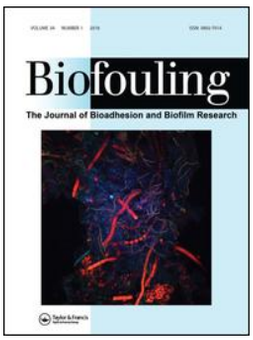

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Biofouling
The Journal of Bioadhesion and Biofilm Research

ISSN: 0892-7014 (Print) 1029-2454 (Online) Journal homepage: https://www.tandfonline.com/loi/gbif20

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To cite this article: Rodnei Dennis Rossoni, Patrícia Pimentel de Barros, Janaina Araújo de Alvarenga, Felipe de Camargo Ribeiro, Marisol dos Santos Velloso, Beth Burgwyn Fuchs, Eleftherios Mylonakis, Antonio Olavo Cardoso Jorge & Juliana Campos Junqueira (2018) Antifungal activity of clinical *Lactobacillus* strains against *Candida albicans* biofilms: identification of potential probiotic candidates to prevent oral candidiasis, Biofouling, 34:2, 212-225, DOI: 10.1080/08927014.2018.1425402

To link to this article: https://doi.org/10.1080/08927014.2018.1425402

BIOFOULING, 2018
VOL. 34, NO. 2, 212–225
https://doi.org/10.1080/08927014.2018.1425402

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Antifungal activity of clinical *Lactobacillus* strains against *Candida albicans* biofilms: identification of potential probiotic candidates to prevent oral candidiasis

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ABSTRACT

This study isolated *Lactobacillus* strains from caries-free subjects and evaluated the inhibitory effects directly on three strains of *C. albicans*, two clinical strains and one reference strain. Thirty *Lactobacillus* strains were isolated and evaluated for antimicrobial activity against *in vitro C. albicans* biofilms. *L. paracasei* 28.4, *L. rhamnosus* 5.2 and *L. fermentum* 20.4 isolates exhibited the most significant inhibitory activity against *C. albicans*. Co-incubation between these microorganisms resulted in deterrence of biofilm development and retardation of hyphal formation. The hindrance of biofilm development was characterized by the downregulated expression of *C. albicans* biofilm-specific genes (*ALS3*, *HWP1*, *EFG1* and *CPH1*). *L. paracasei* 28.4, *L. rhamnosus* 5.2 and *L. fermentum* 20.4 demonstrated the ability to exert antifungal activity through the inhibition of *C. albicans* biofilms.

ARTICLE HISTORY
Received 11 September 2017
Accepted 3 January 2018

KEYWORDS
Biofilm; mycology; pathogenesis; *Candida albicans*; *Lactobacillus*

Introduction

Candida albicans is a commensal yeast commonly found in the oral cavity of humans, isolated from ~62% of healthy individuals. This dimorphic fungus is considered an opportunistic pathogen that can cause severe and recurrent infections in the mucosa and even fatal systemic infections (Sellam and Whiteway 2016; Noble et al. 2017). The impact of candidiasis on human health has significantly increased in recent decades, in particular because of the growing number of immunocompromised patients resulting from the acquired immunodeficiency syndrome (AIDS) epidemic, organ transplantation, and cancer chemotherapies that affect the human microbiome (Sellam and Whiteway 2016).

Infections in the oral cavity by *Candida* spp., such as pseudomembranous, erythematous, and hyperplastic candidiasis, occur due to the formation of biofilms composed of yeast and hyphae (da Silva Dantas et al. 2016; Millsop and Fazel 2016). Whilst *C. albicans* can exist in a planktonic form, it inhabits humans predominantly as biofilms. Structured biofilms are three-dimensional microbial communities attached to a solid surface, embedded in a matrix of extracellular polymeric substances (Darrene and Cecile 2016; Tsui et al. 2016). Due to the greater resistance of biofilms to antifungals and host defenses compared to planktonic cultures, biofilm formation is an important virulence attribute of *C. albicans* (Alcazar-Fuoli and Mellado 2014). Since biofilms can be so difficult to eradicate due to the induction of efflux pump expression, physical barring of antimicrobial therapeutic agents and the harboring of persister cells that lead to chronic infections, it is important to develop biofilm prevention methods for susceptible individuals. Several antimicrobial agents and new therapeutic strategies, such as the use of probiotics in the oral cavity, have been investigated to control oral biofilms (do Carmo et al. 2016; Oliveira et al. 2016; Salas-Jara et al. 2016; Schwendicke et al. 2017).

The World Health Organization defines probiotics as live microorganisms that confer health benefits to the host when administered in adequate amounts. These benefits are mainly due to the regulation of the resident microbiota and modulation of the immune system by activation of lymphoid cells. Several microorganisms have been used as probiotics, including *Bifidobacterium*, *Saccharomyces*, *Bacillus*, and *Lactobacillus* (Guarner et al. 2012; Herbel

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Supplemental material for this paper is available online at https://doi.org/10.1080/08927014.2018.1425402

et al. 2013; Vilela et al. 2015). Lactobacilli are bacteria that naturally colonize the oral cavity and gastrointestinal tract of healthy humans. In the oral cavity, certain strains of lactobacilli can cause caries through their acidogenic and aciduric characteristics and are frequently detected in lesions of deep cavities. However, some studies suggest an additional beneficial role for oral lactobacilli. *Lactobacillus paracasei*, *Lactobacillus plantarum*, and *Lactobacillus rhamnosus* strains isolated from caries-free individuals inhibited the *in vitro* growth of oral pathogens species such as *Streptococcus mutans* and *Streptococcus sobrinus* more efficiently than the *Lactobacillus* strains isolated from patients with active caries (Simark-Mattsson et al. 2007; Belda-Ferre et al. 2012; Million and Raoult 2013).

Moreover, previous studies in the literature showed that some probiotic strains of *Lactobacillus* inhibited *C. albicans* biofilm formation through the production of bacteriocins, immunomodulatory effects, or even by mechanical inhibition of adhesion receptors (Orsi et al. 2014; Vilela et al. 2015; Matsubara et al. 2016; Wannun et al. 2016; Ribeiro et al. 2017). Despite the large number of studies with probiotics against *C. albicans* biofilms, it is still unclear whether the inhibition of fungal biofilm development is dependent on a direct interaction with probiotic bacterial cells or the secretion of factors produced by the probiotics (ie supernatants), and it is also unknown which of the *C. albicans* genes involved in this prokaryote–eukaryote association can affect and destabilize biofilm formation.

Despite growing interest in probiotics in the past few years, most probiotic strains used and known were isolated from the gastrointestinal tract and have become 'standard strains' (Simark-Mattsson et al. 2007; Rivera-Espinoza and Gallardo-Navarro 2010; Shanahan et al. 2012). The present authors' studies are based on the hypothesis that the oral cavity of healthy individuals may house alternative beneficial *Lactobacillus* isolates with inhibitory activity against fungal pathogens. Healthy oral microbiota contains a highly diverse species population, which results in balance, functional redundancy, and resistance to disease (Grady et al. 2016). Therefore, resident oral microbiota from healthy individuals could provide new strains with probiotic efficacy (Belda-Ferre et al. 2012; Shanahan et al. 2012; Olle 2013).

Thus, the aim of this study was to isolate and to identify *Lactobacillus* isolates from the oral cavity of caries-free individuals and evaluate their antifungal effects on biofilms of *C. albicans*, using three fungal strains, one reference and two clinics. In addition, this study sought to elucidate the mechanisms that *Lactobacillus* isolates use to disrupt *Candida* biofilms by studying the supernatant filtrate of *Lactobacillus* cultures and the effect on the gene expression of *C. albicans*.

