Mycopathologia (2013) 176:33–40
DOI 10.1007/s11046-013-9661-0

Differential Adherence and Expression of Virulence Traits by *Candida albicans* and *Candida parapsilosis* in Mono- and Dual-Species Cultures in Artificial Saliva

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Received: 6 November 2012 / Accepted: 27 April 2013 / Published online: 10 May 2013
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Abstract
Aims To evaluate specific virulence factors of *Candida albicans* and *Candida parapsilosis* clinical oral isolates in mono- and dual-species culture in the presence of artificial saliva.
Methods and Results Two of the strains used in this study were isolated from co-infection (*C. albicans* AM and *C. parapsilosis* AM2), and the other two were isolated from single infection (*C. albicans* AC and *C. parapsilosis* AD). The number of adhered yeast cells was measured and their enzymatic activity was determined simultaneously. In mono-species culture, *C. parapsilosis* strains adhered to a higher extent to the surface in comparison with the *C. albicans* strains. In dual-species culture, the *C. parapsilosis* strains adhered more in the presence of *C. albicans* AM. Interestingly, *C. albicans* AM and *C. parapsilosis* AD adhered to a higher extent when compared with all other co-cultures. In dual-species culture, the enzymatic activity of *C. parapsilosis* strains in the presence of *C. albicans* AC was higher than in the presence of *C. albicans* AM.
Conclusions The virulence factors of *C. albicans* and *C. parapsilosis* differ from strain to strain and are influenced by the presence of other species in culture.
Significance and Impact of the Study To understand the expression of virulence factors in *Candida* dual-species systems.

Keywords Co-infection · Adhesion · Enzymatic activity · Saliva

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Introduction

The oral cavity comprises diverse microenvironments, containing a range of surfaces to which microbial cells can adhere. In nature, most microorganisms do not exist as pure cultures of free-living cells, but are associated in multispecies cultures, where more than 200 microbial species, such as *Candida* species or bacteria, coexist in a unique habitat and are attached to a surface [1, 2]. These surfaces present in oral cavity include dental prostheses, teeth enamel and epithelial mucosa [3, 4]. In the oral cavity, since most surfaces are soaked in saliva, the binding of microorganisms to salivary proteins is of paramount significance to colonization [5]. *Candida* species are oral commensals found in 50–60 % of the population either as transient or as permanent colonizers [2], and they can be isolated from dentures, dental plaque and patients with periodontal disease [4]. A study performed at a dental

clinic [6] located in Braga, Portugal, demonstrated that *Candida parapsilosis* is emerging as a notable pathogenic agent of the oral mucosa, either in co-infection with *Candida albicans* or occurring in single-species infection.

In order to persist in the oral cavity, *Candida* species must adhere to the oral surfaces; otherwise, they would be washed out by the salivary flow. *Candida* species have the ability to adhere using a variety of mechanisms and specific strategies, allowing the yeast cells to anchor at a precise site favoring the initiation of tissue colonization [7–9]. It is important to mention that a key virulence factor of *Candida* species is the release of hydrolytic enzymes into the local environment, such as hemolysin, proteinases and phospholipases. Iron is extremely important for *Candida* species' survival; therefore, they secrete hemolysin to the surrounding medium, which will degrade the hemoglobin of the host cells releasing the iron [10, 11]. The ability to produce phospholipase is a very important virulence factor as this enzyme will degrade the phospholipid components of the host cell membrane, leading to changes in its properties in order to facilitate infection or even leading to the disruption of the cell membrane [12]. A similar effect is achieved by the expression of secreted aspartyl proteinases (SAP), which facilitates the invasion and colonization of host tissues by disruption of the host mucosal membranes and by degrading important immunologic and structural defense proteins [7, 13].

As mentioned previously, the oral cavity has several microorganisms living in community and it is known that the interaction of different *Candida* species can suppress each other's growth, due to the competition for nutrients and/or as a result of the presence of metabolites produced by one or both species present. Competitive inhibition between two *Candida* species in co-culture has been observed even during the initial step of adhesion onto a substrate [14]. Therefore, it is postulated that the adhesion ability of individual *Candida* species and the competition for adhesion sites between two *Candida* species regulate the formation and colony expansion in biofilms [1, 14].

So, the aim of this study was to evaluate the expression of virulence factors (adhesion and enzymatic activity) of *C. albicans* and *C. parapsilosis* clinical isolates in mono- and dual-species cultures.

