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Research Article

RESEARCH ARTICLE

Candida tropicalis affects the virulence profile of *Candida albicans*: an *in vitro* and *in vivo* study

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One sentence summary: *Candida tropicalis* by reducing *Candida albicans* virulence profile may limit the ability of this pathogenic fungus to cause infection.
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ABSTRACT

Candida albicans and *Candida tropicalis* are commensal microorganisms occurring in the oral cavity of approximately 50%–70% of healthy individuals. However, these microbes can become pathogenic through changes in the environment or weakened host immune system. Thus, the aim of this investigation was to evaluate the interaction between species of the genus *Candida* in the biofilm formation, filamentation, gene expression and virulence in *Galleria mellonella*. Coincubation of *C. albicans* with *C. tropicalis* cells after 48 h resulted in significant reduction of biofilm formation by decreasing viable cell counts, metabolic activity and hyphal growth. The *C. albicans* genes (*BCR1*, *CPH1*, *EFG1*, *UME6*, *HWP1*, *ALS3*, *SAP5* and *PLB2*) were quantified by quantitative real-time polymerase chain reaction and most of genes were downregulated. Regarding *in vivo* assay, the groups that the larvae received *C. albicans* and *C. tropicalis* had a significant survival increase compared to the control group of *C. albicans* ($P = 0.0001$) in agreement with the *in vitro* results. In conclusion, *C. tropicalis* colonization was associated with a decrease in the growth of *C. albicans*, suggesting an antagonistic relation between these two species. Therefore, *C. tropicalis* by reducing *C. albicans* virulence profile may limit the ability of this pathogenic fungus to cause infection.

Keywords: *Candida albicans*; *Candida tropicalis*; biofilm; gene expression; microbial interaction; *Galleria mellonella*

INTRODUCTION

The fungal community (mycobiome) is an essential and integral component of the oral microbiome (Zaura *et al.* 2009; Ghannoum *et al.* 2010; Huffnagle and Noverr 2013). This community resides in the oral cavity and generally is composed of commensal fungi, but some of them can be pathogenic and/or opportunistic, therefore they are considered critical components in the generation of oral diseases in immunocompetent and immunocompromised individuals (Cui, Morris and Ghedin 2013; Underhill and Iliev 2014; Zakaria *et al.* 2017). In this context, factors related to the fungal pathogenicity associated with intrinsic factors in susceptible patients are responsible for the high number of *Candida* infections, which has become a disease of great importance worldwide (Colombo *et al.* 2013; Cui, Morris and Ghedin 2013; Krom, Kidwai and Ten Cate 2014; Vallabhaneni *et al.* 2016; de Mello *et al.* 2017).

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Candida genus has been the most prevalent in the oral cavity colonization and is present in 45%–65% of infants and 30%–55% of healthy adults (Millsop and Fazel 2016). *Candida albicans* is a dimorphic yeast, which can exist in both hyphal and yeast forms depending on the environment and has been isolated in more than 80% of oral lesions (Reichart, Samaranayake and Philipsen 2000; Ramesh *et al.* 2011). The pathogenicity of *C. albicans* is facilitated by the production of an arsenal of virulence factors. Specifically, the ability to switch morphologically and form biofilms play a key role on causing diseases (Nantel *et al.* 2002; Nobile and Johnson 2015; Gulati and Nobile 2016; Tsui, Kong and Jabra-Rizk 2016). *Candida tropicalis* has been widely considered the second most virulent *Candida* species, only preceded by *C. albicans*. It is considered a strong biofilm producer species and is highly adherent to epithelial and endothelial cells (Junqueira *et al.* 2011; Silva *et al.* 2012; Marcos-Zambrano *et al.* 2014; Zuza-Alves, Silva-Rocha and Chaves 2017). In addition, several studies have been reported that some *C. tropicalis* exhibit resistance to the antifungal drugs, such as the azoles derivatives, amphotericin B, and echinocandins (Choi *et al.* 2016; Seneviratne *et al.* 2016; Zuza-Alves *et al.* 2016; Zuza-Alves, Silva-Rocha and Chaves 2017).

In microbiomes, microbes rarely exist as single-species planktonic forms (Peters *et al.* 2012). Most microorganisms live in complex communities, known as polymicrobial biofilms (Galvan, Mateyca and Ielpi 2016). Interactions within these biofilms can be mutualistic, commensal, or antagonistic and microorganisms have evolved highly defined responses to sense and adapt to neighboring species (Willems, Xu and Peters 2016). Genetic diversity of biofilm communities increases the fitness of the residing community, making the species better able to survive to environmental pressures, resulting in accelerated growth, increased stress resistance, immune evasion, passive resistance and metabolic cooperation (Kolenbrander *et al.* 2010; Elias and Banin 2012; Dixon and Hall 2015; Gabrilska and Rumbaugh 2015; O'Donnell *et al.* 2015).

In this context, in order to understand the mechanisms involved when two pathogenic *Candida* species interact in the same environment, the present study evaluated the influence of *C. tropicalis* on the pathogenicity of *C. albicans* by means biofilm formation, filamentation, gene expression and virulence in *Galleria mellonella*.

MATERIALS AND METHODS

Samples

In this study, we used the reference strains *C. albicans* ATCC 18804 and *C. tropicalis* ATCC13803 and two clinical strains, *C. albicans* (Ca60) and *C. tropicalis* (Ct11), which were obtained from oral candidiasis lesions in a previous study (Junqueira *et al.* 2012). Samples were grown in a chromogenic HiCrome *Candida* medium (HiMedia, Mumbai, India) and were subsequently confirmed via molecular methods (PCR Multiplex).