Materials and methods

Subjects

The study group comprised 41 caries-free individuals (mean age 19 ± 2.1 years) with no history of systemic disease or antibiotic therapy one month prior to sampling. All patients agreed to participate in the study by signing the informed consent form approved by the Research Ethics Committee of the Institute of Science and Technology, UNESP – Univ Estadual Paulista (protocol 560.479).

Participants were recruited from February to August 2014 and subjected to an oral clinical examination. The presence of a cavity and/or restorations due to caries was considered an exclusion factor for the study. The same examiner performed all clinical evaluations.

Sampling of saliva and Lactobacillus *identification*

All subjects were investigated for the presence of lactobacilli in saliva. Saliva samples were collected by oral rinses in 10 ml of phosphate buffered saline (PBS, pH 7.2) for 1 min. The samples were centrifuged for 10 min at 5,000 RPM and the supernatant was discarded. Next, 2.5 ml of PBS were added to the pellet. Tenfold serial dilutions were carried out, and aliquots of 100 µl were seeded onto Man–Rogosa–Sharpe (MRS) agar (Difco, Detroit, MI, USA) and Rogosa SL agar (Difco) using the conventional pour plate methodology. The plates were incubated for 72 h under microaerobic conditions with 10% CO_2 at 37°C.

After incubation of the plates in microaerobic conditions (10% CO_2) for 72 h, colonies with different morphologies (MRS agar) and characteristic discoid colonies (Rogosa SL agar) were Gram stained, and only the Gram-positive and rod-shaped bacteria were isolated for identification using molecular methods. The chromosomal DNA of each isolate was extracted using a PureLink® Genomic DNA kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. PCR amplification of the intergenic segment between the 16S and 23S rRNA subunits was carried out as described by Song et al. (2000). For this experiment, reference samples of *Lactobacillus* were included as positive controls for the PCR reaction (*Lactobacillus plantarum* ATCC 8014, *Lactobacillus paracasei* subsp. *paracasei* ATCC 335 and *Lactobacillus rhamnosus* ATCC 9595).

Strains and growth conditions

In this study, the reference strain *C. albicans* ATCC 18804 was used, as well as two clinical strains, *C. albicans* 60 (CA60) and *C. albicans* 230S (CA230S) from the Laboratory of Microbiology of the Institute of Science and Technology of São José dos Campos/UNESP

(São Paulo State University). The clinical strains were isolated from oropharyngeal candidiasis lesions of HIV-positive patients from Emílio Ribas Infectology Institute (Instituto de Infectologia Emílio Ribas, São Paulo, Brazil), with the approval of the Ethics Committee (Protocol 051/2009-PH/CEP). In addition, 30 clinical isolates of *Lactobacillus* were identified according to the section above. The clinical strains of *Candida* were isolated and identified according to Junqueira et al. (2012).

All *C. albicans* strains were cultured for 18 h at 37°C in yeast nitrogen base broth (YNB; Difco) supplemented with 100 mM glucose. *Lactobacillus* isolates were cultured in *Lactobacillus* MRS broth (MRS broth; Difco) for 24 h at 37°C under microaerophilic conditions. The suspension densities were determined with a spectrophotometer (B582, Micronal, Sao Paulo, Brazil) and diluted to a concentration of 10^7 cells ml^{-1}. The quantification of the cell number of the inoculum was confirmed by counting CFU ml^{-1} after plating in Sabouraud dextrose agar (SDA; HiMedia, Mumbai, India) with chloramphenicol (0.05 g l^{-1}) for *C. albicans* and MRS agar (Difco) for *Lactobacillus*.

The preparation of the *Lactobacillus* supernatant was performed according to Ribeiro et al. (2017). An inoculum of 1 ml of the standard suspension was seeded into 6 ml of MRS broth and incubated at 37°C for 24 h under microaerophilic conditions. After this incubation, the broth was centrifuged (5,000 RPM for 10 min) and filtered with a 0.22 µm pore size membrane (MFS, Dublin, CA, USA).

Antibacterial activity of Lactobacillus *strains against* C. albicans *in planktonic cultures*

The antibacterial activities of the *Lactobacillus* strains against *C. albicans* ATCC 18804 in planktonic cultures were assessed according to the methodology previously described (Lin et al. 2015) with some modifications. Standardized cell suspensions of *C. albicans* and *Lactobacillus* were prepared as described above. Next, 250 µl of a *C. albicans* suspension and 250 µl of a *Lactobacillus* suspension (or culture filtrate) were mixed with 1.5 ml of BHI broth. In the control group, microbial suspensions of *Lactobacillus* were replaced by PBS. All the cultures were incubated at 37°C for 24 h (5% CO_2). After incubation, the cultures were diluted and plated on Sabouraud dextrose agar (Difco) supplemented with chloramphenicol (0.05 g l^{-1}) for *C. albicans* growth. The plates were incubated at 37°C for 48 h and the number of colony-forming units (CFU ml^{-1}) were determined. This assay was performed as three independent experiments with four separate cultures per group.

Biofilm formation

The anti-biofilm activity of the *Lactobacillus* strains were tested against *C. albicans* ATCC 18004, CA60 and CA230S. This test was performed in 96-well microtiter plates (TPP®, Trasadingen, Switzerland) following the methodology described by Vilela et al. (2015) and Ribeiro et al. (2017), with some modifications. Briefly, 100 µl of all *C. albicans* standard suspension (10^7 cells ml^{-1}) were pipetted into 96-well microtiter plates and the plates were placed on a 75-rpm shaking incubator (Quimis, Diadema, São Paulo, Brazil) at 37°C for 90 min. Each well was washed twice with PBS, and 50 µl of *Lactobacillus* cells or 50 µl of supernatant were added into the wells of each plate. For the control groups, 50 µl of PBS or MRS broth were added. To promote biofilm growth, 70 µl of YNB supplemented with 100 mM glucose and 30 µl of BHI broth were added to each well. The plate was incubated for 48 h at 37°C with shaking at 75 rpm. The liquid medium was replaced after 24 h.

Analysis of biofilms by CFU counting

The quantification of the number of viable cells in the biofilms was based on the methodologies described by Thein et al. (2006) and Vilela et al. (2015). After 48 h, plate contents were aspirated, and the wells were washed twice with 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, CT, USA) for 30 s with 25% amplification. After homogenization, serial dilutions of the biofilm suspension were performed, and 100 µl aliquots of these dilutions were inoculated into Petri dishes containing SDA. The plates were incubated at 37°C for 48 h. After incubation, the colonies were counted to calculate the CFU ml^{-1} values. The cellular quantification was performed with $n = 10$ biofilm replicates per group.

Analysis of biofilms by total biomass quantification

After biofilm formation, the biofilm biomass was quantified utilizing an assay previously described by Peeters et al. (2008), with modifications. For fixation of the biofilms, 100 µl of 99% methanol were added to the wells (Sigma-Aldrich, São Paulo, Brazil). After 15 min, the supernatants were removed and the plates were air dried. Then, 100 µl of a 1% crystal violet (CV) solution were added to all wells. After 20 min, the residual CV solution was removed by washing with PBS. Finally, bound CV was released by adding 150 µl of 33% acetic acid (Sigma-Aldrich). The absorbance was measured at 540 nm. All steps were carried out at room temperature. The CV assay

was performed as two independent experiments with $n = 6$ biofilms per group.

Analysis of biofilms by scanning electron microscopy (SEM)

Acrylic resin disks, measuring 8 mm in diameter, were placed on a 24-well plate for biofilm formation, following the methodology of Barbosa et al. (2016). After biofilm formation, the specimens were fixed in 1 ml of 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 total drying of the specimens.

After drying, the specimens were transferred to aluminum stubs and sputter coated with gold for 160 s at 40 mA (Denton Vacuum Desk II, Denton Vaccum LLC, Moorestown, NJ, USA). The specimens were examined and imaged using a JEOL JSM-5600 scanning electron microscope (JEOL USA, Inc., Peabody, MA, USA) at the Institute of Science and Technology, UNESP – Univ Estadual Paulista. These experiments were performed at two different times with $n = 3$ biofilms per group.

