Archives of Oral Biology 64 (2016) 92–101

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/aob

Influence of *Candida krusei* and *Candida glabrata* on *Candida albicans* gene expression in *in vitro* biofilms

Patrícia Pimentel Barros*, Felipe Camargo Ribeiro, Rodnei Dennis Rossoni, Juliana Campos Junqueira, Antonio Olavo Cardoso Jorge

Departament of Biosciences and Oral Diagnosis, Institute of Science and Technology, UNESP—Univ. Estadual Paulista, São José dos Campos, Brazil

A R T I C L E I N F O

Article history:
Received 5 May 2015
Received in revised form 17 December 2015
Accepted 12 January 2016

Keywords:
Yeast
Mixed biofilms
Virulence genes
Competitive interaction

A B S T R A C T

Objective: The present study aimed to evaluate the interactions between the species *Candida albicans*, *Candida krusei* and *Candida glabrata* in monotypic and mixed biofilm models formed *in vitro* as well as the relative expression of the *ALS1*, *ALS3*, *HWP1*, *BCR1*, *EFG1*, *TEC1*, *SAP5*, *PLB2* and *LIP9* genes.
Material and methods: Mixed (*C. albicans*/*C. krusei* and *C. albicans*/*C. glabrata*) and monotypic biofilms were cultured for 0, 12 and 24 h. Gene expression was analyzed in the same biofilm model in which the number of CFU/mL was counted.
Results: The *C. albicans* CFU/mL values were lower at the 12 and 24 h time points in the mixed biofilms compared with the monotypic biofilms, and decreases of 56.23% and 64.4% in *C. albicans* were observed when this species was associated with *C. glabrata* and *C. krusei*, respectively. In the presence of *C. krusei*, the expression of the *ALS3*, *HWP1*, *BCR1*, *EFG1* and *TEC1* genes of *C. albicans* was completely inhibited, indicating both transcriptome and the phenotypic antagonism between these two species, but genes related to the secretion of enzymes were stimulated. In the presence of *C. glabrata*, *C. albicans* showed a similar gene expression profile to that obtained in association with *C. krusei*, though it was altered to a lesser degree.
Conclusion: We conclude that *C. krusei* and *C. glabrata* may alter or inhibit the mechanisms involved in the *in vitro* adherence and formation of *C. albicans* biofilms, influencing the pathogenicity of this species and suggesting a competitive interaction with *C. krusei* and *C. glabrata* in biofilm formation.
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1. Introduction

The *Candida* genus can be found in approximately 25–75% of healthy individuals as a commensal organism, neither causing any apparent damage nor inducing inflammation in surrounding tissues. In fungal infections caused by these yeasts, *Candida albicans* is the predominant species found in the oral cavity of humans, representing 50–70% of clinical isolates (Hube, 2004; Mayer, Wilson, & Hube, 2013). Recently, the incidence of infections by non-albicans species, especially *Candida glabrata*, *Candida krusei*, *Candida parapsilosis* and *Candida tropicalis*, has increased significantly. Junqueira et al. (2012) collected samples from the oral cavities of 60 HIV-positive patients and found that *C. albicans* was the most prevalent species, representing approximately 51% of the isolates. In addition, 42% of these patients presented mixed *Candida* infections, formed by associations between *C. albicans* and *C. krusei*, *C. albicans* and *C. glabrata*, or *C. albicans* and *C. tropicalis*. Despite this increase in infections by non-*albicans* species, little is known about the ecological interactions between different species of this genus.

The study of microbial interactions in a given niche is extremely important for obtaining knowledge of the pathogenicity of microbes in the host and for the development of effective treatments without relapses. Few studies have evaluated the interactions between *C. albicans* and non-*albicans* species (Agwu et al., 2012; Cenci et al., 2008; El-Azizi, Starks, & Khardori, 2004; Kirkpatrick, Lopez-Ribot, McAtee, & Patterson, 2000; Thein, Samaranayake, & Samaranayake, 2007; Thein, Seneviratne, Samaranayake, & Samaranayake, 2009). According to Thein et al. (2007) high *C. krusei* concentrations are able to inhibit *C. albicans* in *in vitro* biofilm models, which reveals an antagonistic relationship between these two species. In contrast, Cenci et al. (2008) found no competitive relationship between *C. albicans* and *C. glabrata* in

* Corresponding author at: Departament of Biosciences Oral Diagnosis, Institute of Science and Technology, UNESP—Univ. Estadual Paulista, Av. Engenheiro Francisco José Longo 777, São José dos Campos CEP: 12245-000, SP, Brazil.
Fax: +55 12 39479010.
E-mail addresses: barrosdnapp@yahoo.com.br, patricia.barros@ict.unesp.br (P.P. Barros).

http://dx.doi.org/10.1016/j.archoralbio.2016.01.005
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biofilms that formed on the surface of dental materials. Given that all of these studies were carried out by observing only phenotypic aspects of the interactions between *Candida* species, little is currently known about the behavior of *C. albicans* genes in the formation of mixed biofilms.

C. albicans exhibits specific disease development mechanisms that overcome the defenses and allow the colonization of mucosal tissue. The expression of *C. albicans* virulence factors may vary depending on the type of infection, whether local or systemic, the stage of the disease and the response of the host (Naglik et al., 2003). The ability to form biofilms in catheters and prostheses (abiotic) and on mucosal surfaces (biotic) is one of the most important characteristics of this species because it facilitates the adhesion, proliferation and the spreading of this microorganism to other infection sites. Biofilm formation is a complex sequential process that includes several stages: adhesion of yeast cells to a substrate, proliferation of these cells, the formation of hyphae on the most superficial layer of the biofilm, the production and accumulation of extracellular matrix and, ultimately, dispersion of these cells (Nobile and Mitchell, 2006).