Materials and Methods

Clinical Isolates and Growth Conditions

In this study, four clinical isolates (two strains of *Candida albicans* and two strains of *Candida parapsilosis*) recovered from the oral cavity of patients with candidosis were used. The clinical isolates had different origins: *C. albicans* AM and *C. parapsilosis* AM2 were isolated from the same patient (dual-species colonization), whereas *C. albicans* AC and *C. parapsilosis* AD were isolated from different patients (mono-species colonization). These oral isolates were obtained from Biofilm Group collection of the Centre of Biological Engineering, University of Minho (Braga, Portugal) [6].

Candida species stored at −80 °C were propagated by streaking a loop full of cells onto Sabouraud dextrose agar (SDA, Liofilchem, Italy) medium and incubated at 37 °C for 24 h.

For each experiment, batches of Sabouraud dextrose broth (SDB, Liofilchem, Italy) were inoculated with freshly grown *Candida* cells and incubated in an orbital shaker at 1.61×10^{-2} g at 37 °C. After 18 h, the cells were harvested by centrifugation at 6,654*g* for 5 min at 4 °C and washed twice with phosphate saline buffer (PBS, 0.1 mol l^{-1}, pH 7).

Surface Coating

The 6 wells of polystyrene plates were coated with artificial saliva for 4 h at 37 °C in an orbital shaker at 1.61×10^{-2} g, adapted from Guggenheim et al. [15]. Artificial saliva was prepared using a modified protocol described by Lamfon et al. [16]: 2 g l^{-1} yeast extract (Liofilchem, Italy), 5 g l^{-1} peptone (Liofilchem, Italy), 2 g l^{-1} glucose (Applichem, Germany), 1 g l^{-1} mucin from bovine submaxillary glands—Type I–S (Sigma-Aldrich, USA), 0.35 g l^{1} NaCl (Applichem, Germany), 0.2 g l^{-1} $CaCl_2$ (Riedel-de-Häen, Germany), and 0.2 g l^{-1} KCl (Pronalab, Portugal) (pH 6.8–7). Then, the wells were washed with PBS and stored in the same buffer at 4 °C until needed (1 week maximum). The storage of the plates was optimized and analyzed in order to ensure the same properties for 1 week.

Adhesion Assay

As mentioned previously, for each mono-species culture, 2 ml of each yeast cell suspension with a

concentration of 1×10^7 cells ml^{-1} was prepared. Regarding the dual-species adhesion assays, 1 ml of *Candida albicans* and 1 ml of *Candida parapsilosis* at a concentration of 2×10^7 cells ml^{-1} each were added. This fact allowed the comparison of growth kinetics between mono-species culture and dual-species culture. Both cultures (mono-species and dual-species) were prepared in artificial saliva. Afterward, the suspensions were placed on the pre-coated plate and incubated at 37 °C in an orbital shaker at 1.61×10^{-2}g. In order to determine whether the behavior of *Candida* species varies according to their origin, besides the single cultures, several combinations were tested: *C. albicans* AM + *C. parapsilosis* AM2, *C. albicans* AC + *C. parapsilosis* AD, *C. albicans* AC + *C. parapsilosis* AM2 and *C. albicans* AM + *C. parapsilosis* AD.

The number of adhered yeast cells was determined by counting the colony-forming units (CFUs). Briefly, after 2 h of yeast cells' contact with the wells (37 °C, 1.61×10^{-2} g), the medium was removed and the wells were firstly washed with PBS to remove loosely attached cells and then scraped to resuspend adhered cells in 2 ml of PBS by pipetting up and down several times. Suspensions containing the yeast cells were sonicated with a probe of diameter 0.3 cm, for 45 s at 30 W (Ultrasonic Processor, Cole-Parmer, USA), followed by a vigorous homogenization for 30 s using a vortex. Viable counts of each species were obtained by decimal serial dilutions in PBS and plated on CHROMagar *Candida* medium (CHROMagar™ *Candida*, France) for 48 h at 37 °C, which allows species distinction.

Experiments were performed in triplicate and repeated in three independent assays.

Determination of Enzymatic Activity

When analyzing the enzymatic activity of mono-species and dual-species cultures, it is impossible to determine the contribution of each particular strain. In order to overcome this problem, the initial concentration of the yeast cells on the mono-specie culture was kept as 2×10^7 cells ml^{-1}, which is the same concentration as the total concentration of the yeast cells on dual-species cultures (2×10^7 cells ml^{-1}). So, the results presented are related to the same initial concentration on both conditions.

After 2 h of adhesion, cells were washed with PBS to remove loosely attached cells, scraped, resuspended, sonicated, and vortexed as described previously.