Biofilm formation

Suspensions of each *Candida* sample were adjusted to 10^7 viable cells/mL using a hemocytometer. The methods used to form the monotypic and mixed biofilms were described previously (Costa *et al.* 2013). Initially, 100 μL of the standardized microorganism suspension was pipetted into the wells of 96-well microtiter plates (TPP®, Trasadingen, Switzerland) to form monotypic biofilms, and 100 μL of each species were used to form mixed biofilms. The plates were incubated with stirring at 75 rpm (Quimis, Diadema, Brazil) and 37°C for 90 min for the initial adhesion phase. After this period, the supernatant of the microorganism cultures was gently aspirated, and each well was rinsed with 200 μL of sterile 0.9% NaCl saline. The wells were washed two additional times with sterile 0.9% NaCl saline to remove non-adherent cells. Subsequently, 200 μL of YNB broth (Difco Laboratories Inc., Detroit, USA) with 100 mM glucose were pipetted into the wells, and the plates were incubated at 37°C for 48 h under stirring. After 24 h, the broth was changed. The experiments were performed in triplicate at different times.

Quantification of biofilms cell number

The quantification of cell numbers was performed following a methodology described by (Costa *et al.* 2013). After 48 h, the plate contents were aspirated and washed twice with phosphate-buffered saline (PBS). Subsequently, 200 μL of PBS were added to each well, and the biofilm was disrupted using an ultrasonic homogenizer (Vibra Cell, Sonics & Materials, Inc., Newtown, USA) for 30 s with 25% amplification. After homogenization, decimal dilutions of the biofilm suspension were made, and 100 μL aliquots of these dilutions were inoculated into *Petri* dishes containing HiCrome *Candida* medium. The plates were incubated at 37°C for 48 h. After incubation, the colonies were counted to calculate the CFU/mL.

Analysis of biofilm cell viability using the XTT colorimetric assay

The biofilms formed were evaluated by a metabolic assay based on the reduction of XTT, a tetrazolium salt (Sigma-Aldrich, São Paulo, Brazil). The methodology described by Jin *et al.* (2004) was used in this assay. Briefly, XTT salt was dissolved in PBS at a final concentration of 1 mg/mL. Immediately before each assay, a menadione (Sigma-Aldrich) solution was prepared at a final concentration of 0.4 mM and filter-sterilized. The XTT solution was thawed prior to each assay and mixed with the menadione solution at a ratio of 20:1 (v:v). Each well was washed two times with 200 μL of PBS to remove any non-adherent cells. Next, 158 μL of PBS, 40 μL of XTT and 2 μL of menadione were added to each of the pre-washed wells. The plates were incubated in the dark at 37°C for 3 h. Afterwards, 100 μL of the solution was transferred to a new well, and any colorimetric change in the solution was measured using a microtiter plate reader (Tp Reader; Thermo Plate, Shenzhen, China) at 490 nm.

Analysis of biofilm formation by SEM

Acrylic resin discs measuring 8 mm in diameter were placed on a 24-well microtiter plate (TPP®, Trasadingen, Switzerland) for biofilm formation as previously described. After biofilm formation, the specimens were fixed in 1 mL 2.5% glutaraldehyde for 1 h. The specimens were then dehydrated in an increasing ethanol series (10%, 25%, 50%, 75% and 90%) for 20 min each, followed by immersion in 100% alcohol for 1 h. The plates were kept in an oven at 37°C for 24 h to permit complete drying of the specimens.

After drying, the specimens were transferred to aluminum stubs and sputtered with gold for 160 s at 40 mA (Denton Vacuum Desk II, Denton Vaccum LLC, Moorestown, USA). The specimens were examined and photographed under a JEOL JSM5600 (JEOL USA, Inc., Peabody, USA) scanning electron microscope at the Institute of Science and Technology, UNESP—Univ. Estadual

Paulista. These experiments were performed at two different times with three biofilms per group.

Total count of *Candida* filaments

The filamentation assay was performed in 24-well microtiter plates following a methodology described previously (Ribeiro *et al.* 2017). For this assay, the following groups were evaluated: *C. albicans* + PBS (n = 5), *C. tropicalis* + PBS (n = 5) and *C. albicans* + *C. tropicalis* (n = 5). These groups were used for the references (total of n = 15) and clinical strains (total of n = 15). The experiment was performed independently in triplicate.

In a 24-well culture plate, 1 mL of distilled water was mixed with 10% fetal bovine serum and 50 μL of the standardized *C. albicans* suspension (10^7 viable cells/mL). For the experimental group, 50 μL of standardized *C. tropicalis* suspension (10^7 viable cells/mL) was also added. In the control groups, 50 μL of PBS was added to each well. The plates were incubated at 37°C under a partial pressure of 5% CO_2. After 6 h of incubation, 50 μL of the inoculum was transferred to glass slides with 10 previously demarcated fields on the back of the slide and observed under a light microscope at 40× magnification. The images were analyzed with regard to *C. albicans* morphology, and 10 microscopic fields per slide were chosen for the quantification of hyphae. The following scores were assigned for the number of hyphae present in each microscopic field: 0, no hyphae; 1, 1–10 hyphae; 2, 11–20 hyphae; 3, 21–30 hyphae; 4, 31–40 hyphae; and 5, more than 41 hyphae. The germination percentage was calculated for the mixed groups formed by *C. albicans* + *C. tropicalis* with respect to the monotypic *C. albicans* group.

