



## Effects of *Bacillus subtilis* on *Candida albicans*: biofilm formation, filamentation and gene expression

Efeitos de *Bacillus subtilis* sobre *Candida albicans*: formação de biofilme, filamentação e expressão gênica

Michelle Peneluppi SILVA<sup>1</sup>, Patrícia Pimentel de BARROS<sup>1</sup>, Adeline Lacerda JORJÃO<sup>1</sup>, Rodney Dennis ROSSONI<sup>1</sup>, Juliana Campos JUNQUEIRA<sup>1</sup>, Antonio Olavo Cardoso JORGE<sup>1</sup>.

1 – Univ Estadual Paulista (Unesp) - Institute of Science and Technology, São José dos Campos - Department of Biosciences and Oral Diagnosis – São José dos Campos – SP - Brazil.

### ABSTRACT

**Objective:** The aim of this study was evaluate the effect of *Bacillus subtilis* on *Candida albicans* biofilm formation and filamentation by evaluating the gene expression of *ALS3*, *HWP1*, *BCR1*, *EFG1* and *TEC1*. **Material and Methods:** Mixed (*C. albicans* / *B.subtilis*) and monotypic biofilms were cultured in plates at 37°C for 48 h under shaking for counting viable cells (CFU / mL) and analysis of gene expression by real-time PCR. The *C. albicans* filamentation assay was performed in medium containing 10% fetal bovine serum at 37°C for 6 hours. Data was analysed by *t*-Student and Mann–Whitney tests. **Results:** *B. subtilis* reduced the biofilm formation of *C. albicans* in 1 log when cultured in the same environment ( $p < 0.0001$ ). In addition, it significantly reduced the yeast - hypha transition affecting the morphology of *C. albicans*. Among all of the analyzed genes, the *ALS3* and *HWP1* genes were the most affected, achieving 111.1- and 333.3-fold decreases in the *C. albicans* biofilms associated with *B. subtilis*, respectively. **Conclusion:** *B. subtilis* reduced the biofilm formation and filamentation of *C. albicans* by negatively regulating the *ALS3*, *HWP1*, *BCR1*, *EFG1* and *TEC1* genes that are essential for the production of biofilm and hyphae.

### KEYWORDS

*Bacillus subtilis*; *Candida albicans*; Biofilme; Filamentation; Gene expression.

### RESUMO

**Objetivo:** O objetivo deste estudo foi avaliar o efeito de *Bacillus subtilis* sobre a formação de biofilme e filamentação de *Candida albicans* através da avaliação da expressão dos genes *ALS3*, *HWP1*, *BCR1*, *EFG1* and *TEC1*. **Material e métodos:** Biofilmes monotípicos e mistos (*C. albicans* / *B.subtilis*) foram cultivados em placas a 37°C por 48 h sob agitação, para a contagem de células viáveis (UFC/mL) e para a análise da expressão gênica por PCR em tempo real. O ensaio de filamentação de *C. albicans* foi realizado em meio contendo 10% de soro fetal bovino a 37°C por 6 h. Os dados obtidos foram analisados por testes *t*-Student e Mann–Whitney. **Resultados:** *B.subtilis* reduziu em 1 log a formação de biofilme por *C. albicans* quando cultivados no mesmo ambiente ( $p < 0.0001$ ). Além disso, reduziu significativamente a transição de levedura para hifa, afetando assim, a morfologia de *C. albicans*. Em relação aos genes analisados, os genes *ALS3* e *HWP1* foram os mais regulados negativamente, com uma diminuição de 111,1 e 333,3 vezes, respectivamente, na sua expressão em biofilmes de *C. albicans* associados a *B. subtilis*. **Conclusão:** *B. subtilis* reduziu a filamentação e a formação de biofilme de *C. albicans* através da regulação negativa dos genes *ALS3*, *HWP1*, *BCR1*, *EFG1* e *TEC1*, que são essenciais na produção de hifas e de biofilme.

### PALAVRAS-CHAVE

*Bacillus subtilis*; *Candida albicans*; Biofilme; Filamentação; Expressão gênica.

## INTRODUCTION

*Candida albicans* is a commensal fungus found on mucosal surfaces of the oral cavity, gastrointestinal and genitourinary tracts. This yeast causes severe and recurrent mucosal infections as well as fatal invasive infections in both immunocompromised and immunocompetent individuals [1].

Some virulence factors such as dimorphism and the ability to adhere and form biofilm on medical devices and/or the host mucosal epithelium, enhance the pathogenicity of *C. albicans*. The cells in biofilms exhibit distinct phenotypes that are similar to their free-living counterparts, including extreme resistance to many antimicrobial agents. Advances in genetic manipulation and expression profiling have helped define the regulatory pathways and mechanisms that govern *C. albicans* biofilm development [1,2,3]. Several classes of genes, such as specify transcription factors (*EFG1*, *BCR1*, *TEC1*) and adhesion (*HWP1*, *ALS3*), control biofilm development on abiotic and biotic surfaces [4,5].