Analysis of C. albicans gene expression using RT-PCR

Biofilms were formed in 24-well microtiter plates (TPP®) using the same concentrations and conditions for biofilm formation described above. After biofilm formation, 1 ml of TRIzol® (Ambion, Inc., Carlsbad, CA, USA) was added to each well to remove and collect the biofilm. The yeast/TRIzol® suspension was added to a 2 ml microtube and incubated at room temperature for 10 min. Subsequently, 200 µl of chloroform were added (Sigma-Aldrich, St Louis, MO, USA), and the microtubes were centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatants were transferred to new microtubes, and 500 µl of isopropanol (Sigma-Aldrich) were added. After centrifugation, the resulting sediment was washed with 70% ethanol (Sigma-Aldrich), centrifuged again and resuspended in 50 µl of RNA storage buffer (Ambion, Inc.). The RNA concentration, purity and quality were measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and agarose gel electrophoresis (Invitrogen™, Carlsbad, CA, USA). The gel was stained with ethidium bromide (Invitrogen™) and visualized on a transilluminator.

The total extracted RNA (1 µg) was treated with DNase-I (TURBO DNase Treatment and Removal Reagents, Ambion, Inc.) and was transcribed into complementary DNA (cDNA) using the SuperScript® III First-Strand Synthesis SuperMix Kit for qRT-PCR (Invitrogen™) according to the manufacturer's recommendations.

The primers for the genes analyzed in the present study were described and used as indicated by Nailis et al. (2010), Hnisz et al. (2012) and Granger (2012). The primer specificity was confirmed for *C. albicans* (Barros et al. 2016).

Transcribed cDNAs were amplified for the relative quantification of the *ALS3*, *HWP1*, *CPH1*, *EFG1*, and *YWP1* gene expression levels in relation to the concentration of the reference gene (*RPP2B*). In the present study, four reference genes (*RPP2B*, *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 RPP2B.

The qPCR method was used to evaluate the amount of the cDNA product in the exponential phase of the amplification reaction. The SYBR® Green fluorophore (Platinum® SYBR® Green qPCR SuperMix-UDG Applied Biosystems, Framingham, MA, USA) was used as the detection system as recommended by the manufacturer. As a negative control for the reaction, all reagents except cDNA were added to the last wells of the plates and the wells were sealed with optical adhesive (Invitrogen™). Next, plates were placed in a StepOnePlus™ System (Applied Biosystems), and the following cycling parameters were used: 50°C for 2 min, followed by an initial denaturation at 95°C for 2 min, 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. 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).

Statistical analysis

The Student's *t*-test was used to compare the CFU ml^{-1} results from the *in vitro* biofilm formation assay, the CV assay, and the relative quantification of gene expression. All analyses were performed using the GraphPad Prism 6 Program (GraphPad Software, Inc., La Jolla, CA, USA) and a level of significance of 5% was adopted.

Results

Among the 41 caries-free individuals evaluated, 27 (66%) had cultures that were positive for *Lactobacillus* in the oral cavity. From these individuals, 30 clinical isolates of *Lactobacillus* were identified including the following species: *Lactobacillus paracasei* (23), *Lactobacillus rhamnosus* (5) and *Lactobacillus fermentum* (2). The distribution of the identified species is shown in Table 1.

Table 1. Clinical strains of *Lactobacillus* identified in this study.

Species	Strain designation
L. paracasei	1.1
L. paracasei	3.1
L. paracasei	4.2
L. rhamnosus	5.2
L. paracasei	6.2
L. paracasei	7.5
L. paracasei	8.4
L. paracasei	10.5
L. paracasei	11.6
L. rhamnosus	13.1
L. fermentum	14.5
L. paracasei	15.8
L. paracasei	16.4
L. paracasei	17.1
L. rhamnosus	19.3
L. rhamnosus	19.9
L. paracasei	20.3
L. fermentum	20.4
L. paracasei	21.4
L. paracasei	23.4
L. paracasei	24.1
L. paracasei	25.4
L. paracasei	26.1
L. paracasei	27.1
L. paracasei	28.4
L. paracasei	30.1
L. fermentum	31.4
L. rhamnosus	36.4
L. paracasei	37.1
L. paracasei	39.2

First, all the strains were screened for antibacterial activity against *C. albicans* ATCC 18804 using planktonic cultures. For this purpose, each *Lactobacillus* strain was co-cultured with *C. albicans* for 24 h. Moreover, the indirect effects of *Lactobacillus* were analyzed using only the *Lactobacillus* culture filtrate that was obtained after its growth in MRS broth. To determine whether the MRS broth of the *Lactobacillus* culture could exert an effect on *C. albicans* and interfere with the results, a control group consisting only of *C. albicans* and MRS broth was included. After 24 h, the growth of *C. albicans* was evaluated by counting the CFU ml^{-1}.

Among the 30 *Lactobacillus* strains analyzed, 26 (86%) showed antibacterial activity against *C. albicans*. *L. paracasei* 30.1, 37.1 and 39.2 strains and *L. rhamnosus* strain 36.4 were the strains that had no inhibitory effects on *C. albicans* ATCC 18804. For other *Lactobacillus* strains, the percentage reductions in *C. albicans* growth ranged from 82 to 98% depending on the strain analyzed (Figure 1A).

In addition, 86% of the *Lactobacillus* strains had an inhibitory effect on *C. albicans* ATCC 18804 growth when only their supernatant was placed in contact with *C. albicans* (Figure 1A). The results showed that the MRS broth used to prepare the *Lactobacillus* supernatant did not interfere with the growth of *C. albicans*. The CFU ml^{-1} count of *C. albicans* was 8.43 Log for the *C. albicans* + PBS control group and 8.36 Log for the *C. albicans* + MRS broth control group (Student's *t*-test, $p = 0.8927$). These data indicated that the anti-*Candida* activity of the supernatant could be attributed to some metabolites produced by the *Lactobacillus* strains.

Based on these results, these strains were selected for the *in vitro C. albicans* biofilm studies. 30 clinically derived isolates were screened to select those which were most capable of reducing the CFUs of *C. albicans* ATCC 18804 in the mixed biofilms (*C. albicans* and *Lactobacillus*) in comparison to the single biofilms (only *C. albicans*, control group). The growth of *C. albicans* ATCC 18804 was inhibited by most *Lactobacillus* isolates when interacting directly with the *Lactobacillus* cells and even with the supernatant of *Lactobacillus* cultures (Figure 1B). To verify whether the composition of the MRS broth of the *Lactobacillus* cultures could affect *C. albicans* and interfere with the assay testing the supernatants of these lactic acid bacteria, another control group was included, *C. albicans* and MRS broth. The results showed that addition of MRS broth alone to the supernatant did not inhibit the growth of *C. albicans* for any of the studied strains (Supplemental material Table 1).

The three isolates that exhibited the highest antifungal activity against *C. albicans* ATCC 18804 were *L. rhamnosus* 5.2, *L. fermentum* 20.4 and *L. paracasei* 28.4. The recovered number of *C. albicans* CFUs after exposure to the *Lactobacillus* strains were subjected to statistical analysis and the results are presented in Figure 2A. *C. albicans* exhibited a significant decrease in growth after interaction with *Lactobacillus* cells for the three analyzed bacterial isolates. Exposure to *L. rhamnosus* 5.2 resulted in a 0.5 Log reduction in the number of recovered fungal CFUs ($p = 0.0001$). This was followed by a 0.4 Log reduction in fungi after exposure to *L. fermentum* 20.4 ($p = 0.0001$). The most significant reduction in the number of recovered fungal CFUs was attributed to *L. paracasei* 28.4 that reduced fungal cells by 0.72 Log ($p = 0.0001$). *Lactobacillus* supernatant decreased the *C. albicans* growth by 0.4 Log for *L. rhamnosus* 5.2 ($p = 0.0001$), 0.6 Log for *L. fermentum* 20.4 ($p = 0.0001$) and 0.6 Log for *L. paracasei* 28.4 ($p = 0.0001$).