Mature biofilms are far more resistant to antifungal therapy and immunological factors of the host compared with planktonic yeast cells (Fanning and Mitchell, 2012). The ability to form biofilms has been associated with the presence of transcriptional regulatory genes in *C. albicans* (Finkel and Mitchell, 2011). These genes include *BCR1*, *TEC1* and *EFG1*. The *ALS* (agglutinin-like sequence) family of adhesins, which includes eight members (*ALS1*–*ALS9*) are expressed from *ALS* genes encoding glycosylphosphatidylinositol (GPI)-anchored cell surface glycoproteins. Within this group, *ALS3* is the most important gene because it actively contributes to biofilm formation and is positively regulated (highly expressed) during *in vitro* infection of epithelial cells of the oral mucosa (Murciano et al., 2012; Zordan & Cormack, 2012). Other important proteins involved in the process of adhesion and invasion have been described in the literature, such as *HWP1*, *EAP1*, *SSA1* and *SAPS* (Wächtler et al., 2001). Other *C. albicans* genes include proteases (SAPs), lipases (LIP) and phospholipases (PLBs), which contribute to colonization and infection *via* degrading components of the host cell membrane (Barros et al., 2008).

Because few studies have addressed the interactions between different species of the *Candida* genus and the importance of determining the specific mechanisms underlying the development of the disease and the high incidence of these lesions in immunocompromised patients, the aim of the present study was to evaluate the interactions between *C. albicans*, *C. krusei* and *C. glabrata* in monotypic and mixed biofilm models formed *in vitro*. Furthermore, we assessed the relative expression of the *ALS1*, *ALS3*, *HWP1*, *BCR1*, *EFG1*, *TEC1*, *SAP5*, *PLB2*, and *LIP9* genes between these species.

2. Materials and methods

2.1. Samples

The samples used in the present study were isolated from a single HIV-infected patient with oropharyngeal candidiasis lesions induced by an association between *C. albicans*, *C. krusei* and *C. glabrata*. The samples were collected at the Emílio Ribas Infectious Diseases Institute (*Instituto de Infectologia Emílio Ribas*) under the approval of the Ethics Committee (Protocol 051/2009-PH/CEP) and were stored in the Microbiology Laboratory of the Institute of Science and Technology of São José dos Campos/São Paulo State University (*Universidade Estadual Paulista* "*Júlio de Mesquita Filho*"—UNESP). The samples were grown in a chromogenic *HiCrome Candida* medium (Himedia, Mumbai, India), and the species they contained were identified using biochemical methods (API20C System-BioMérieux, Paris, France) by Junqueira et al. (2012) and subsequently confirmed *via* molecular methods (PCR Multiplex).

2.2. Biofilm formation

Suspensions of each *Candida* sample were prepared from overnight cultures performed in 5 mL of YNB broth (Difco Laboratories Inc., Detroit, MI, USA) at 37 °C for 18 h. Subsequently, the cells were centrifuged at 2.000 × *g* for 10 min, and the supernatant was discarded. The pellet was resuspended in 0.9% NaCl and mixed in a tube mixer for 30 s. The cells were washed two additional times. The cell densities were adjusted to 10^7 viable cells/mL using a hemocytometer.

To form the monotypic and mixed biofilms, the methods described by Seneviratne, Silva, Jin, Samaranayake, and Samaranayake (2009) and Costa, Pereira, Freire, Junqueira, and Jorge (2013) were applied, with some modifications. 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 was used to form mixed biofilms. The plates were incubated with stirring at 75 rpm (Quimis, Diadema, São Paulo) at 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, MI, USA) with 100 mM glucose were pipetted into the wells, and the plates were incubated at 37 °C for 0, 12 or 24 h under stirring (Quimis, Diadema, São Paulo). For the 0 h time point, the biofilm was removed after the initial adhesion phase. The experiments were performed in triplicate at different times, with 10 repetitions, totaling 270 assays.

2.3. Quantification of biofilm by viable cell count (CFU/mL)

Following biofilm formation, the contents of the plates were aspirated and washed twice with sterile 0.9% NaCl saline. Subsequently, 200 μL of sterile 0.9% NaCl saline was transferred to each well, and the biofilm adhered to the bottom of the plate was disrupted *via* homogenization for 30 s in an ultrasonic homogenizer (Vibra Cell—Sonics & Materials, Inc., Newtown, USA) with 25% amplification. A 100-μL volume of the inoculum was transferred to 1.5-mL microtubes containing 900 μL of sterile 0.9% NaCl saline. From the solution obtained in the microtubes, decimal dilutions of the biofilm suspension were performed, and 100-μL aliquots of these dilutions were inoculated into *Petri* dishes containing chromogenic HiCrome *Candida* medium (Himedia, Mumbai, India), followed by incubation of the plates at 37 °C for 48 h. After this time, the density of the yeast in CFU/mL was calculated. The *Candida* species in the mixed biofilms were differentiated based on the color of the colony using HiCrome *Candida* medium: light green for *C. albicans*, off-white for *C. glabrata* and purple for *C. krusei*.

2.4. Quantitative RT-PCR

Total RNA was extracted using a TRIzol kit (Ambion, Inc., Carlsbad, CA, USA) as recommended by the manufacturer. A 1.0 mL volume of TRIzol was added to a 2.0-mL microtube containing the collected yeast, followed by incubation at room temperature (RT) for 10 min. Subsequently, 200 μL of chloroform (Sigma–Aldrich, St. Louis, MO, USA) was added, and the microtubes were centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatant was then transferred to a new microtube, and 500 μL of isopropanol (Sigma–Aldrich, St. Louis, MO, USA) was added. After

centrifugation, the obtained pellet was washed with 70% ethanol (Sigma–Aldrich, St. Louis, MO, USA), centrifuged again and resuspended in 50 μL of RNA Storage buffer (Ambion Inc., Carlsbad, CA, USA). The concentration, purity and quality of the RNA was verified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and through agarose gel electrophoresis (Invitrogen™, Carlsbad, CA, USA) with staining using ethidium bromide (Invitrogen™, Carlsbad, CA, USA) and visualization on a transilluminator.