In the recent past, microbial secondary metabolites have engrossed great attention of researchers as antifungal compounds. Most of the antifungal compounds currently in use are synthetic derivatives with known serious side effects and toxicity [6]. Emergence of drug resistant *Candida* has augmented the desire for new biocompatible antifungal drugs [7,8].

The genus *Bacillus* includes a great diversity of industrially important strains, including *Bacillus subtilis*. This spore-forming bacterium has been established as industrial bacteria in the production of biological indicators for sterilization, in studies of biodefense and astrobiology methods as well as disinfection agents, in treatment evaluation and as potential adjuvants or vehicles for vaccines, among other applications. The metabolic diversity and lack of reported incidence of pathogenicity makes this bacterium the most suitable for the

development of marketable products [9].

*Bacillus* strains could produce some metabolites that inhibit pathogens, since some *Bacillus* species used in commercially available products have the ability to produce antimicrobials, such as aminocoumarin A [10] and bacteriocin [11,12]. Zheng and Slavik [11] isolated and characterized a bacteriocin (molecular weight of approximately 34 kDa and a pI value of approximately 47) of *B. subtilis* 22. Firstly, the precipitated supernatant from the *B. subtilis* 22 culture had inhibitory activity in nine strains of Gram-negative and Gram-positive bacteria by agar diffusion assay. After partial purification of supernatant, the bacteriocin was named bacillocin 22 making this peptide potentially useful against the food-borne pathogens. In a recent study, Mayer and Kronstad [13] have identified the soil bacterium, *Bacillus safensis*, as a potent inhibitor of virulence factor production by two major fungal pathogens of humans, *Cryptococcus neoformans*, and *C. albicans*. They determined that the anti-virulence factor mechanism was, at least in part, based on production of bacterial chitinases that target and destabilize the fungal cell surface.

In this context, in an attempt to investigate new compounds and/or metabolites with antifungal properties produced by a sporulated bacillus, the current study was evaluate the effect of *B. subtilis* on *C. albicans* biofilm formation and filamentation by evaluating the gene expression of *ALS3*, *HWP1*, *BCR1*, *EFG1* and *TEC1*.

## MATERIAL AND METHODS

### Microorganisms and growth conditions

In this study, we used the reference strains of *B. subtilis* (ATCC 19659) and *C. albicans* (ATCC 18804); these strains were plated on Brain Heart Infusion Agar (Difco, Detroit, USA) and Sabouraud Dextrose Agar (Difco, Detroit, USA), respectively, and were

incubated at 37 °C for 24 h. After growth, bacterial colonies were transferred and cultured in Brain Heart Infusion Broth (BHI) (Difco Laboratories Inc., Detroit, MI, USA) and the yeast in Yeast Nitrogen Base - YNB (Difco Laboratories Inc., Detroit, MI, USA) at 37 °C for 24 h.

### Biofilm formation

Suspensions of each microorganism sample were adjusted to 10<sup>7</sup> cells/mL using a spectrophotometer (B582, Micronal, São Paulo, Brazil). The formation of monotypic and mixed biofilms was based on the methodology described by Seneviratne et al. [14] and Bridier et al. [15] with modifications. Biofilms were formed in the bottom of a 96-well microtiter plate (TPP®, Trasadingen, Switzerland), by pipetting 100 µL of a standardized suspension containing 10<sup>7</sup> cells/mL of each microorganism, *C. albicans* and *B. subtilis*, associated in each well to the formation of a mixed biofilm. For monotypic biofilm formation, 100 µL of only one microorganism and 100 µL BHI broth were pipetted in each well. The plates were incubated under shaking at 75 rpm rotation (Quimis, Diadema, Brazil) for 90 min at 37 °C with only the microorganisms to facilitate initial adhesion.

After adhesion phase, the suspension of non-adhered microorganisms was aspirated, and each well was washed with 200 µL of 0.85% NaCl to remove the non-adhered cells; 200 µL of BHI broth was added in all formed biofilms, both monotypic and mixed groups. All plates were incubated at 37°C for 48 h under shaking at 75 rpm rotation. After 24 h, the broth was changed and after 48 h, the biofilms were aspirated and washed with 0.85% NaCl to remove the non-adhered cells. Biofilms were evaluated in relation to cell viability (quantification of the colony forming units - CFU/mL) and gene expression in interspecies interaction. For each of these evaluations the experiment was performed in triplicate at two different times with 10 trials for each experimental group, resulting in 180 trials.

### Quantification of viable cells (CFU/mL)

After washing the biofilm, 200 µL of 0.85% NaCl was added in each well for homogenization during 30 s in an ultrasonic homogenizer (Vibra Cell-Sonics & Materials, Inc. Newtown, Connecticut, USA), with 25% amplification to detach the biofilms. Serial dilutions of suspensions were performed, and 100 µL aliquots of each dilution were plated in the culture medium, according to the microorganism: BHI Agar supplemented with fluconazole for *B. subtilis* and HiCrome *Candida* Differential Agar (Himedia, Mumbai, India) for *C. albicans*. The plates were incubated at 37 °C for 48 h to count the CFU/mL and converted into logarithm to base 10 (Log).