Quantitative real-time polymerase chain reaction

Biofilms were formed in 24-well microtiter plates with the same concentration and conditions as in the biofilm formation section. Total RNA was isolated with the TRIzol reagent (Ambion, Inc., Carlsbad, USA) following the manufacturer's instructions. The extracted total RNA (1 μg) was treated with DNase I (Turbo DNase Treatment and Removal Reagents—Ambion Inc., Carlsbad, USA) and transcribed into complementary DNA (cDNA) using the SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR Kit (Invitrogen, Carlsbad, USA), according to the protocols recommended by the manufacturer.

The primers for all analyzed genes in the present study were described and used in the same way as indicated by Nailis *et al.* (2010) and Hnisz *et al.* (2012). Their specificity was confirmed for *C. albicans*, and not for the other species of this genus. Thus, no fragment was obtained when the primers were tested with *C. tropicalis* alone (data not show). Transcribed cDNAs were amplified for the relative quantification of *BCR1*, *CPH1*, *EFG1*, *UME6*, *HWP1*, *ALS3*, *SAP5* and *PLB2* gene expression levels in relation to the concentration of the reference gene (*ACT1*). In our study, four reference genes, *ACT1*, *PMA1*, *RIP1* and *LSC2*, were tested in all experimental groups. The results were analyzed at http://www.leonxie.com/referencegene.phpe, and the reference gene chosen was *ACT1* (data not show).

Quantitative real-time polymerase chain reaction (qPCR) was conducted using the Platinum® SYBR® Green qPCR SuperMix-UDG Kit (Applied Biosystems, Framingham, USA) in the StepOnePlus™ apparatus (Applied Biosystems). The $2^{-\Delta\Delta CT}$ method was used to analyze the relative changes in gene expression from the quantitative RT-qPCR experiment (Livak and Schmittgen 2001).

Galleria mellonella survival

For this study, the methodologies described by Mylonakis *et al.* (2005) and Rossoni *et al.* (2017) were used with some modifications. *Galleria mellonella* larvae were maintained in the laboratory as described by Jorjao *et al.* (2018) and the larvae weighing approximately 250 mg without color alterations were selected. Sixteen randomly chosen *G. mellonella* larvae were used per group in all assays. Two control groups were included in the assays that form part of this study: one group was inoculated with PBS to enable us to observe the demise of the larvae due to physical trauma, and the other received no injection as a control for general viability.

Each of the *Candida* strain suspensions was prepared from cultures in 5 mL of YPD liquid medium at 37°C for 18 h. From that, cells were centrifuged at 2000× *g* for 10 min, and the supernatant discarded. The cell pellet was dissolved in PBS and homogenized in tube shaker for 30 s. This cell-cleansing procedure was further repeated twice. Cell densities were adjusted using a hemocytometer.

For mixed infections, the larvae were infected with 1×10^6 CFU/larva of *C. albicans* and 1×10^6 CFU/larva of *C. tropicalis* in different prolegs. For single infections, the larvae also received two injections: one with 1×10^6 CFU/larva of *Candida* (*C. albicans* or *C. tropicalis*) and other with PBS. In addition, another group of single infection by *C. albicans* was made, in which the larvae were infected with 2×10^6 CFU/larva.

The *Candida* suspensions were injected into the haemolymph of each larva via last two prolegs, using a Hamilton 10 μL syringe. Cell densities of *Candida* inoculum were confirmed by CFU/mL measurements in Sabouraud Dextrose Agar. After inoculation, the larvae were stored in Petri dishes at 37°C, and the number of *G. mellonella* killed was recorded daily for a period of 5 days. The larvae were considered dead when they did not react to touch.

Statistical analysis

Student's *t*-test was used to compare the CFU/mL results from the *in vitro* biofilm formation assay, the XTT assay and the relative quantification of gene expression. The scores obtained from the *in vitro* filamentation analysis were compared using the Mann–Whitney test. Percent survival and killing curves of *G. mellonella* were plotted, and statistical analysis was performed by the Kaplan–Meier test. All analyses were performed using the GraphPad Prism 6 Program (GraphPad Software, Inc., USA), and a 5% level of significance was adopted.

RESULTS

In biofilms formed at the bottom of 96-well plates, we found that *C. albicans* exhibited higher CFU/mL in monotypic biofilms than in mixed biofilms with *C. tropicalis* (Fig. 1). The number of viable *C. albicans* cells in monotypic biofilms (7.31 Log for ATCC18804 and 7.41 Log for Ca60) was significantly different ($P > 0.0001$) from those in mixed biofilms, where *C. albicans* was reduced (5.8 Log for ATCC18804 and 6.24 Log for Ca60) in the presence of *C. tropicalis*.

In the group with mixed biofilms, the number of *C. tropicalis* was also determined (Fig. 1). The mixed biofilms showed similar CFU/mL results compared to the monotypic biofilm formed by *C. tropicalis*.