The extracted total RNA (2 μg) was treated with DNase I (Turbo DNase Treatment and Removal Reagents—Ambion Inc., Carlsbad, CA, USA) and transcribed into complementary DNA (cDNA) using the SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR Kit (Invitrogen™, Carlsbad, CA, USA), according to the protocols recommended by the manufacturer.

The primers for all genes analyzed in the present study were described and used in the same way as indicated by Nailis, Coenye, Van Nieuwerburgh, Deforce, and Nelis (2006) and Nailis et al. (2010) and Hnisz, Bardet, Nobile, Petryshyn, and Glaser (2012). Their specificity was confirmed for *C. albicans*, and not for the other species of this genus.

The transcribed cDNAs were amplified for relative quantification of the expression of the *EFG1*, *TEC1*, *BCR1*, *ALS1*, *ALS3*, *HWP1*, *SAP5*, *LIP9* and *PLB2* genes in relation to the concentration of the reference gene (*ACT1*). In the present study, four reference genes, *ACT1*, *PMA1*, *RIP1* and *LSC2*, were tested in all experimental groups. The obtained results were analyzed at http://www.leonxie.com/referencegene.phpe, and the selected reference gene was *ACT1*.

The qPCR method was applied to evaluate the amount of the cDNA product in the exponential phase of the amplification reaction. As a detection system, the SYBR® Green fluorophore (Platinum® SYBR® Green qPCR SuperMix-UDG Applied Biosystems, Framingham, MA, USA) was used, in the following reaction mixture: 12.5 μL of Super mix Platinum SYBR Green, 1 μL of ROX (reference dye), 300 nM of the forward primer, 300 nM of the reverse primer, 3.4 μL of cDNA solution (diluted 1:5) and 2.1 μL of DEPEC water (Invitrogen™, Carlsbad, CA, USA), to obtain a final volume of 20 μL in each well of a 96-well plate (Invitrogen™, Carlsbad, CA, USA). As a negative control for the reaction, all of the reagents were added to the last wells of the plates except for cDNA, and the wells were sealed with optical adhesive (Invitrogen™, Carlsbad, CA, USA). Subsequently, the plate was placed in a StepOnePlus™ System (Applied Biosystems, Framingham, MA, USA) device, and the following cycling parameters were used: 50 °C for 2 min, followed by an initial denaturation at 95 °C for 2 min and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. After the end of the last cycle, the samples were subjected to dissociation (melting) curve analysis, and the absence of any bimodal curve or abnormal amplification signal was observed and analyzed every 0.1 °C. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative changes in gene expression from the quantitative RT-PCR experiment (Livak and Schmittgen, 2001).

2.5. Statistical analysis

The data are expressed as the means ± standard deviation (SD) of the results obtained in each experimental group and for each time point of biofilm development and were analyzed for a normal distribution using the Kolmogorov–Smirnov test (SigmaPlot™ Systat Software, Inc., San Jose, CA, USA). The data obtained in the CFU/mL assay showed a normal distribution compared with the control (0 h time monotypic biofilm) and were analyzed using analysis of variance (ANOVA) and Tukey test. The statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc., California, CA, USA). *P* values <0.05 were considered significant (Tables 1 and 2).

3. Results

When counting the CFU/mL of the yeast in biofilms formed *in vitro* at the 12 and 24 h time points, we observed that *C. albicans* showed higher CFU/mL values in monotypic biofilms compared with mixed biofilms associated with *C. krusei* and *C. glabrata* (Fig. 1). These data suggest that *C. albicans* establishes competitive relationships with *C. krusei* and *C. glabrata* during biofilm formation. More specifically, when comparing the results obtained from mixed *versus C. albicans* monospecies biofilms, we observed reductions of 52.6% and 64.4% for *C. albicans* in association with *C. krusei* at 12 and 24 h, respectively, while in association with *C. glabrata*, reductions of 69.8% and 56.23% were observed. The numbers of CFU/mL in the *C. albicans* monotypic biofilms were 6.40 ± 0.19, 6.92 ± 0.24 and 6.77 ± 0.09 $\log_{10}$ for the 0, 12 and 24 h time points of biofilm formation, respectively. Compared with the 0 h time point (control), the values obtained at 12 and 24 h showed significant differences ($p = 0.001$). In the presence of *C. krusei*, 6.39 ± 0.14, 6.72 ± 0.16 and 6.29 ± 0.19 CFU/mL were recorded, and in the presence of *C. glabrata*, 6.24 ± 0.09, 6.52 ± 0.16 and 6.39 ± 0.14 CFU/mL were recorded at the 0, 12 and 24 h time points, respectively. Significant values ($p = 0.001$) were found for the association with *C. glabrata* at 12 h and for those both with *C. krusei* and *C. glabrata* at 24 h ($p = 0.0182$). There was also an increase in the CFU/mL of *C. albicans* in both the monotypic and mixed biofilms observed at the 12 h time point, indicating that this yeast had reached the log phase of the growth curve.

For the analysis of gene expression, the primers employed in this study were specific for the targeted *C. albicans* genes, amplifying in the expected PCR fragment, which were subjected to agarose gel electrophoresis to confirm the molecular weight (Fig. 1—Supplementary material). The efficiency of PCR amplification was between 95% and 100% for each primer tested, indicating validation of the primers and the standardization of the qPCR assays. The analysis of the obtained melting curves confirmed the presence of a single peak, demonstrating the specificity of the tested primers.

Table 1
Values of relative quantification (Log) and standard deviation for all genes analyzed in monotypic and mixed biofilms of *Candida albicans* clinical strain 60 at 12 h.