For each enzyme studied, a specific culture medium was used, as described in the next subsections.

Each experiment was conducted in triplicate and repeated in three independent assays and the result was normalized *per* CFUs [(%/CFUs) $\times 10^6$], in order to compare the effect caused by the same number of yeasts from each strain.

Hemolytic Activity

The hemolytic activity was evaluated using a modification of the blood plate assay described by Luo et al. [17]. The medium was prepared by adding 18.75 g l^{-1} agar (Applichem), 12.5 g l^{-1} peptone (Liofilchem, Italy), 350 g l^{-1} glucose (Applichem, Germany), and 7 % (v/v) fresh sheep blood (Probiológica, Portugal) (pH 6.8–7).

In brief, a drop of 25 µl of each *Candida* suspension, recovered after adhesion, was placed on blood agar medium, let to dry at room temperature and incubated at 37 °C for 48 h. The presence of hemolysin was determined by the formation of an opaque zone around the yeast colonies, and the enzyme activity (Pz) was calculated by the ratio of the colony diameter to the colony diameter plus the halo zone as described by Price et al. [18]. To determine the enzymatic activity, the following formula was used: (1-Pz) × 100 %.

Phospholipase Activity

The adhered *Candida* were screened for phospholipase activity in egg yolk medium, which consisted of 43 g l^{-1} peptone (Liofilchem, Italy), 12.85 g l^{-1} glucose (Applichem, Germany), 36.84 g l^{-1} NaCl (Applichem, Germany), 0.354 g l^{-1} $CaCl_2$ (Riedel-de-Häen, Germany), 12.86 g l^{-1} agar (Applichem, Germany), and 4 % (v/v) of chicken egg yolk (pH 6.8–7).

In brief, a drop of the resultant suspension (25 µl) was placed on the plate and incubated at 37 °C for 96 h. The presence of phospholipase was measured in the same manner as described for hemolysin activity.

Proteinase Activity

The proteinase activity was analyzed in terms of bovine serum albumin (BSA) degradation according

to Rüchel [19]. In brief, the proteinase agar medium consisted of 20 g l^{-1} agar (Applichem, Germany), 13 g l^{-1} yeast carbon base (Sigma–Aldrich, USA), and 25 g l^{-1} BSA (Sigma-Aldrich, USA) (pH 6.8–7). The procedure for the evaluation of proteinase activity was the same as described previously for phospholipase and hemolysin activity.

Statistical Analysis

Statistical analysis of all the assays was performed using the SPSS software (Statistical Package for the Social Sciences, Inc., Chicago) applying the one-way ANOVA, with Bonferroni as a post hoc test, with a confidence level of 95 % ($P < 0.05$).

Results and Discussion

In this study, four different strains were used, two of which were isolated from the same patient (*C. albicans* AM and *C. parapsilosis* AM2) and other two from different patients (*C. albicans* AC and *C. parapsilosis* AD), for colonizing the oral cavity. In a previous study, it was demonstrated that mono-species colonization is the most common situation [6], and so we hypothesized that a dual-species colonization occurs only when a synergy is established, determining an increase in virulence. The first step is to evaluate the ability of these strains to adhere to a surface coated with artificial saliva, using CHROMagar Candida medium (differential medium). This medium facilitates the detection of species in dual-species culture, due to the differences in the colors of the yeast colonies—green for *C. albicans* strains and white for *C. parapsilosis* strains. So, as shown in Fig. 1a, all strains have the ability to adhere to the surface, which is very important as the initial attachment of *Candida* species to the mucosal surface of the denture can lead to stomatitis. Interestingly, *C. parapsilosis* showed to have a higher affinity to the coated surface than *C. albicans* strains ($P < 0.05$), which is known to be the most prevalent species causing oral infections [20]. However, the mechanism behind the adhesion of these particular strains to the saliva-coated surface is not yet fully clear. Although it is known that hydrophobic interactions and repulsive electrostatic forces may be involved in this phenomenon, it can also be hypothesized that the differences observed may be related to specific cell-wall proteins and receptors. It is known that *C. albicans* has nine agglutinin-like sequence (ALS) genes involved in the adhesion mechanism [21]. While, for *C. parapsilosis*, until now, only five ALS genes were described and six genes were predicted for glycophosphatidylinositol-anchored protein 30 (Pga30) [22], little is known about their role in adhesion [7, 22]. Even though more information regarding the presence of ALS genes of *C. albicans* is available in the literature, there is a high variability within *C. albicans* strains, which makes it difficult to predict the behavior of a specific strain [21, 23, 24]. Therefore, additional experiments are needed to deepen the knowledge regarding the adhesion mechanism of these particular strains.