Regarding the XTT assay, the biofilms formed by *C. albicans* and *C. tropicalis* (interaction group) exhibited a significant

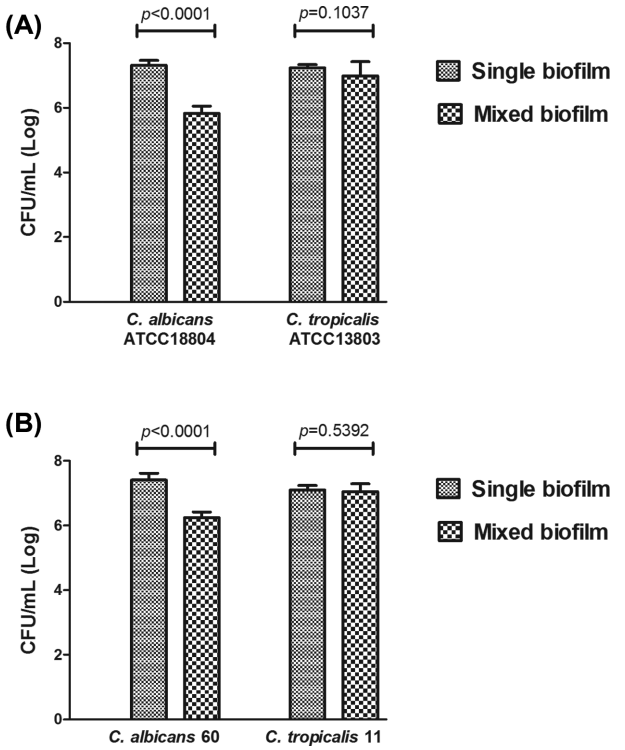

Figure 1. Quantitative analysis of *in vitro* biofilm formation by CFU/mL count. (**A**) Means and standard deviations of *C. albicans* and *C. tropicalis*, reference strains, CFU/mL (Log) values in the following groups: monotypic, formed by *C. albicans* (Control group); mixed, formed by *C. albicans* + *C. tropicalis*, monotypic, formed by *C. tropicalis;* mixed, formed by *C. albicans* + *C. tropicalis*. (**B**) Means and standard deviations of *C. albicans* and *C. tropicalis*, clinical strains, CFU/mL (Log) values for the following groups: monotypic, formed by *C. albicans* (Control group); mixed, formed by *C. albicans* + *C. tropicalis*, monotypic, formed by *C. tropicalis;* mixed, formed by *C. albicans* + *C. tropicalis*. Tukey's test, $P < 0.05$.

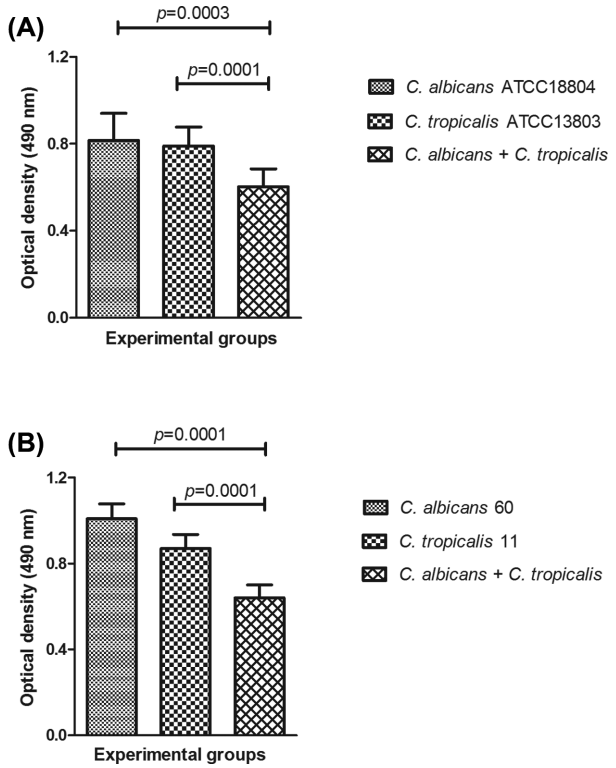

Figure 2. Means and standard deviations of the metabolic activity obtained in the XTT assay for the following groups. (**A**) Means and standard deviations of *C. albicans* and *C. tropicalis*, reference strains, metabolic activity values in the following groups: monotypic, formed by *C. albicans* (Control group); monotypic, formed by *C. tropicalis;* mixed, formed by *C. albicans* + *C. tropicalis*. (**B**) Means and standard deviations of *C. albicans* and *C. tropicalis*, clinical strains, metabolic activity values in the following groups: monotypic, formed by *C. albicans* (Control group); monotypic, formed by *C. tropicalis;* mixed, formed by *C. albicans* + *C. tropicalis*.Tukey's test, $P < 0.05$.

decrease in metabolic activity compared to the control groups formed only by *C. albicans* or *C. tropicalis* (Fig. 2).

The biofilms formed *in vitro* were also analyzed by scanning electron microscopy (SEM), in which we observed the formation of a mature biofilm on acrylic resin discs after 48 h of incubation. The biofilms formed only by *C. albicans* strains showed a thick and dense hyphal structure, but a lower number of yeast and water channels responsible for the biofilm's nutrition (Fig. 3A). The *C. tropicalis* strains produced biofilms with a hyphal structure, but a higher number of yeast compared to the *C. albicans* control group (Fig. 3B). Mixed biofilms exhibited fewer hyphae, gaps between cell clusters and a profuse distribution of yeast compared with the monotypic biofilms (Fig. 3C and D). These results demonstrated that in the mixed biofilm a reduction of hyphal formation occurred for both species.

