with both regulatory and nutritional roles (Hube 2000; Monod et al. 2002), which present broad substrate specificity and hydrolytic activity at neutral and alkaline pH (de Souza et al. 2015). Corroborating these premises, the serine peptidases of the *C. haemulonii* complex showed activity across a wide range of pH and temperature. Moreover, the pH of maximum activity (7.0) is close to the pH range of the sole of the foot (pH 5.0–7.82) (Marshall et al. 1987), blood (pH 7.35–7.45) (de Melo et al. 2007), and bronchoalveolar lavage (pH 5.96–6.58) (Lozo Vukovac et al. 2014), sources from which the fungal isolates were recovered. Regarding temperature, the range of maximum peptidase activity observed (37–42 °C) suggests the effectiveness of these enzymes during the infection in an individual with normal body temperature (36.5–37 °C) or someone in feverish condition (> 37 °C).

Several studies have suggested the importance of serine peptidases as virulence factors in human pathogenic fungi (Jousson et al. 2004; Behnsen et al. 2010). Rodier et al. (1994) described two main cellular serine peptidases of 50 and 60 kDa found in *C. albicans* samples recovered from urine, feces, vagina, and mouth, which degraded gelatin at 37 °C and over a wide pH range (5.0–8.0). Ito et al. (2010) reported the presence of a 64-kDa, PMSF-sensitive serine peptidase in the cellular extract of *C. glabrata* and which had optimum enzymatic activity over casein at pH 7.0 and 37 °C. Secreted serine peptidases have also been studied in *Candida* genus. Our group reported, for the first time, the presence of extracellular acidic serine peptidases (30–58 kDa) in oral clinical isolates of *C. albicans* recovered from HIV-positive and healthy children (de Brito Costa et al. 2003). Santos and Soares (2005) identified a 50-kDa serine peptidase secreted by *C. guilliermondii* isolated from bronchoalveolar lavage which was able to hydrolyze relevant human proteins, including HSA, IgG, fibronectin, and laminin, at 37 °C and physiological pH. A 50-kDa serine peptidase was also identified in the supernatant of an isolate of *C. albicans* recovered from urine, which was active within a wide pH range (5.0–7.2) at 37 °C, and was able to hydrolyze human serum proteins and extracellular matrix components (Santos and Soares 2005). Extracellular serine peptidases possessing an ability to cleave key host proteins were also reported in clinical isolates of *C. rugosa* (50, 94, and 120 kDa), *C. lipolytica* (60 kDa) (de Melo et al. 2007), *C. tropicalis* and *C. dubliniensis* (44 to 104 kDa) (Portela et al. 2010), and *C. parapsilosis* (60 kDa) (Vermelho et al. 2010).

Collectively, our results reveal the capability of clinical isolates of the *C. haemulonii* species complex to produce serine peptidases that can hydrolyze a broad spectrum of protein substrates, some of which have great significance in the fungi-host interface (e.g., IgG, HSA, and Hgb). Moreover, these peptidases are active across a wide range of pH and temperature, which may explain the ability of the *C. haemulonii* species complex to colonize several sites of the human body. Future in vivo investigations are needed to determine the biological significance of these peptidases and, additionally, to explore the possibility of using these enzymes as new targets for the development of antifungal drugs based on peptidase inhibitors.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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