The biofilms associated with *L. paracasei* strain 28.4 showed the best reduction in *C. albicans* ATCC 18804 cells. Then, this *Lactobacillus* strain was tested with two clinical isolates of *C. albicans*, CA60 and CA230S, as previous studies demonstrated that these fungal isolates exhibited high *in vitro* expression of virulence genes (*ALS1*, *ALS3*, *HWP1*, *BCR1*, *EFG1*, *TEC1*, *SAP5*, *PLB2*, and *LIP9*) and pathogenicity in an animal model (de Barros et al. 2017). In biofilms with *C. albicans* clinical strains, exposure to *L. paracasei* 28.4 cells resulted in a 0.77 Log reduction in the number of recovered fungal CFUs ($p = 0.0001$) for CA60 and 1.20 Log reduction for CA230S ($p = 0.0001$).

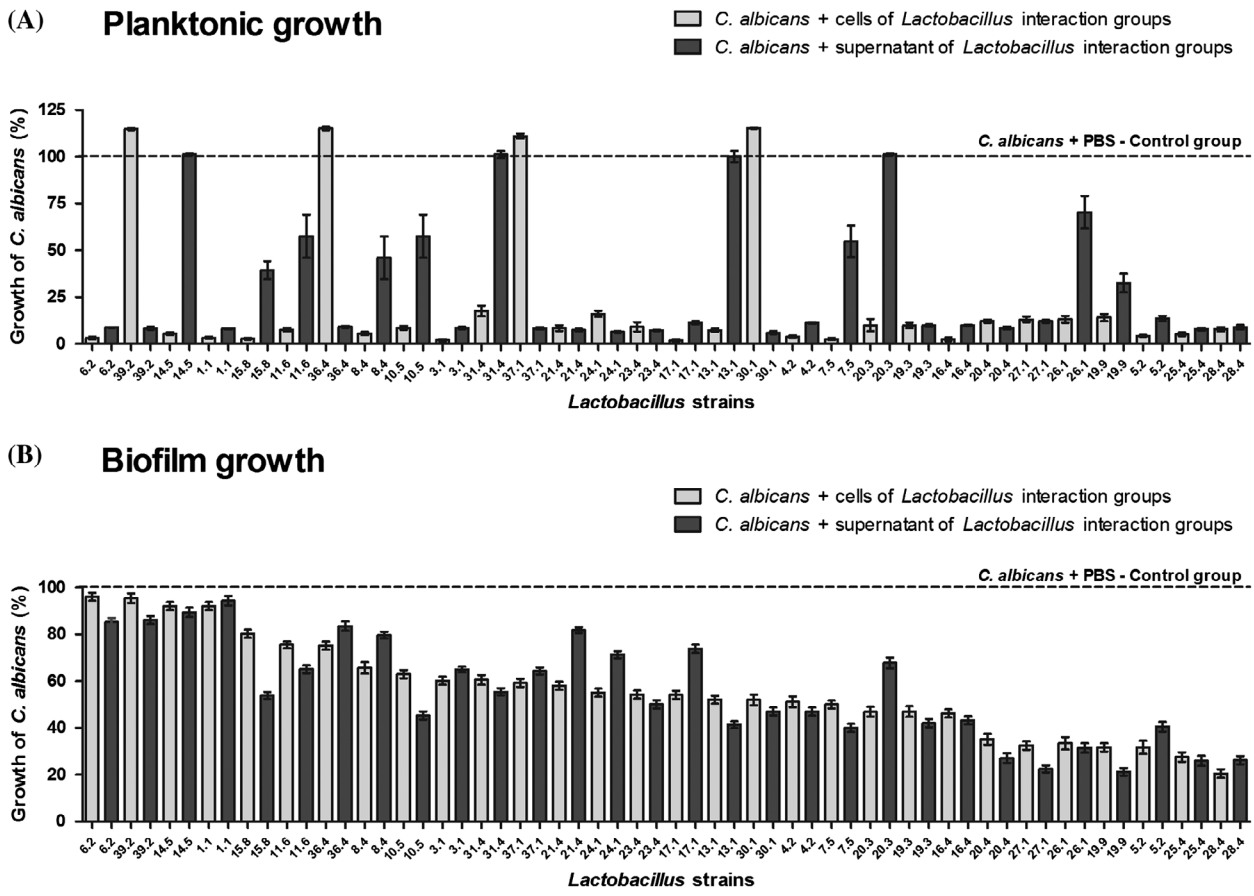

Figure 1. (A) Growth percentages of *C. albicans* ATCC 18804 obtained by counting the CFU ml^{-1} in planktonic culture. (B) Growth percentages of *C. albicans* ATCC 18804 obtained by counting the CFU biofilm^{-1} in a biofilm model. Percentage reduction expressed as mean values (CFU ml^{-1} or CFU biofilm^{-1}) show the viability of *C. albicans* in the cell and supernatant groups of the *Lactobacillus* spp. in relation to the untreated control group.

This was followed by a 0.96 and 1.92 Log reduction in the fungus after exposure to *L. paracasei* 28.4 supernatant ($p = 0.0001$) for CA60 CA230S, respectively (Figure 2B).

To quantify the difference in the total amount of biofilm present among co-cultured samples, the biofilms were stained with CV. Prioritizing the *Lactobacillus* isolates that inhibited growth most significantly through the reduction in the number of cells present in the biofilm, the isolates *L. rhamnosus* 5.2, *L. fermentum* 20.4 and *L. paracasei* 28.4 were used to determine the effect on quantitative biofilms *C. albicans* ATCC 18804. The biofilms formed by *C. albicans* ATCC 18804 in the presence of *Lactobacillus* cells exhibited a significant reduction compared to the control group that lacked *Lactobacillus* cells. In relation to the indirect effects of *Lactobacillus* on *C. albicans* ATCC 18804, the presence of *Lactobacillus* supernatant also reduced the total biofilm formed by *C. albicans* (Figure 3A). Regarding *L. paracasei* strain 28.4 in association with clinical strains of *C. albicans*, the biofilms formed by *C. albicans* in the presence of *Lactobacillus* cells or its supernatant exhibited a significant reduction compared to the control group of *C. albicans* (Figure 3B).

The biofilms formed were also evaluated by SEM, enabling observation of mature biofilm formation on acrylic resin disks after incubation for 48 h. The *C. albicans* cells observed in the biofilms showed morphological variations according to the experimental group. The biofilms formed by *C. albicans* in the absence of *Lactobacillus* were characterized by the presence of numerous yeasts and hyphae for all tested strains (Figure 4A). In mixed microbe populations involving co-incubation of *C. albicans* with *Lactobacillus* isolates, the adherence of bacterial cells to *C. albicans* yeasts was verified and also inhibition of hyphal formation (Figure 4B). However, for the biofilms composed of *C. albicans* and *Lactobacillus* supernatant, a reduction in yeast was observed, together with a lack of hyphal formation and alterations in the morphology of the fungal cells (Figure 4C).

Therefore, SEM images confirmed the results obtained from the CFU count and CV assays, showing that the cells and supernatants of *Lactobacillus* isolates influenced *C. albicans* viability and reduced the total amount of biofilm. In addition, SEM showed that *Lactobacillus* strains were capable of inhibiting *C. albicans* filamentation.