Groups	*ALS1*	*ALS3*	*HWP1*	*BCR1*	*TEC1*	*EFG1*	*SAP5*	*LIP9*	*PLB2*
Control	1.0^{A}	1.0^{A}	1.0^{A}	1.0^{A}	1.0^{A}	1.0^{A}	1.0^{A}	1.0^{A}	1.0^{A}
Ca12h	0.03 ± 0.08^{B}	3.81 ± 1.30^{B}	16.41 ± 4.47^{B}	1.95 ± 0.53^{B}	2.81 ± 0.40^{B}	1.49 ± 0.24^{B}	3.49 ± 0.92^{B}	3.73 ± 1.10^{B}	2.30 ± 0.50^{B}
Ca/Ck12h	0.30 ± 0.64^{C}	0.07 ± 0.26^{C}	0.13 ± 0.68^{C}	0.23 ± 0.32^{C}	0.23 ± 0.03^{C}	0.07 ± 0.026^{C}	9.45 ± 3.99^{C}	8.74 ± 2.28^{C}	2.99 ± 1.03^{C}
Ca/Cg12h	0.39 ± 0.22^{D}	3.87 ± 0.97^{D}	13.7 ± 5.16^{D}	1.94 ± 0.37^{D}	1.45 ± 0.43^{D}	0.86 ± 0.16^{D}	18.75 ± 3.15^{D}	2.06 ± 0.70^{D}	3.89 ± 1.16^{D}

Control: time immediately after adherence (0 h); A, B, C and D statistically significance difference (ANOVA and Tukey test $p < 0.05$). Ca—*C. albicans*; Ck—*C. krusei*; Cg—*C. glabrata*.

Table 2
Values of relative quantification (Log) and standard deviation for all genes analyzed in monotypic and mixed biofilms of *Candida albicans* clinical strain 60 at 24 h.

Groups	*ALS1*	*ALS3*	*HWP1*	*BCR1*	*TEC1*	*EFG1*	*SAP5*	*LIP9*	*PLB2*
Control	1.0^{A}	1.0^{A}	1.0^{A}	1.0^{A}	1.0^{A}	1.0^{A}	1.0^{A}	1.0^{A}	1.0^{A}
Ca24h	0.45 ± 0.10^{B}	2.82 ± 1.11^{B}	27.59 ± 1.81^{B}	1.64 ± 0.35^{B}	0.87 ± 0.19^{A}	0.69 ± 0.05^{B}	14.86 ± 2.9^{B}	2.40 ± 0.51^{B}	1.30 ± 0.28^{B}
Ca/Ck24h	0.96 ± 0.34^{A}	0.15 ± 0.05^{C}	0.19 ± 0.85^{C}	0.90 ± 0.32^{A}	1.03 ± 0.23^{A}	0.22 ± 0.04^{C}	6.33 ± 3.0^{C}	6.89 ± 1.96^{C}	3.04 ± 0.57^{C}
Ca/Cg24h	0.27 ± 0.07^{C}	1.36 ± 0.86^{A}	5.28 ± 1.21^{D}	0.44 ± 0.16^{C}	0.26 ± 0.48^{B}	0.18 ± 0.05^{D}	10.36 ± 3.8^{D}	4.60 ± 1.20^{D}	2.55 ± 0.44^{D}

Control: time immediately after adherence (0 h); A, B, C and D statistically significance difference (ANOVA and Tukey test $p < 0.05$). Ca—*C. albicans*; Ck—*C. krusei*;Cg—*C. glabrata*.

The expression levels of adhesion genes (*ALS1*, *ALS3* and *HWP1*), transcriptional regulatory genes (*TEC1*, *BCR1* and *EFG1*), and hydrolase genes (*SAP5*, *PLB2* and *LIP9*) were quantified in cells from the Ca60 sample at the 0, 12 and 24 h time points during the development of monotypic and mixed biofilms using quantitative real-time PCR (qPCR), as shown in Figs. 2–4 .

In the association with *C. krusei*, the *ALS1*, *ALS3*, *HWP1* and *EFG1* genes were downregulated; however, the *SAP5*, *PLB2* and *LIP9* genes were upregulated at 12 and 24 h, showing significant differences ($p = 0.001$) compared with control. The *TEC1* and *BCR1* genes were also downregulated, but these differences were significant only at the 12 h time point. Among all of analyzed genes, *ALS3* and *HWP1* were the most downregulated genes in the presence of *C. krusei*, in agreement with the results obtained for the CFU/mL counts in the present study, which suggests that *C. krusei* affects the adhesion of *C. albicans* during biofilm formation and reduces the development of *C. albicans* filamentation.

In the association with *C. glabrata*, the *ALS3*, *HWP1*, *SAP5*, *PLB2* and *LIP9* genes were upregulated at 12 and 24 h, and the *TEC1* and *BCR1* genes were upregulated only at 12 h compared with the control. All of the values were significant ($p = 0.001$). The *ALS1* and *EFG1* genes were downregulated at 12 and 24 h, and the *TEC1* and *BCR1* genes were downregulated at 24 h; all values were significant. Most of the *C. albicans* genes analyzed in the present study were stimulated in the presence of *C. glabrata*, in agreement with the results obtained for the CFU/mL counts, as the reduction of *C. albicans* was smaller in the presence of *C. glabrata* compared with *C. krusei*.

4. Discussion

In nature, biofilms are generally composed of a multitude of microbial species. This is particularly true of the oral cavity, where more than 200 microbial species coexist in a unique habitat. *Candida* species are oral commensals found in 50–60% of the population either as transient or permanent colonizers (Samaranayake, 2006). Oral carriage of more than one yeast species is relatively frequent. For instance, Samaranayake, MacFarlane, and Williamson (1987) in a pioneering study showed an incidence of 15% multiple species *Candida* carriage intraorally in a cohort of dental hospital patients. Subsequent workers have also shown that multispecies *Candida* colonization is becoming an increasing problem especially in debilitated patients with malignancies and bone marrow transplant recipients and those with candidemia (Agwu et al., 2012; Cenci et al., 2008; Junqueira et al., 2012; Thein et al., 2007, 2009). This study is unprecedented in the literature because we evaluate the interaction of different *Candida* species in biofilm formation and confirmed with gene expression at different times.

The present study evaluated the influence of *C. krusei* and *C. glabrata* when associated with *C. albicans* on a model of *in vitro* biofilm production at 12 and 24 h based on CFU/mL counts and quantification of the *ALS1*, *ALS3*, *HWP1*, *BCR1*, *EFG1*, *TEC1*, *SAP5*, *PLB2*, and *LIP9* genes of *C. albicans*, which contribute to the development of diseases and to protection against host defenses, allowing the invasion and destruction of colonized tissue.