After verifying that *C. tropicalis* reduced the formation of hyphae by *C. albicans* in biofilms formed *in vitro*, another experiment focusing on *C. albicans* filamentation was conducted. In this experiment, we verified a significant reduction on the total hyphae formation when *C. albicans* strains were incubated in the presence of *C. tropicalis* strains compared to *C. albicans* monotypic groups (Fig. 4). Although we did not differentiate the filaments of each *Candida* species analyzed in this study, there was a significant reduction in the total number of hyphae formed in the interaction groups compared to the control.

To elucidate the mechanisms involved in this interaction, we quantified the *C. albicans* genes related to the formation of biofilms, morphogenesis and hydrolytic enzymes by qPCR. All primers were specific for the species *C. albicans*, showing no amplification in *C. tropicalis*, confirming the accuracy of nucleotide sequences designed. All genes of the *C. albicans* strains were downregulated in the presence of *C. tropicalis* strains, demonstrating the inhibitory effect of *C. tropicalis* on the transcriptional profile of *C. albicans* when associated in the same environment (Fig. 5). Among all of the analyzed genes, *ALS3*, *CPH1*, *EFG1*, *HWP1* and *UME6* were the most downregulated genes with 4.0-, 7.9-, 4.0-, 3.0- and 8.5-fold decreases, respectively, in mixed biofilms formed by the clinical strains ($P = 0.0001$). These results agreed with those obtained for the CFU/mL counts and filamentation assay in the present study, which suggests that *C. tropicalis* affects biofilm formation and reduces the development of *C. albicans* filamentation by downregulating the expression of the *ALS3*, *CPH1*, *EFG1*, *HWP1* and *UME6* genes.

Based on the *in vitro* results, we studied the *in vivo C. albicans* + *C. tropicalis* interaction using *G. mellonella* model by means of the survival analysis. In the control groups of *C. albicans*, 100% of the larvae died within 24 h, showing that the infection by this species is lethal to these insects. In contrast, the groups that the larvae received *C. albicans* and *C. tropicalis* had a significant survival increase compared to the control group of *C. albicans* ($P = 0.0001$). These data show that mixed infection is less pathogenic to larvae than infection by *C. albicans* alone, in

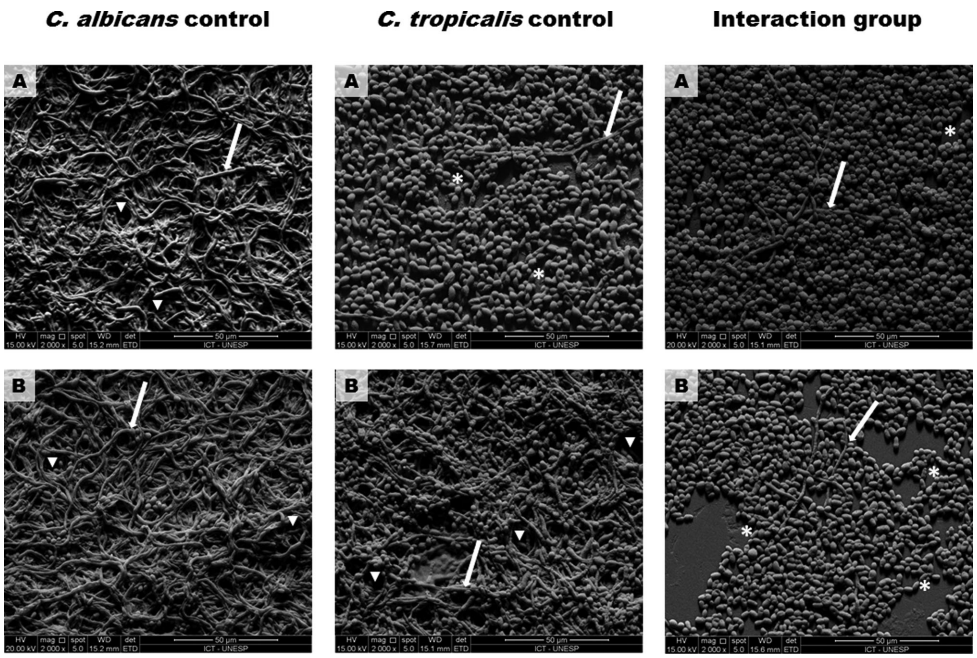

Figure 3. Representative SEM images of a mixed *Candida* biofilm compared with a monotypic *C. albicans* biofilm. (**A**) Reference strains. (**B**) Clinical strains. Note that the *C. albicans* biofilms (Control) consisted of a dense layer of hyphae whereas in mixed *Candida* biofilms single cells (yeast) predominated. Biofilms formed only by *C. tropicalis* and PBS have multiple yeasts and hyphae. Hyphae of *C. albicans* (arrow), yeasts (asterisk) and water channels (filled inverted triangle) are highlighted in the biofilm MEV. Original magnification 2000×.