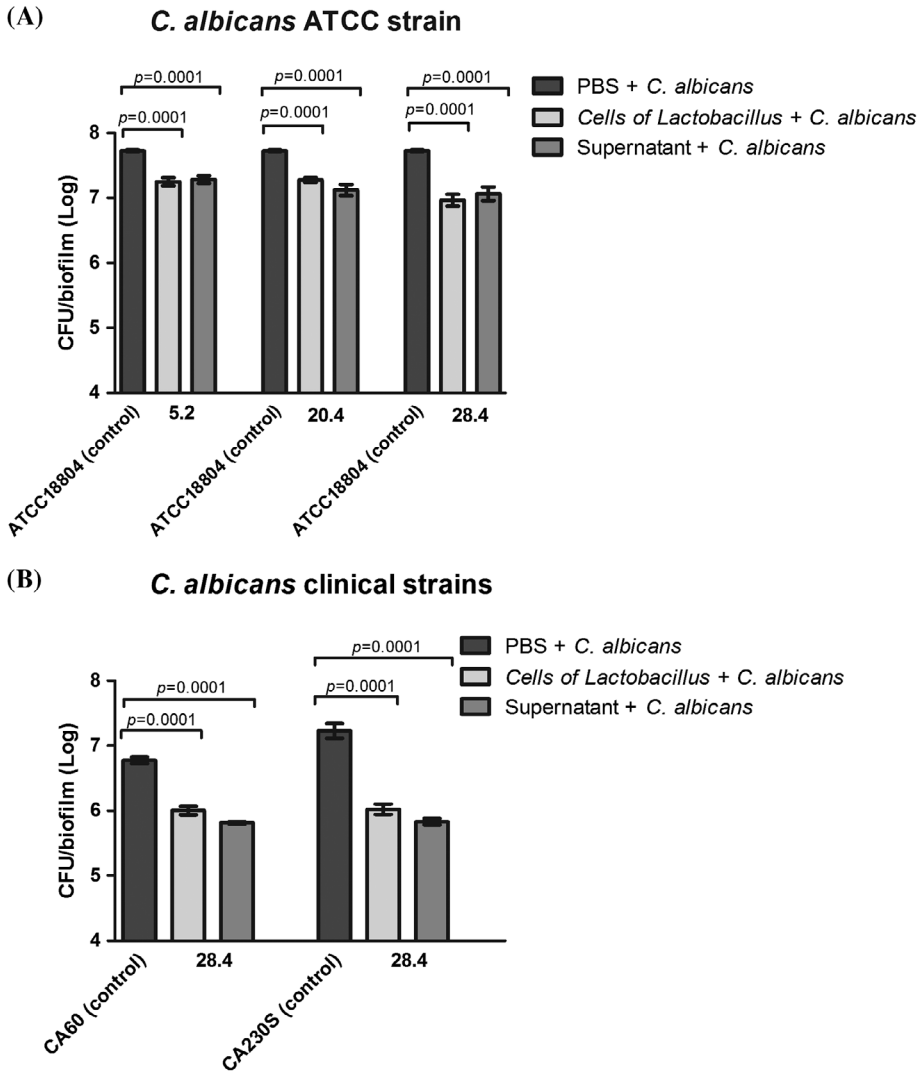

Figure 2. CFU counting of *C. albicans* in biofilms formed *in vitro*. Mean and SD for the number of CFU ml^{-1} of *C. albicans* (Log) in the control group biofilm (*C. albicans* + PBS) and in the groups with *Lactobacillus* cells or their supernatant. (A) Interaction of *C. albicans* ATCC 188804 and *Lactobacillus*. There was a statistically significant difference between all analyzed groups ($p = 0.0001$). (B) Interaction of *C. albicans* clinical strains (CA60 and CA230S) and *Lactobacillus*. There was a statistically significant difference between all the groups analyzed ($p = 0.0001$). The Student's *t*-test was used.

To elucidate the mechanisms used by *Lactobacillus* strains to inhibit *C. albicans* biofilms this study was extended to analyze *C. albicans* gene expression, concentrating on genes known to be important to biofilm formation. The expression levels of the adhesion genes (*ALS3*, *HWP1*, and *YWP1*) and transcriptional regulatory genes (*EFG1* and *CPH1*) were quantified in *C. albicans* cells from single and mixed biofilms using qPCR (Figure 5).

The largest reductions in the expression of the analyzed *C. albicans* genes were obtained with the 28.4 strain. For the *C. albicans* ATCC 18804, the adhesion (*ALS3*) and filamentation (*HWP1*) genes were the most affected, achieving a 333- and 100-fold decrease in the *C. albicans* biofilms associated with 28.4 cells, respectively. Regarding the biofilms treated with 28.4-strain supernatant, 33- and 20-fold decreases in the *ALS3* and *HWP1* genes, respectively, were verified. This reduction was also observed with the clinical strains of *C. albicans* used in this study for association with *Lactobacillus* cells or their supernatant. For the CA60 strain, a significant fold change in the genes was observed: *ALS3* (5,000-fold decrease), *HWP1* (10,000-fold decrease) and *CPH1* (500-fold decrease). Regarding CA230S, the most downregulated genes were *EFG1* (5.88-fold decrease) and *HWP1* (3.84-fold decrease).

Regarding *YWP1* that is a marker of the yeast form of *C. albicans* linked covalently to glucans of the wall matrix, all groups with *Lactobacillus* strains (cells or supernatant) were positively regulated to this gene compared to the *C. albicans* control group. The largest increases in the expression of this gene were obtained with the supernatant