Regarding the obtained *C. albicans* CFU/mL counts, higher CFU/mL values were obtained at 12 h in all groups compared with the 0 and 24 h time points. These findings coincide with a study by Thein et al. (2007), wherein the authors analyzed the quantity of the biofilm formed, based on CFU/mL counts, on acrylic surfaces during the interaction between *C. albicans* and *C. krusei* at time points of 0, 3, 6, 9 and 12 h, observing the greatest growth at 12 h. Chandra et al. (2001) temporally analyzed the ultrastructure of *C. albicans* biofilms formed in test specimens and defined three distinct phases of development. These authors observed the highest biomass production between 12 and 30 h, classifying this period as the intermediate stage in biofilm production.

In polymicrobial biofilms formed by fungi and bacteria, microbial diversity may directly influence the survival and proliferation of microorganisms (Morales and Hogan, 2010; Park, Han, Park, Choi, & Lee, 2014). These interactions may be beneficial for the microorganisms. For example, mixed biofilms containing *C. albicans* and *Streptococcus* spp. are more resistant to antimicrobial agents (Martin, Wächtle, Schaller, Wilson, & Hube, 2011) conversely; there are also antagonistic interactions, in which one species is impaired in relation to another. A clear example of such antagonism is the interaction between *C. albicans* and *Pseudomonas aeruginosa*, wherein the latter accumulates on the filaments of the yeast, feeding on the hyphae, and consequently causing the death of *C. albicans* (Hogan, Vik, & Kolter, 2004). The first reports of an interaction between two types of *Candida* were published by Kirkpatrick et al. (2000), who demonstrated that the association between *C. albicans* and *C. dubliniensis*, in both planktonic cells and biofilms, was antagonistic because the growth of both species was impaired. One of the most probable causes of such an effect would be competition between these species for nutrients and inhibition by toxic products generated from the metabolism of one species, preventing the growth of the other.

The present study is the first to report the inhibitory influence of *C. krusei* on the expression of the *ALS1*, *ALS3*, *HWP1*, *BCR1*, *EFG1* and *TEC1* genes of *C. albicans* during *in vitro* biofilm production. The presence of *C. krusei* during the *in vitro* development of a *C. albicans* biofilm resulted in an inhibitory and antagonistic relationship, which was observed based not only on a decrease in CFU/mL counts in mixed biofilms but also on the reduced expression of genes related to adherence and transcriptional regulation of the biofilm formation process. In the present study, reductions of 52.6% and 64.4% in the *C. albicans* count were observed at the 12 and 24 h time points. This antagonism between these two species agrees with the findings of Thein et al. (2007), who studied the *in vitro* interaction between *C. albicans* and *C. krusei* and observed competition between these two species, including an 85%