In dual-species culture, we used CHROMagar Candida medium for the identification, by difference in color of colonies, of the number of adhered cells of *C. albicans* and *C. parapsilosis* in the culture. So, in the dual-species culture of *C. albicans* AC and *C. parapsilosis* AD, strains isolated from different patients (Fig. 1b), the adhesion of the latter was inhibited, as it presents a lower number of adherent cells than when cultured in mono-species culture ($P < 0.05$). In fact, this inhibition is in accordance with [1], who showed that *C. parapsilosis* strains have a less tendency to adhere to surfaces in a dual-species culture with *C. albicans* than in single culture. But, in opposition, the results obtained for the other combinations show an enhanced or not affected extent of adhesion of *C. parapsilosis* in comparison with its behavior alone. These results point out that the interaction between two *Candida* species is highly dependent on the strain under study.

The number of *C. parapsilosis* AM2 adhered cells when cultured with its counterpart in co-culture (*C. albicans* AM) is higher than when cultured alone ($P < 0.05$). However, when these two strains were separated and *C. parapsilosis* AM2 was cultured in the presence of strain AC, the number of adhered cells is lower than in the previous combination, but similar to single adhesion ($P < 0.05$). This result suggests that in dual-species cultures, the adhesion of *C. parapsilosis* AM2 is favored by the presence of *C. albicans* AM, with which it was co-isolated. In fact, it is known that in multispecies systems, cells respond selectively to a diversity of molecular signals present in the microenvironment [2, 14, 25]; therefore, the presence or absence of specific molecular signals can affect the

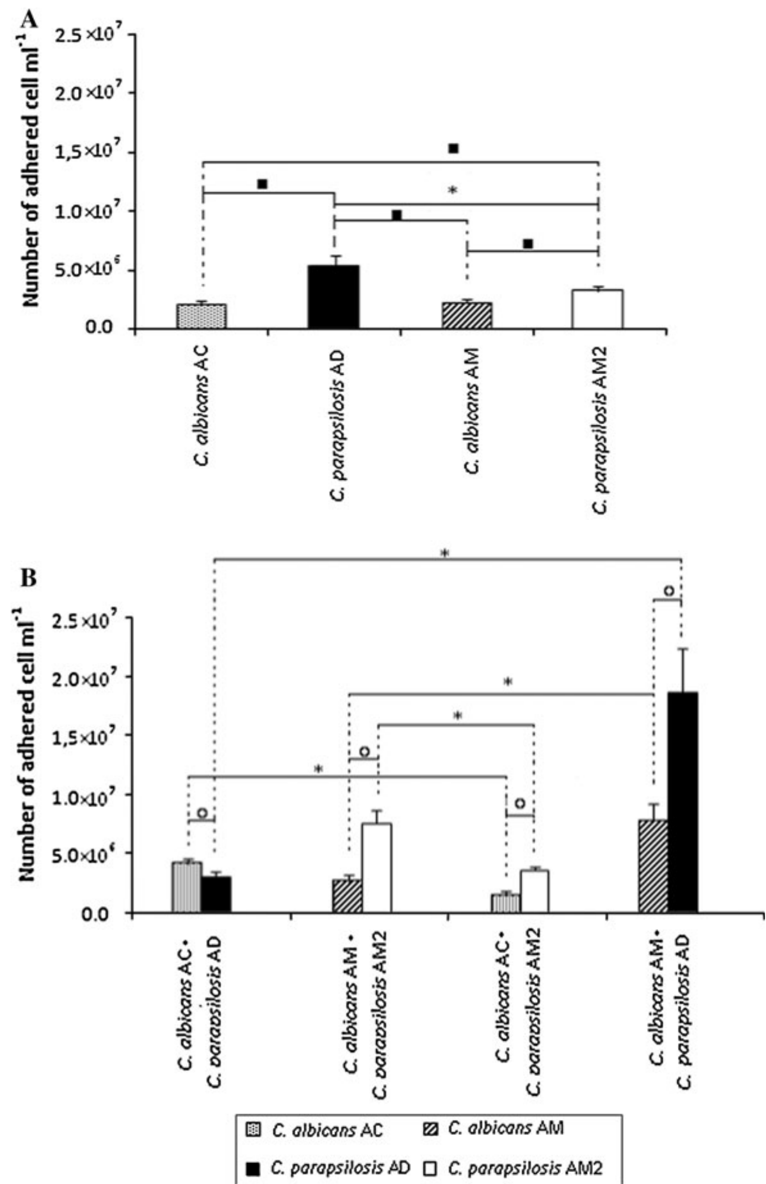

Fig. 1 Number of *Candida* adhered in (**a**) mono-species and (**b**) dual-species cultures to surfaces coated with artificial saliva. *Error bars* represent standard error. $P < 0.05$; *strains of the same species that are significantly different; *filled square* statistical differences between species; *open circle* species that are significantly different in the same culture