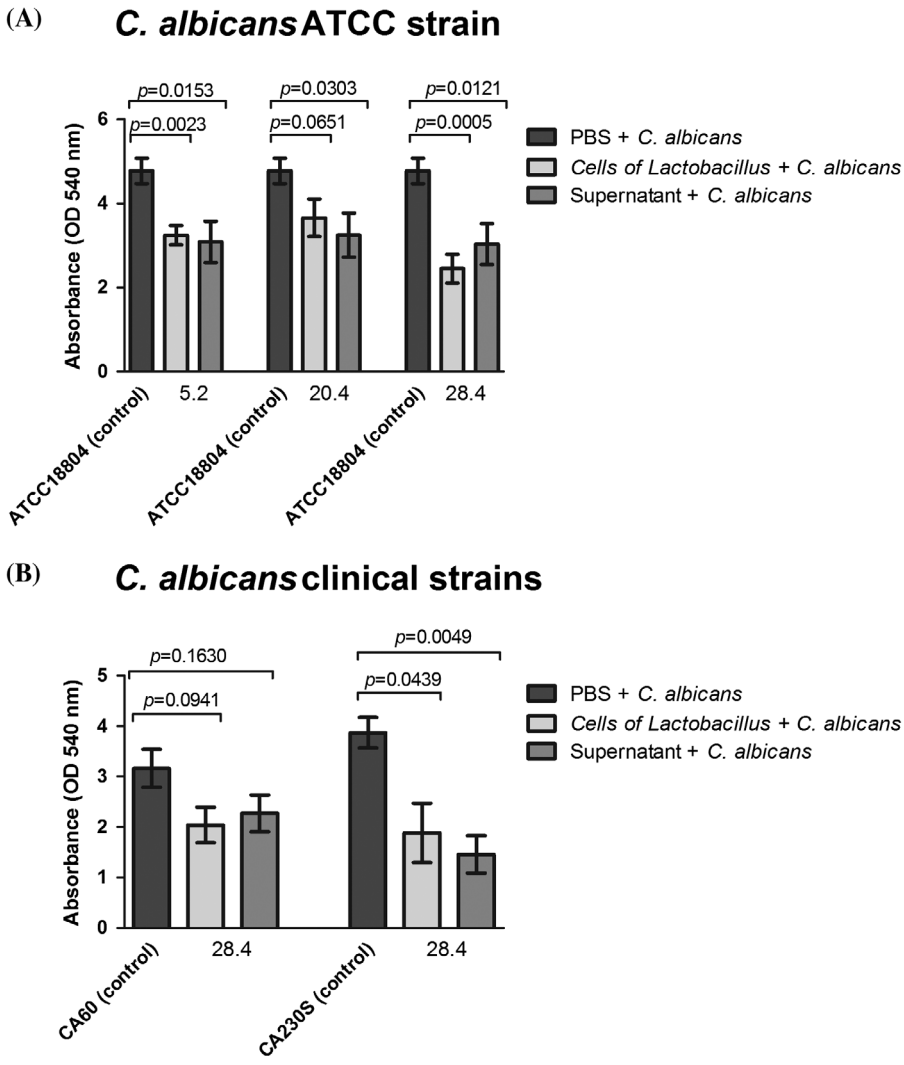

Figure 3. Evaluation of biomass formed by biofilms of *C. albicans*. Mean and SD of the absorbance value of control group biofilms (*C. albicans* + PBS) and in groups with *Lactobacillus* cells or their supernatants. (A) Interaction of *C. albicans* ATCC 18804 and *Lactobacillus*; (B) interaction of *C. albicans* clinical strains (CA60 and CA230S) and *Lactobacillus*. The Student's *t*-test was used.

of the 28.4 strain (fivefold increase for *C. albicans* ATCC and threefold increase for clinical strains).

Considering the results observed by the qPCR assay, it can be confirmed that these three strains of *Lactobacillus* affect biofilm formation of *C. albicans* by downregulating the expression of the *ALS3*, *HWP1*, *CPH1*, and *EFG1* genes.

Discussion

The oral cavity was evaluated for colonization of *Lactobacillus* spp. For this endeavor, this study investigated the presence of the genus *Lactobacillus* in 41 caries-free participants. It was verified that only 66% of caries-free individuals were colonized by *Lactobacillus* species. These data agree with Ramamurthy et al. (2014), who studied the prevalence of *Lactobacillus* in 50 children aged between 2 and 5 years, with 25 caries-free and 25 caries-affected children. These authors found a significant increase in the *Lactobacillus* colonization rate in the oral cavity from children with active caries (88%) in comparison to caries-free children (60%). The presence of *Lactobacillus* in the oral cavity is correlated with active caries or with a greater predisposition to future caries. This is due to some *Lactobacillus* strains having acidogenic properties and the ability to co-aggregate with other cariogenic microorganisms during tooth colonization (Ahola et al. 2002; Badet and Thebaud 2008).

Thirty *Lactobacillus* isolates were identified, and it was found that *L. paracasei* was the most prevalent species, corresponding to 76% of the total population. In a similar study, Koll-Klais et al. (2005) isolated and identified *Lactobacillus* species from 15 chronic periodontitis patients and found 31 isolates; however, the most prevalent strains were *Lactobacillus gasseri* and *Lactobacillus fermentum*. Shimada et al. (2015) investigated the distribution of oral

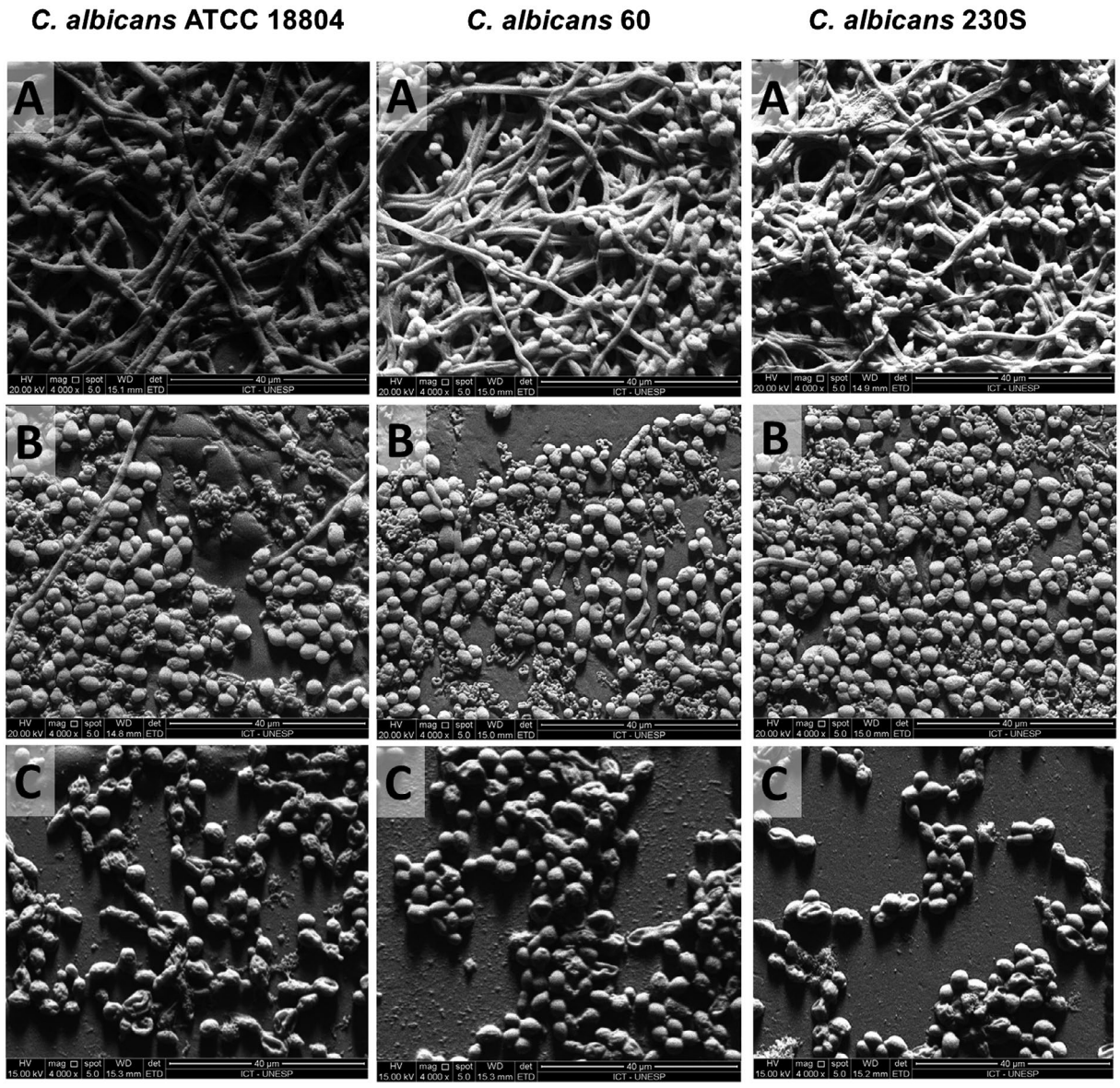

Figure 4. SEM of biofilms formed *in vitro*. (A) Control group of *C. albicans* + PBS: the presence of numerous yeast and hyphae is verified. (B) Group interaction of *C. albicans* + *L. paracasei* 28.4: in all mixed biofilms, it is possible to observe a reduction in the number of yeast cells and hyphae of *C. albicans* and an intimate interaction between *C. albicans* and *Lactobacillus* cells. (C) Group interaction of *C. albicans* + supernatant of *L. paracasei* 28.4: in all supernatant-treated biofilms, the number of yeast and hyphae of *C. albicans* are reduced, and the yeast morphology is altered. Magnification: 4,000×.

Lactobacilli among Japanese preschool children with various prevalence levels of caries. The authors verified that *L. gasseri* and *L. salivarius* were the most detected species in the dental cavity and carious lesions, suggesting that these species strongly contribute to the development of dental caries. The present results differ from previous reports because the strains isolated in this study came from caries-free individuals without any apparent oral disease.