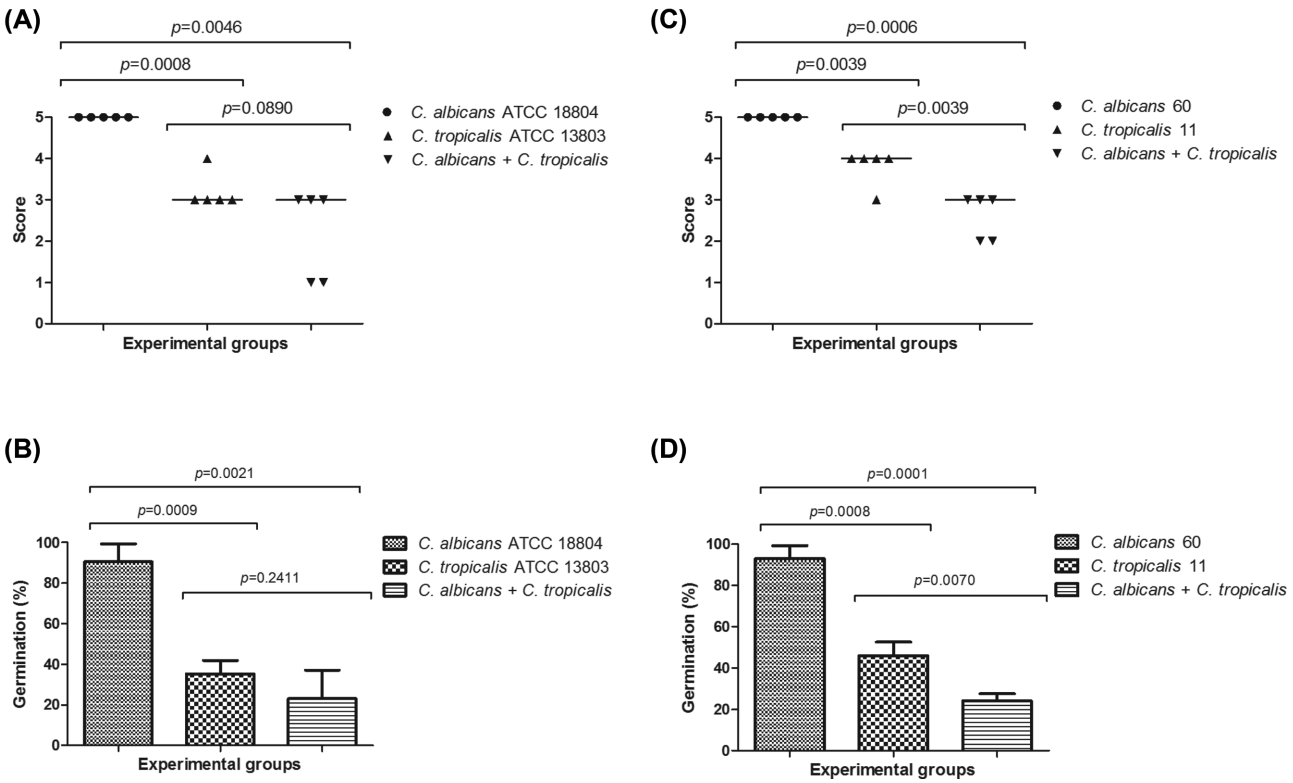

Figure 4. *Candida tropicalis* (reference and clinical strains) reduce *C. albicans* filamentation. (**A** and **C**) Median scores obtained by determining the number of hyphae in the *in vitro Candida*, filamentation assay for the following groups: *C. albicans* control group; *C. tropicalis* control group; interaction group with *C. tropicalis*. A significant hyphal reduction was observed in the interaction group compared to the control groups (Mann–Whitney test, $P \leq 0.05$). (**B** and **D**) Percentage germination, expressed as the mean values of hyphae and pseudohyphae, in the viability of *C. albicans* when associated with *C. tropicalis* relative to the control groups.