behavior and metabolism of cells. In this particular case, it seems that the adhesion of *C. parapsilosis* strains may be influenced by molecular signals produced by *C. albicans* AM, which enhances its ability to adhere to the surface.

On the other hand, the adhesion ability of *C. albicans* AM does not seem to be significantly affected by the presence of *C. parapsilosis* AM2 (both species were isolated from co-infection), but it is favored by *C. parapsilosis* AD. In the presence of *C. parapsilosis* AM2, *C. albicans* AM may be fully adapted to their microenvironment and therefore have a similar behavior as the one obtained when cultured alone. These results suggest that *C. albicans* AM may have receptors that recognize and respond specifically to the signaling molecules produced by *C. parapsilosis* AD, which may trigger the adhesion receptors, as reported in the literature [25].

The next step taken to evaluate strains' virulence was to assess their enzymatic activity. As it can be seen in Fig. 2a, no phospholipase activity was detected for *C. parapsilosis* strains, which is in

accordance with some reports [13, 26], referring that most *C. parapsilosis* strains do not have the ability to produce phospholipases. However, this is a controversial issue because other authors have demonstrated that different strains of *C. parapsilosis* have the ability to secrete phospholipase [27]. These discrepancies may be attributed to strain variation or differences in the methods used to detect phospholipase secretion in these strains [27]. Phenotype switching has been reported in *C. albicans* and *C. parapsilosis*, and it has been demonstrated that this phenomenon regulates a number of phenotypic characteristics involved in pathogenesis such as secretion of phospholipases [28]. As an example, it has been demonstrated that *C. albicans* colonies with the highest percentage of hyphae have the highest phospholipase activity [28].