To identify the isolates of *Lactobacillus* with the most prominent antifungal effects, the collection of isolates was subjected to interrogation in the presence of planktonic cultures and biofilms models in order to evaluate any reduction in the fungal cell population. All *C. albicans* strains used in this study were evaluated in association with *Lactobacillus* cells or with supernatant of *Lactobacillus* culture. Most *Lactobacillus* isolates exerted an antimicrobial activity against *C. albicans* ATCC 18804 when this yeast was placed in contact with the cells or supernatant of *Lactobacillus*. The percentage reductions varied between the 30 *Lactobacillus* strains that were tested, indicating that the antifungal effect was *Lactobacillus* species- and strain-specific. The number of *C. albicans* ATCC 18804 cells was reduced by up to 98% for planktonic cultures.

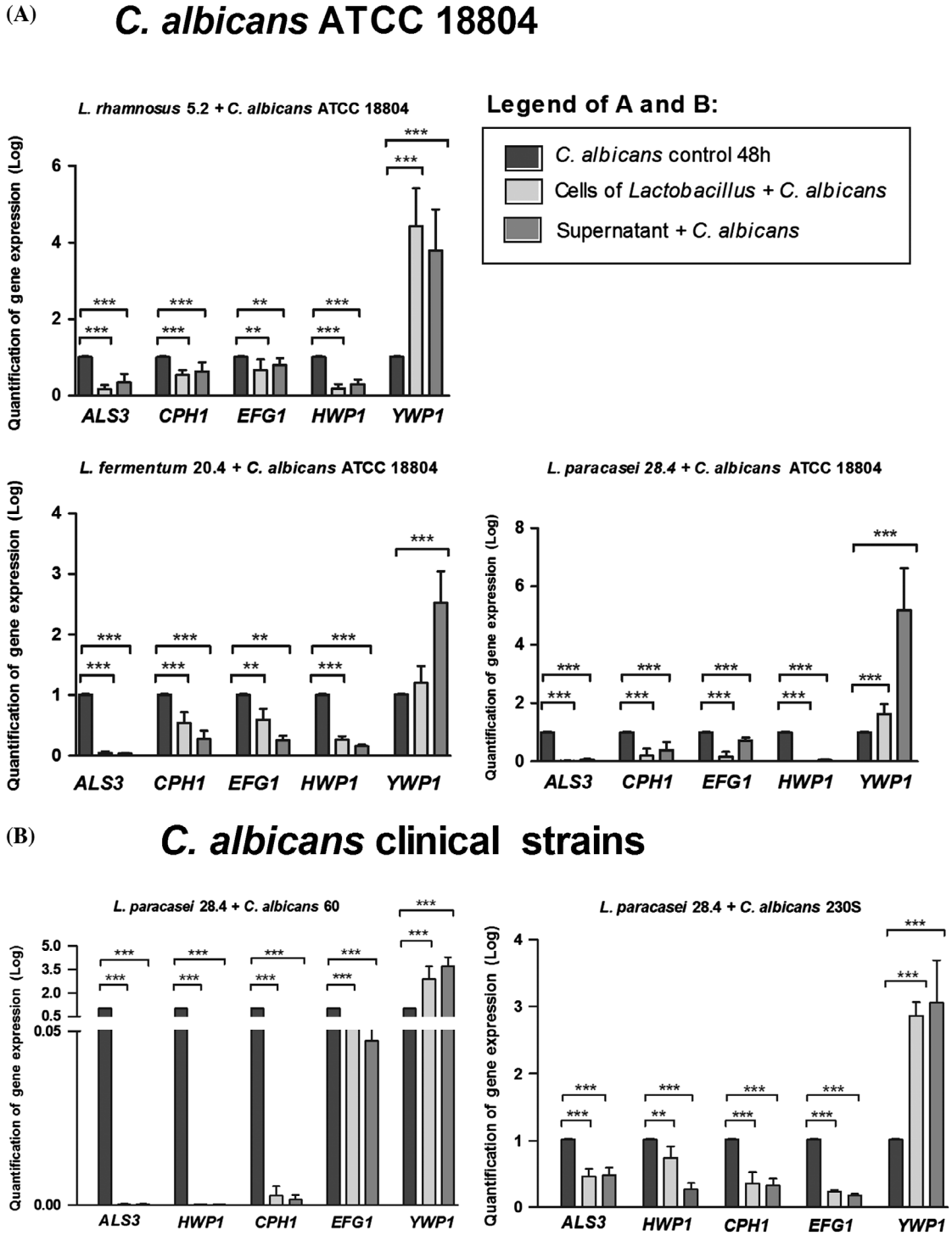

Figure 5. Relative quantification of *ALS3*, *HWP1*, *CPH1*, *EFG1*, and *YWP1* in monotypic and mixed biofilms of *C. albicans* associated with *Lactobacillus* cells and their supernatants. (A) *C. albicans* ATCC 18804 associated with *L. rhamnosus* 5.2; *C. albicans* biofilms associated with *L. fermentum* 20.4; *C. albicans* biofilms associated with *L. paracasei* 28.4. (B) *C. albicans* strains CA60 and CA230S associated with *L. paracasei* 28.4. For the adhesion gene (*ALS3* and *YWP1*), biofilm genes (*CPH1* and *EFG1*) and filamentation gene (*HPW1*): each evaluated group was normalized and compared with *C. albicans* + PBS (control). Values were expressed as the mean and SD. The Student's *t*-test was used to compare gene expression among the studied groups ($p \leq 0.05$). **Indicates $p \leq 0.01$. ***Indicates $p \leq 0.0001$.

In general, the reduction in *C. albicans* ATCC 18804 biofilms associated with *Lactobacillus* cells was 78%. Similar results were found by Vilela et al. (2015) who evaluated the influence of *L. acidophilus* ATCC 4356 cells or its supernatant on biofilm formation of *C. albicans*. A reduction in the number of *C. albicans* cells compared to

the control group was observed for both groups, with a reduction of 57.5% for biofilms associated with probiotic cells and 45.1% for the corresponding supernatant. van der Mei et al. (2014) investigated biofilm formation by *C. albicans* in silicon pads with different strains of *Lactobacillus*. To form the biofilms, silicone pads were submerged in a suspension of the microbial species for 5 h and, after that time, the biofilm was incubated for eight days at 37°C. The authors found a 99% reduction in *C. albicans* due to an association with *L. crispatus*, 98% for *L. acidophilus* and 95% for *L. paracasei*.

A significant reduction in the CFU ml^{-1} count of *C. albicans* ATCC 18004 with the supernatants from the *Lactobacillus* cultures was also observed. These data demonstrated a competitive interaction between the two tested species and suggested that the presence of substances in the bacterial metabolites converted the medium into a hostile environment for fungal biofilm development. In a recent study, Matsubara et al. (2016) evaluated the effect of *L. rhamnosus*, *L. casei*, and *L. acidophilus* supernatants on different stages of biofilm formation, such as after incubation for 90 min, 24 h and 48 h. They found that the supernatants from the first phase of incubation (90 min) were unable to inhibit *C. albicans* biofilm development, and the inhibitory effect was observed only with the 24- and 48-h supernatants of the lactobacilli. Their findings strengthen the results found in the present study which show that the 24-h *Lactobacillus* culture supernatant was sufficient for an inhibitory effect. Moreover, these data suggest that the exometabolites produced by the *Lactobacillus* strains that inhibited *C. albicans* biofilm formation require a mature probiotic growth. In addition, the supernatant can inhibit *C albicans* due to the high levels of organic acid produced by *Lactobacillus* metabolism and consequently reduce the final pH of the environment (Simark-Mattsson et al. 2009; Matsubara et al. 2016; Ribeiro et al. 2017).