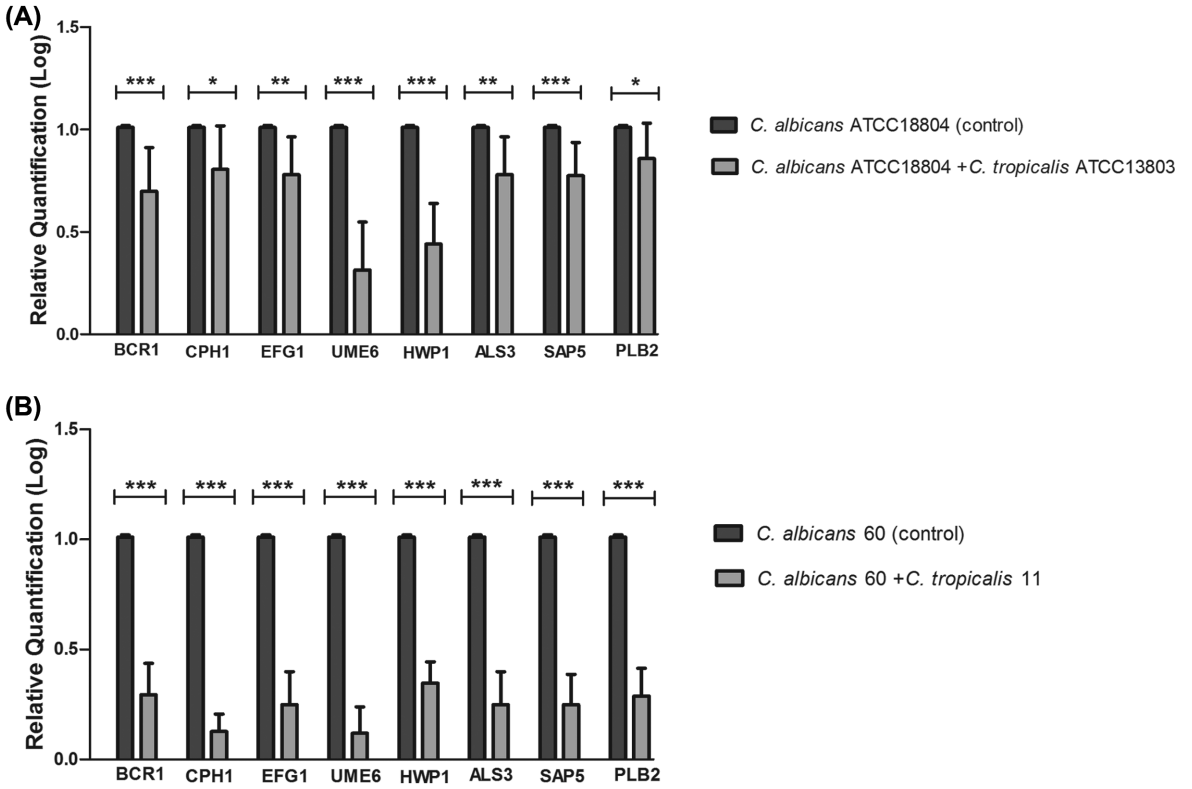

Figure 5. *Candida tropicalis* reference and clinical strains decreased the expression of *C. albicans* virulence genes. Relative quantification (Log) of *BCR1*, *CPH1*, *EFG1*, *UME6*, *HWP1*, *ALS3*, *SAP5* and *PLB2* genes in monotypic and mixed biofilms formed by *C. albicans* reference (**A**) and clinical (**B**) strains. The units in the Y-axis were calculated based on the $2^{-\Delta\Delta CT}$ method, and are expressed as the means and standard deviations. Each gene was normalized and compared with the expression of monotypic biofilms of *C. albicans* (Control groups) using the reference gene β-actin. Student's *t*-test was used to compare gene expression between the groups ($P \leq 0.05$).

agreement with *in vitro* results. Regarding the control groups of *C. tropicalis*, the clinical strain killed 100% of the larvae within 120 h of infection. On the other hand, the larvae infected by the reference strain presented a survival of up to 30% in the same period evaluated (Fig. 6).

DISCUSSION

The different ecological relationships existing in the oral microbiome are critical components of health and disease. Changes in the resident microbiota may indicate, trigger, or influence the course of diseases, especially among immunocompromised patients (e.g. patients with HIV or cancer) (Jenkinson and Lamont 2005). Although fungi, particularly Candida, are important components of the oral microbiome, most studies have focused on the bacterial components (Ghannoum *et al.* 2010; Peleg, Hogan and Mylonakis 2010). The full extent of the microbial diversity of the oral cavity and the microbial interactions in this environment can affect the survival, competitiveness and morphogenesis of C. albicans. In this context, we aimed to evaluate the effects of *C. tropicalis* on the pathogenicity of *C. albicans* by *in vitro* and *in vivo* methods.

In an attempt to understand the effects of *C. tropicalis* on the growth of *C. albicans* in mixed biofilms, we used three different assays to evaluate the biofilms formed *in vitro*: CFU/mL count, XTT assay and SEM. In the CFU/mL analyses, the results showed a lower *C. albicans* count in the mixed biofilms compared to the monotypic biofilms, indicating that *C. tropicalis* was able to limit the growth of *C. albicans*. These findings were consistent with the results published by Santos *et al.* (2016) demonstrating a reduction in *C. albicans* in mixed biofilms formed with *C. tropicalis* by CFU/mL count.

In XTT assay, we observed that mixed *C. albicans* + *C. tropicalis* biofilms exhibited significantly different metabolic activity after 48 h compared to the monotypic *C. albicans* group. The analyzed *C. tropicalis* strains formed a large number of biofilms in all the assays. In agreement with previous studies, *C. tropicalis* was more successful at forming biofilms compared with non-*albicans Candida* species (Silva *et al.* 2012; Marcos-Zambrano *et al.* 2014; Martins *et al.* 2016).

SEM observations were used to analyze particular morphologies of *C. albicans* in mixed biofilms with *C. tropicalis*. In monotypic *C. albicans* biofilms, we found a thick and dense hyphal structure but a lower number of blastospores, in agreement with previous studies (Ramage *et al.* 2005; Barbosa *et al.* 2016; Martins *et al.* 2016). However, the mixed biofilms formed by *C. albicans* and *C. tropicalis* showed a large number of blastoconidia and very rarely hyphae. In the case of *C. tropicalis* control group, a dense network of yeast cells with evident filamentous morphologies was found, which usually characterizes mature biofilms. SEM images tended to confirm the CFU/mL counts and XTT assay, and these may suggest that competitive inhibition may occur even in the initial step of adhesion onto a substrate during dual-species *Candida* biofilm development.

In previous study, competitive interactions were also observed when *C. albicans* was associated with *Pichia* (yeast) in HIV-infected patients undergoing oral analysis mycobiome (Mukherjee *et al.* 2014). These authors showed that the anti-*Candida*