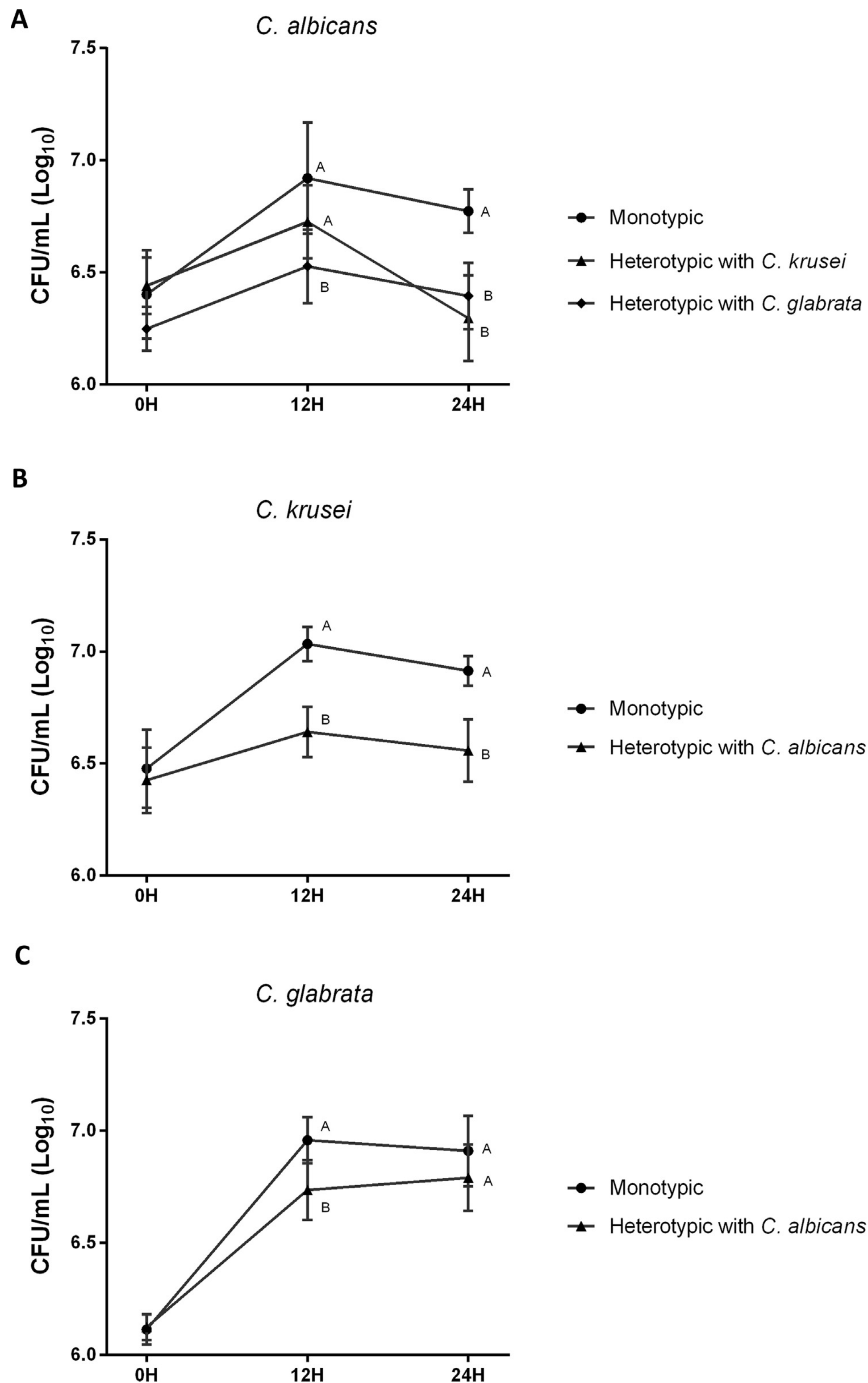

Fig. 1. Temporal quantification (0, 12 and 24 h) of fungal cells in biofilms formed at the bottom of 96-well plates for each *Candida* species. (A) Mean and standard deviation of *C. albicans* CFU/mL (Log) values in the following groups: monotypic formed only by *C. albicans*; mixed formed by *C. albicans* and *C. krusei* (0 h: $p = 0.6047$; 12 h: $p = 0.0344$; 24 h: $p = 0.0001$); mixed formed by *C. albicans* and *C. glabrata* (0 h: $p = 0.0416$; 12 h: $p = 0.0002$; 24 h: $p = 0.0001$); (B) mean and standard deviation of *C. krusei* CFU/mL (Log) values in in the following groups: monotypic formed only by *C. krusei*; mixed formed by *C. krusei and C. albicans* (0 h: $p = 0.6216$; 12 h: $p = 0.0002$; 24 h: $p = 0.0008$); (C) mean and standard deviation of *C. glabrata* CFU/mL (Log) values in the following groups: monotypic formed only by *C. glabrata*; mixed formed by *C. glabrata and C. albicans* (0 h: $p = 0.8878$; 12 h: $p = 0.0179$; 24 h: $p = 0.2488$); different letters represent significant differences between the monotypic and mixed biofilms at the same time (ANOVA, Tukey test, student t test, $p < 0.05$).

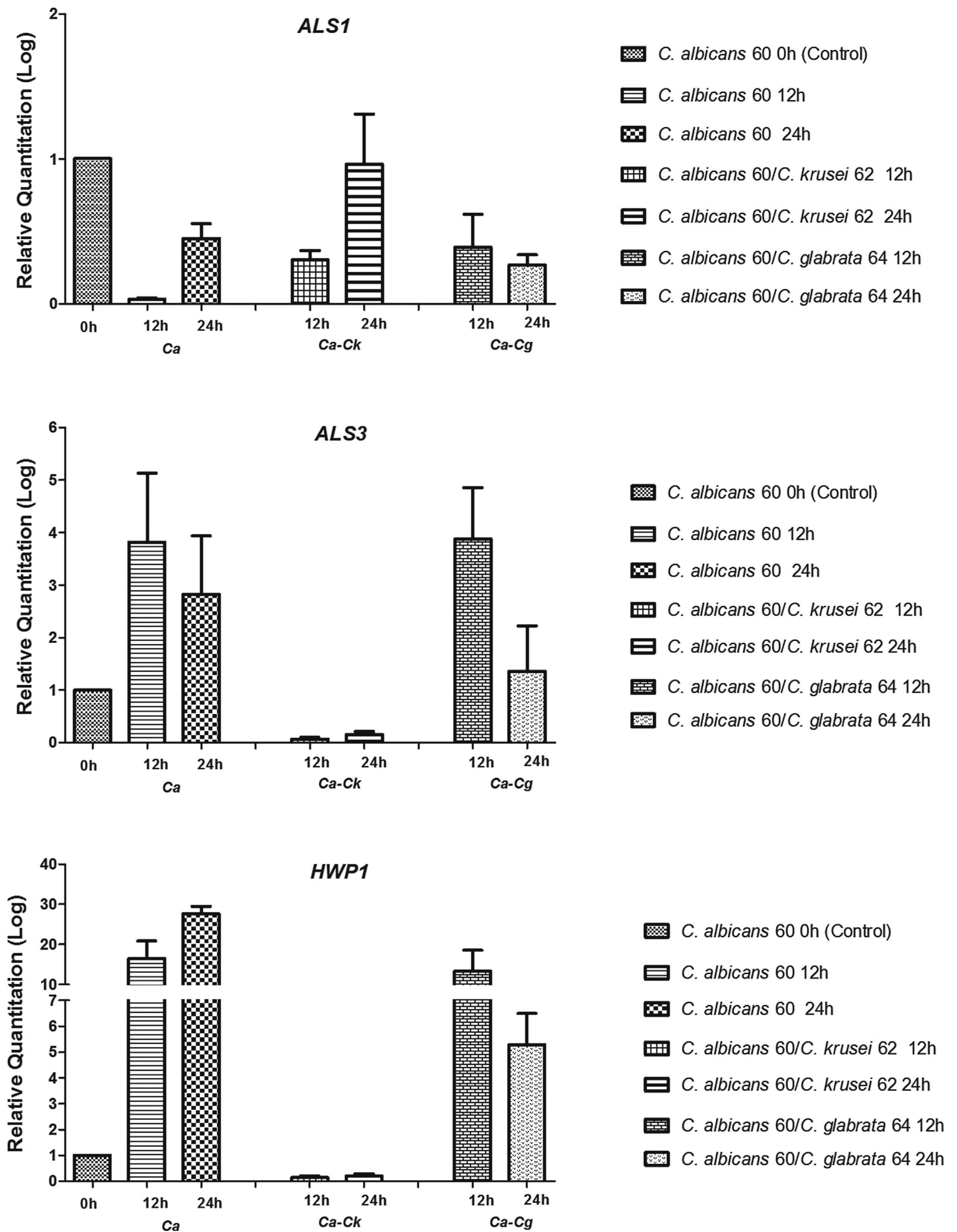

Fig. 2. Relative quantification (Log) of the expression of transcriptional regulatory genes (*ALS1*,*ALS3* and *HWP1*) in *C. albicans* cells in monotypic and mixed biofilms at different development times using quantitative real-time PCR (qPCR), in relation to the control (time 0 h). Values are expressed as the means and standard deviation.