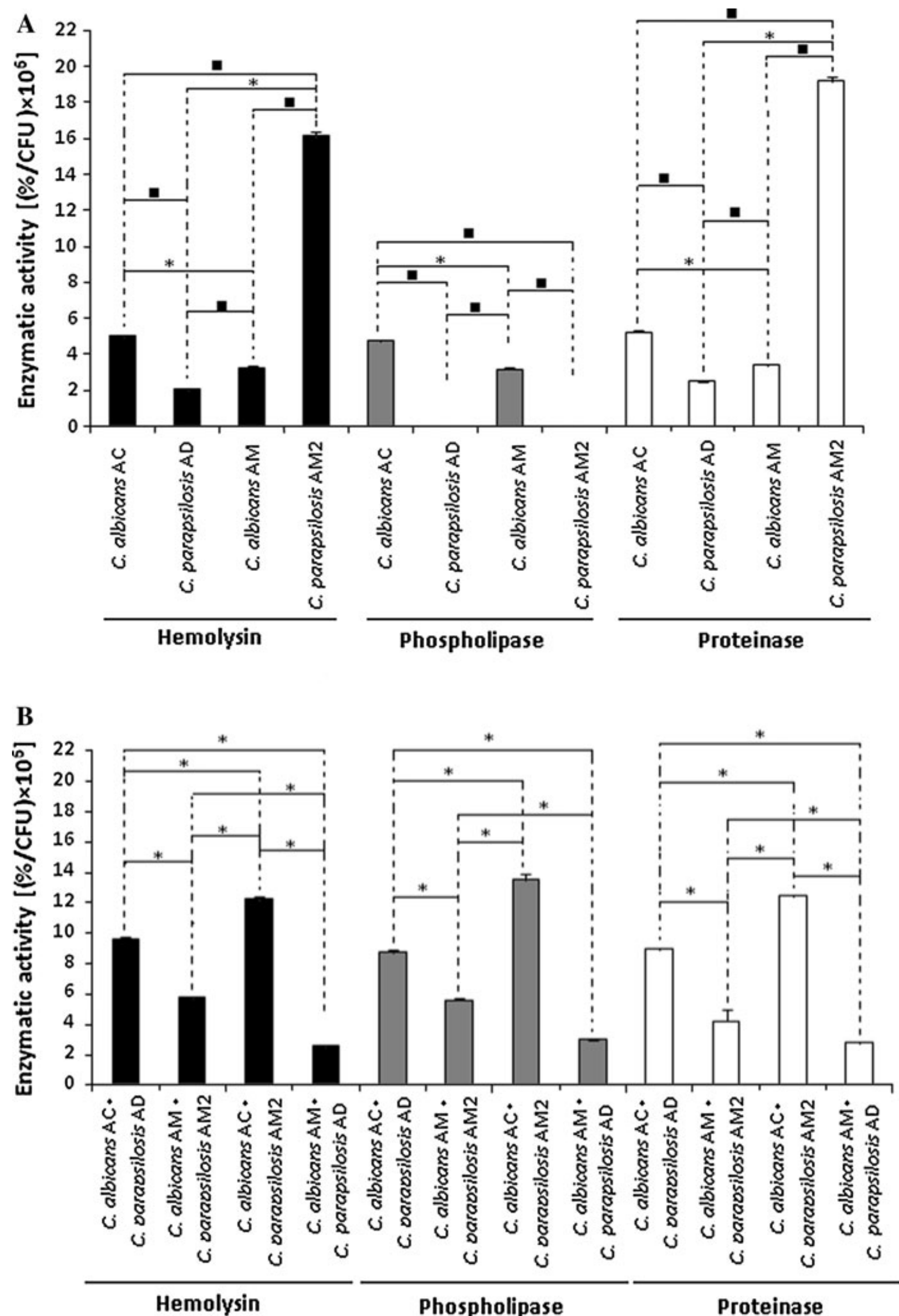

Fig. 2 Enzymatic activity [(%/CFUs) $\times$ 10^6] of *Candida* species in (**a**) mono-species and (**b**) dual-species cultures. *Error bars* represent standard error. $P < 0.05$; *strains of the same species that are significantly different in each enzyme; *filled square* statistical differences between species in each enzyme

Therefore, the role of phospholipase has been shown to be an important factor for *C. albicans* virulence [29], but until now, it is not well studied in *C. parapsilosis*.

According to the results obtained (Fig. 2a), it can be noted that *C. albicans* strains displayed a higher hemolytic and proteinase activity than *C. parapsilosis* AD ($P < 0.05$), which is in accordance with the literature [26]. Although this was not the case for *C. parapsilosis* AM2, which presented a significant higher enzymatic activity than the other strains, this result once again highlights the differences between the strains. It is interesting to notice that this high enzymatic activity decreases in the presence of *C. albicans* (Fig. 2b). Furthermore, when *C. parapsilosis* AM2 adhered in combination with *C. albicans* AM, the enzymatic activity was lower than when *C. parapsilosis* AM2 was cultured with *C. albicans* AC ($P < 0.05$). This result suggests that in the presence of *C. albicans* AM, the enzymatic activity of *C. parapsilosis* AM2 seems to be inhibited. This corroborates the hypothesis that metabolites produced by *C. albicans* AM influence the expression of *C. parapsilosis* AM2 virulence factors. Interestingly, although the number of adhered *C. parapsilosis* AM2 cells increased in the presence of *C. albicans* AM, the production of proteinase and hemolysin was significantly decreased. In dual-species cultures, enzymatic activity may be favored by the presence of *C. albicans* AC, since enzymatic activity was higher when *C. parapsilosis* strains adhered in combination with *C. albicans* AC than when they adhered in combination with *C. albicans* AM ($P < 0.05$). This result corroborates the hypothesis raised earlier that metabolites produced by *C. parapsilosis* strains do not influence *C. albicans* AM, but influence *C. albicans* AC.

It is important to highlight that there is an additive effect for hemolytic and proteinase activity in the co-culture *C. albicans* AC with *C. parapsilosis* AD, but no additive effect was observed for any of the enzymes analyzed in all other dual-species cultures studied. This is particularly evident for phospholipase activity, which was higher in dual-species cultures than the total phospholipase activity of *C. albicans* strains ($P < 0.05$). This result suggests that there is a synergism which promotes the phospholipase activity in dual-species cultures. To our knowledge, this is the first time that this synergism is reported. However, the mechanism is still unknown.

The results obtained in this study are a step forward to understand the expression of virulence factors (adhesion and enzymatic activity) in *Candida* dual-species systems.

Acknowledgments This work was supported by Portuguese Foundation for Science and Technology (FCT) through the grant SFRH/BPD/20987/2004 attributed to Cláudia Botelho.

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