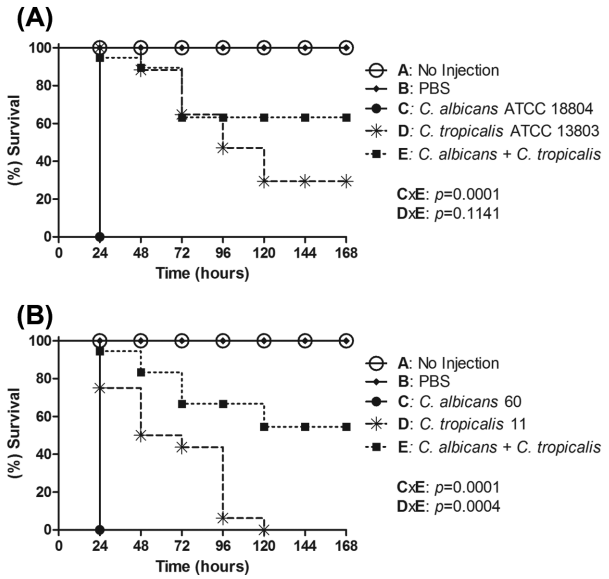

Figure 6. *Candida tropicalis* + *C. albicans* infection is less pathogenic to larvae than infection by *C. albicans* alone. (**A**) There was a significant difference between the *C. tropicalis* + *C. albicans* and *C. albicans* control group ($P = 0.0001$). (**B**) There was a significant difference between the *C. tropicalis* + *C. albicans* and *C. albicans* control group ($P = 0.0001$) and between the *C. tropicalis* + *C. albicans* and *C. tropicalis* control group ($P = 0.0004$). Larvae exhibited a greater survival of approximately 50% to the C. tropicalis + *C. albicans* group compared to the *C. albicans* control group. Log-rank test, $P \leq 0.05$.

activity of *Pichia* is mediated by inhibition of *Candida* growth and virulence factors like germination, adherence and biofilm formation. Moreover, it is well known that interfering with virulence factors decreases the ability of *Candida* to cause infection and this activity also was validated *in vivo* using an experimental murine model of oral candidosis.

To investigate how *C. tropicalis* affects morphogenesis and biofilm formation in *C. albicans*, we also performed the filamentation assays and the quantification of some genes related to these functions. Filamentation is a major contributor to biofilm development and pathogenesis; we found that the total filamentation in mixed groups were reduced compared to the control groups. However, it was not possible to differentiate the hyphae formed by *C. albicans* or *C. tropicalis* in mixed cultures. The ability to switch between proliferation as yeast cells and development into hyphae is a hallmark of *C. albicans* (Maiti *et al.* 2015; Mendelsohn *et al.* 2017). Genetic analysis has elucidated at least part of the regulatory pathways that link extracellular stimuli to morphogenesis (Sudbery 2011). In *C. albicans*, a number of transcription factors were identified that can influence filamentous growth, including *CPH1*, *EFG1*, *CPH2*, *TEC1*, *RIM101*, *NRG1* and *UME6* (Nantel *et al.* 2002; Sudbery 2011; Maiti *et al.* 2015; Gulati and Nobile 2016; Mendelsohn *et al.* 2017). Some of these transcription factors were found to be targets of hypha-inducing signal transduction pathways (Liu 2001).

In our study, we quantified the expression of the *ALS3*, *BCR1*, *CPH1*, *EFG1*, *HWP1* and *UME6* genes in mixed biofilms with *C. tropicalis*. All of these genes were significantly downregulated compared to monotypic biofilms, demonstrating the reduction effect of *C. tropicalis* on morphogenesis and biofilm formation in *C. albicans*. The transcription factor *UME6* lies at the heart of hyphal regulation in *C. albicans* as a necessary and sufficient factor for hyphal growth and as a key transcriptional target for the various signal transduction pathways that mediate hyphal induction stimuli (Carlisle *et al.* 2009; Zeidler *et al.* 2009). Regarding *HWP1* gene, Orsi *et al.* (2014) demonstrated that this gene is involved in the formation of germ tubes and hyphal forms and thus promotes physical contact between the epithelial cells and the fungi; they concluded that *HWP1* is an important effector of *C. albicans* pathogenicity. Moreover, we can highlight the *EFG1* gene, a transcription factor required for biofilm formation. This gene is involved in the regulation of filamentation and biofilm formation, since the deletion of both alleles is critical for these factors (Nobile *et al.* 2008). In this study, *C. tropicalis* reduced the expression of some critical genes in the morphogenesis and formation of biofilms of *C. albicans*, thus affecting virulence factors essential to the pathogenesis of this yeast. Therefore, *C. tropicalis*, by reducing *C. albicans* virulence factors may limit the ability of this pathogenic fungus to cause infection.

The *C. albicans* + *C. tropicalis* interaction was also studied *in vivo* model, we investigated the pathogenicity of these strains in *G. mellonella* model. Firstly, we found that infection by *C. albicans* alone is lethal to larvae within 24 h. These data are similar with previous studies that evaluated the pathogenicity of *C. albicans* in this model (Cotter, Doyle and Kavanagh 2000; Junqueira *et al.* 2011; de Barros *et al.* 2017). In contrast, the larvae that received *C. albicans* and *C. tropicalis* had a significant survival increase compared to the control group of *C. albicans*. These data suggest that *C. tropicalis* may release some metabolite capable of reducing the virulence of *C. albicans* making the infection less lethal to infected larvae, which is consistent with the results obtained in biofilms formed in vitro here presented.

The present study is the first *in vivo* study that attempts to investigate the interactions between *C. albicans* and *C. tropicalis* in the development of experimental candidiasis. However, further studies on molecular mechanisms and molecules identification involving *Candida*/*Candida* interaction should be done in order to understand the metabolic pathway and discover new substances that modulate the virulence of *C. albicans*. In this context, we concluded that the results showed that *C. tropicalis* exerts antagonistic effects on the biofilm formation and morphogenesis of *C. albicans*, in addition to interfering with its transcriptome and virulence profile in *G. mellonella* model.

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Conflict of Interest. None declared.

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