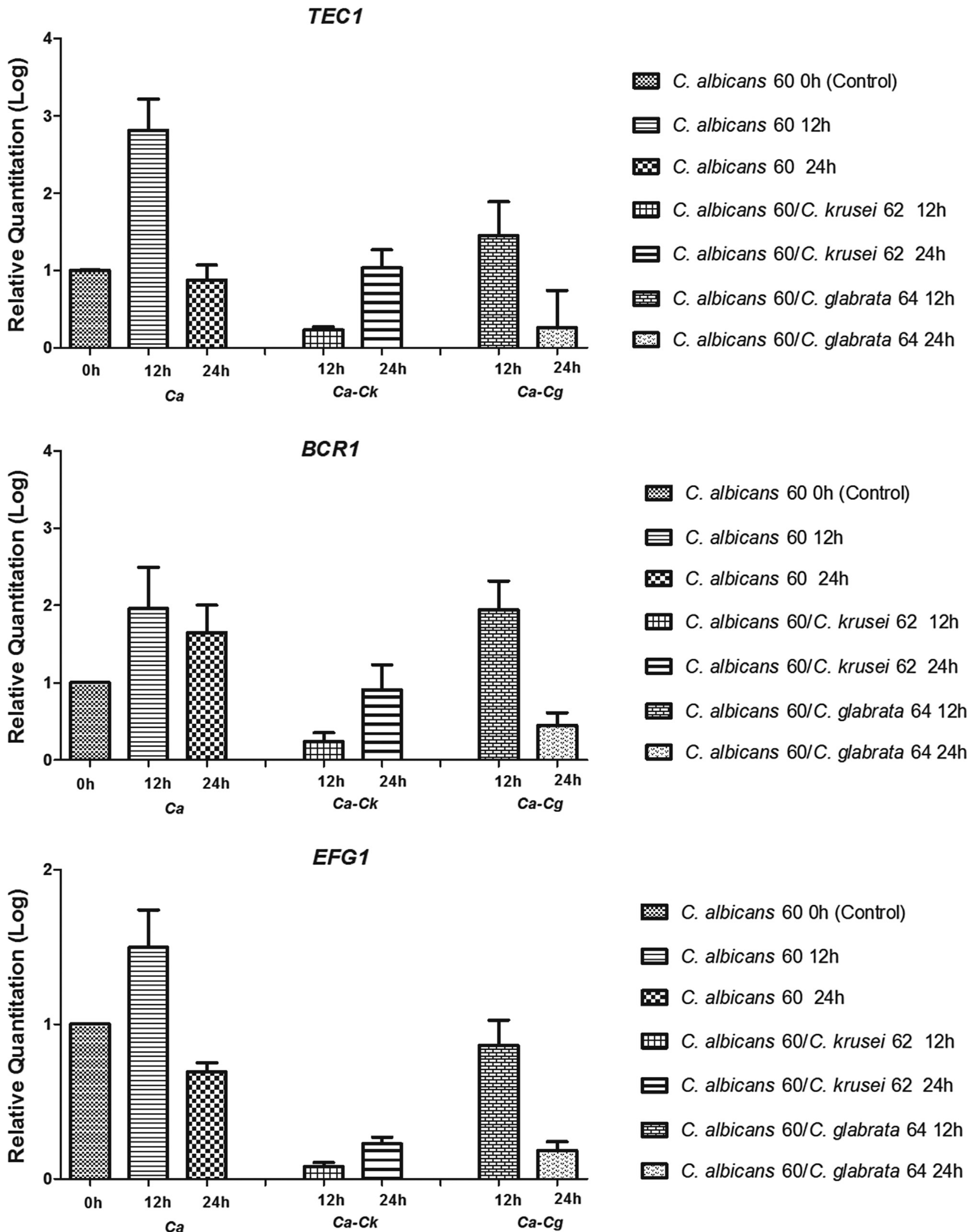

Fig. 3. Relative quantification (Log) of the expression of adhesion genes (*TEC1*, *BCR1* and *EFG1*) in *C. albicans* cells in monotypic and mixed biofilms at different development times using quantitative real-time PCR (qPCR), in relation to the control (time 0 h). Values are expressed as the means and standard deviation.