Based on the CFU counting results of the *C. albicans* ATCC 18804 biofilms, *L. rhamnosus* 5.2, *L. fermentum* 20.4 and *L. paracasei* 28.4 were selected for subsequent experiments, as they showed the greatest ability to reduce biofilm formation by the fungus. Thus, the capacity of these bacterial strains to alter the amounts of biofilm was investigated. This analysis allowed estimation of the quantity of total biofilm cells and extracellular matrix compared to the viable cells counting method (CFU ml^{-1}). In the total biomass quantification assay, a statistically significant difference was found between single *C. albicans* biofilms compared to mixed biofilms with *Lactobacillus* strains. For the mixed group with *C. albicans* ATCC 18804, the greatest reductions were achieved by *L. paracasei* 28.4, *L. rhamnosus* 5.2 and *L. fermentum* 20.4. In the biofilms treated with the supernatant, there was no difference between the *Lactobacillus* strains that were used.

For the CFU count with clinical strains of *C. albicans*, significant differences were also observed when associated with the cells or supernatant of *L. paracasei* 28.4. For the CA60 strain, a reduction of 80% was found in the cell group and 95% in the supernatant group, respectively. The CA230S strain was reduced by 97% when associated with *L. paracasei* 28.4 cells and 99% with their supernatant. Regarding the quantification of the biomass by CV, all the groups associated with the cells or the supernatant of *L. paracasei* 28.4 showed significant reductions compared to the control group.

The biofilms were also evaluated by SEM analysis in which adherence of *Lactobacillus* strains on *C. albicans* yeasts was observed, showing an intimate association between these two microorganisms. In addition, it was possible to verify a reduction in the number of yeast cells and hyphae of *C. albicans* when the single biofilm was compared to the mixed biofilm for all the *C. albicans* strains in this study. According to the SEM images, *L. paracasei* 28.4 reduced the adhesion of *C. albicans* cells to the plastic surface, and this probably caused the reduction in the *C. albican*s CFU counting and total biomass assay. Biosurfactants are microbial compounds (eg exometabolites of lactobacilli) that reduce the hydrophobicity of the surface substratum and consequently alter microbial adhesion (Satpute et al. 2016; Sharma and Saharan 2016). Ceresa et al. (2015) showed that the biosurfactant produced by *Lactobacillus brevis* was able to reduce adhesion and biofilm formation in *C. albicans* by up to 90% on silicone pads. This mechanism may have acted to reduce the adhesion of *C. albicans* to *Lactobacillus*-treated biofilms in this study.

Previous studies have also demonstrated that lactobacilli could alter the architecture of *C. albicans* biofilms and suggested that this effect can be related to the downregulation of genes involved in biofilm formation as well as those associated with DNA replication, translation, glycolysis, and gluconeogenesis (Kohler et al. 2012; Chew et al. 2015; Matsubara et al. 2016). However, to elucidate the mechanisms involved in the reduction of biofilm formation and filamentation of *C. albicans* observed in the analyses by CFU counts, the total amount and the SEM, the present study was expanded at the level of gene expression to determine the involvement of *Lactobacillus* cells and corresponding supernatants on gene expression of *ALS3*, *CPH1*, *EFG1*, *HWP1*, and *YWP1*.

The capacity to form biofilms has been associated with the presence of transcriptional regulatory genes in *C. albicans* and these include the transcriptional regulators *EFG1*, *BCR1*, *TEC1*, *NDT80*, and *ROB1* (Maiti et al. 2015; Gulati and Nobile 2016). Hyphal formation contributes to the overall architectural stability of the biofilm and acts as a support for yeast cells, pseudohyphae and other

microbial cells in the context of polymicrobial biofilms. Thus, the ability to form hyphae as well as the ability of these hyphae to adhere to other cells is critical for biofilm development and maintenance (Gulati and Nobile 2016). For example, the *HWP1* gene is expressed on the hyphal surface and encodes a cell wall protein; according to Fan et al. (2013), biofilms lacking this gene are prone to detachment from the abiotic substratum.

The *ALS3* gene encoding a protein similar to alpha-agglutinin plays an essential role in the adhesion of *C. albicans* and serves as an upstream regulator of both the Ras1-cAMP-Efg1 and MAPK pathways (Maiti et al. 2015). *CPH1* can act in concert with another regulator of morphogenesis, *EFG1*, the transcription factor of the cAMP–PKA pathway. The *cph1efg1Δ* double mutant exhibited loss of function of almost the entire transcription machinery that regulates filamentation, and the mutant was avirulent in a candidiasis mouse model (Lo et al. 1997). Recently, *CPH1* has been implicated in the maintenance of cell wall organization, pseudohyphal formation in response to oxidative stress, biofilm formation and in regulation of the pheromone response in both white and opaque phase cells (Morschhauser 2011; Srinivasa et al. 2012; Maiti et al. 2015). The *YWP1* gene plays a key role in adhesion and dispersal of the yeast form in biofilms. This gene is highly expressed during exponential-phase growth and is strongly suppressed under conditions that promote filamentation. Furthermore, deletions of *YWP1* results in strains with normal growth rates and morphologies, but increased adhesiveness of the yeast forms (Granger et al. 2005; Plaine et al. 2008; Noble et al. 2010; Granger 2012).

In the present expression profiles, most of the *C. albicans* genes that were studied (*CPH1*, *ALS3*, *HWP1*, and *EFG1*) were downregulated after exposure to *Lactobacillus*, either by direct cell–cell contact or *via* supernatants, both in the reference and clinical strains. This result can be associated with the decrease in the CFU counts, the total biomass and, consequently, the development of biofilm. These results agree with previous studies showing the ability of certain bacteria, such as *Lactobacillus*, to induce the downregulation of *C. albicans* genes (Kohler et al. 2012; James et al. 2016; Matsubara et al. 2016; Ribeiro et al. 2017).

YWP1 was the only gene regulated positively in the groups with *Lactobacillus* associated with reference and clinical strains of *C. albicans.* This positive regulation is due to the predominance of yeasts in the biofilms treated with *Lactobacillus* cells or their supernatant in comparison with the large amount of hyphae found in the control biofilm. This suggests a reduction in adhesiveness of the yeast forms and their consequent dispersion in the formed biofilm. However, the exact molecular mechanisms related to the action of *Lactobacillus* against *C. albicans* biofilms are still unclear and need to be further investigated, eg *via* analyses involving fractionation and the characterization of the supernatant components of *Lactobacillus* strains using liquid chromatography-mass spectrometry and nuclear magnetic resonance spectroscopy.

In conclusion, the findings of the present study show that *L. fermentum* 20.4, *L. paracasei* 28.4, and *L. rhamnosus* 5.2 decrease *C. albicans* ATCC 18804 biofilm formation by reducing the number of *C. albicans* cells, by inhibiting hyphal formation, and by destabilizing the biofilm architecture. These effects also occurred when only the supernatant of *Lactobacillus* strains was added to the *C. albicans* biofilms, suggesting that *Lactobacillus* strains can produce acids or exometabolites capable of inhibiting *C. albicans* growth. *L. paracasei* 28.4 also reduced the biofilm formed by CA60 and CA230S strains corroborating its anti-biofilm action. In addition, it was concluded that the inhibitory effects of *Lactobacillus* on *C. albicans* biofilms in this study are associated with the downregulation of the expression levels of the *ALS3*, *HWP1*, *CPH1*, and *EFG1* genes. In summary, *L. fermentum* 20.4, *L. paracasei* 28.4, and *L. rhamnosus* 5.2 have the potential to be used as probiotics in the oral cavity to prevent the development of oral candidiasis.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [grant numbers 2013/26820-4, 2013/25181-8, 2015/09770-9]; and from the Brown/Brazil Initiative.

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