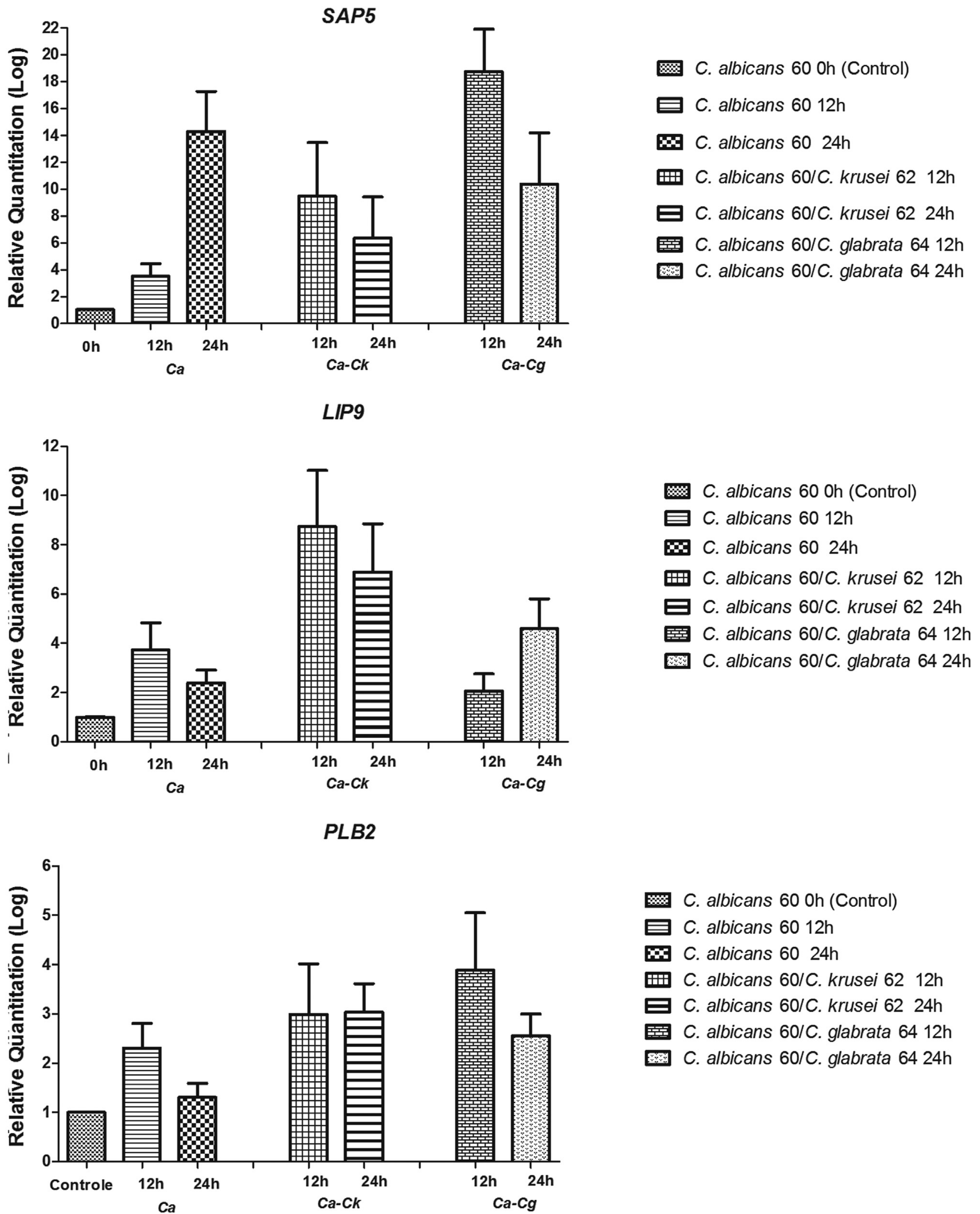

Fig. 4. Relative quantification (Log) of the expression of hydrolase genes (*SAP5*, *LIP9* and *PLB2*) in *C. albicans* cells in monotypic and mixed biofilms at different development times using quantitative real-time PCR (qPCR), in relation to the control (time 0 h). Values are expressed as the means and standard deviation.

reduction of *C. albicans* growth, in addition to inhibition of *C. albicans* filamentation.

Recently, our laboratory developed an *in vivo* study to evaluate the interactions of *C. albicans* (ATCC 18804) with *C. krusei* (ATCC 6258) and *C. glabrata* (ATCC 9030). Rossoni et al. (2015) demonstrated that *C. albicans* was able to establish competitive interactions with non-*albicans* species during infection development processes in different animal models. The authors

demonstrated that inoculation of mixed suspensions of these yeasts in an invertebrate model of *Galleria mellonella* increased the survival rate of larvae for 96 h in relation to *C. albicans* suspension monotypic. Furthermore, monotypic suspensions of *C. albicans* induced lesions typical of oral candidiasis in immunocompromised mice, while the heterotypic suspensions were not capable to cause tissue damage and lesions in the oral cavity.

In the literature, several hypotheses have been proposed to explain this intriguing phenomenon of inhibition between these two species, such as competition for food and space, dissemination of inhibitory chemicals produced in the association and even the existence of mediators or molecular messengers that are able to reduce factors related to the virulence of *C. albicans* (Korres, Buss, Ventura, & Fernandes, 2011; Rossoni et al., 2015; Thein et al., 2007).

Mixed biofilms with *C. glabrata* also showed decreased CFU/mL counts of *C. albicans* at 12 and 24 h; however, there was a slight increase in the expression of the *ALS3*, *HWP1*, *SAP5*, *PLB2* and *LIP9* genes at 12 and 24 h and of *TEC1* and *BCR1* at 12 h. These results may suggest an ecologically neutral relationship or even one of synergism, as described by Pathak, Sharma, and Shrivastva (2012), who evaluated the *in vitro* interaction between these species and observed a positive relationship between them. In contrast, Silva et al. (2013) detected a neutral relationship between these species, in which the number of culturable cells of each species was not affected by the presence of the other. Furthermore, authors such as Cenci et al. (2008) have noted that *C. albicans* in a mixed biofilm with *C. glabrata* shows the same degree of growth as when studied in a monotypic biofilm. In contrast, Agwu et al. (2012) observed a decrease in the development of *C. albicans* hyphae in the presence of *C. glabrata* under some conditions tested.

The present study is a pioneer in the temporal assessment of *C. albicans* genes in the presence of *C. krusei* and *C. glabrata.* However, more complex studies contributing to a greater understanding and elucidation of the genetic and molecular mechanisms involved in the ecological relationships between *Candida* species are required to discover new alternative therapies for the treatment of local and systemic candidiasis.

5. Conclusion

Given the results presented herein, we can conclude that *C. krusei* and *C. glabrata* may alter or inhibit the mechanisms involved in the *in vitro* adhesion and formation of *C. albicans* biofilms, influencing the pathogenicity of this species and suggesting a competitive interaction with *C. krusei* and *C. glabrata* during biofilm formation.

Conflict of interest

There authors declare that there are no conflict of interests.

Sources of funding

São Paulo Council of Research—FAPESP, Brazil (Grants 2011/15194-0 and 2012/15250-0) for supporting this research.

Ethical approval

Approved by the Research Ethics Committee of the São José dos Campos School of Dentistry/UNESP (Protocol 051/2009-PH/CEP).

Acknowledgment

We acknowledge the São Paulo Council of Research—FAPESP, Brazil (Grants 2011/15194-0, 2012/15250-0 and 2012/02184-9) for supporting this research.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.archoralbio.2016.01.005.

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