### *C. albicans* filamentation production

The filamentation assay was performed in 24-well microtiter plates (TPP®, Trasadingen, Switzerland) following a methodology described previously with modifications [16]. The experimental groups formed were: *C. albicans* +PBS (Phosphate Buffered Saline) (control group) and *C. albicans* + *B. subtilis* (interaction group). After 18 hours of growth *C. albicans* and *B. subtilis* were centrifuged to prepare standard suspensions corresponding to 10<sup>7</sup> cells/mL in a spectrophotometer as previously described. In 24-well cell culture plates, 1 mL distilled water supplemented with 10% bovine fetal serum was added to a 100 µL *C. albicans* suspension. Then, a 100 µL *B. subtilis* suspension was added to each well. The control group used 100 µL PBS.

The plate was incubated at 37°C for 6 h and 50 µL of the inoculum contained in each well was placed on microscopic glass slides with 10 fields previously marked on the back and observed under an optical microscope with a magnification of 400 x. The images were analyzed in relation to *C. albicans* morphology and hyphae quantification in 10 microscopic fields per slide and classified as scores from 0 to 4 attributed to each field according to the number of hyphae present:

score 0: no hyphae; score 1: 1 to 20 hyphae; score 2: 21 to 40 hyphae; score 3: 41 to 50 hyphae; and score 4: more than 50 hyphae. Each experimental group had 5 trials at two different times, totaling 20 tests. The percentage of germination was calculated considering the mixed group formed by *C. albicans*-*B. subtilis* in relation to monotypic group of *C. albicans*.

#### Evaluation of *ALS3*, *HWP1*, *BCR1*, *EFG1* and *TEC1* gene expression with qPCR

The evaluation of *C. albicans* virulence genes was based on the methodology described by Barros et al. [17] with modifications. Biofilms were formed in 24-well microtiter plates (TPP®, Trasadingen, Switzerland) with the same concentration and conditions as in the “Biofilm formation” section. The expression of *ALS3*, *HWP1*, *BCR1*, *EFG1* and *TEC1* genes was evaluated in relation to four reference genes, *ACT1* ( $\beta$ -Actin), *RIP1* (Ubiquinol Cytochrome-c Reductase Complex Component), *PMA1* (Adenosine Triphosphatase) and *LSC2* (Succinyl-CoA Synthetase  $\beta$ -Subunit Fragment), described in the literature [18], in all experimental groups. The results were analyzed at <http://www.leonxie.com/referencegene.php>, and the reference gene *RIP1* was selected for this experiment.

Total RNA was extracted using the Trizol reagent (Ambion®, Carlsbad, USA), according to the manufacturer’s recommendations. The purity, quality and concentration of the RNA were analyzed by the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA). To remove contaminating DNA, RNA (1 $\mu$ g) extracted was treated with DNase I (Turbo DNase Treatment and Removal Reagents - Invitrogen™, Carlsbad, USA) and transcribed into complementary DNA (cDNA) using the SuperScript™ III First-Strand Synthesis Super Mix for qRT-PCR Kit with SYBR®Green (Invitrogen™, Carlsbad, USA), according to the protocol recommended by the manufacturer.

Transcribed cDNA was amplified for the relative quantification of *ALS3*, *HWP1*, *BCR1*, *EFG1* and *TEC1* gene expression levels in relation to the concentration of the selected reference gene, *RIP1*. Quantitative real-time PCR was conducted using the Platinum® SYBR® Green qPCR SuperMix-UDG Kit (Applied Biosystems, Framingham, MA, USA) in the StepOnePlus™ apparatus (Applied Biosystems, Framingham, MA, USA). The 2<sup>-DDCT</sup> method was used to analyze the relative changes in gene expression from the quantitative RT-qPCR experiment [19].

#### Statistical analysis

Student’s *t*-test was used to compare the CFU/mL results from the *in vitro* biofilm formation assay and the relative quantification of gene expression. The scores obtained from the filamentation analysis were compared using the Mann–Whitney test. All analyses were performed using the GraphPad Prism 5 Program (GraphPad Software, Inc., USA) and 5% level of significance was adopted.

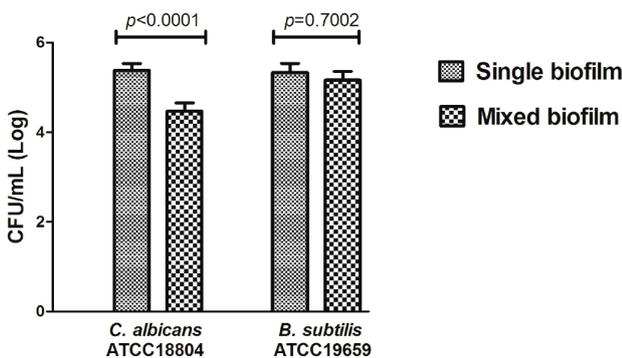
## RESULTS

The CFU/mL count of *C. albicans* in monotypic biofilms showed higher values compared to mixed biofilms formed by associations between *C. albicans* and *B. subtilis* (Figure 1). Comparing the results obtained in monotypic biofilms with mixed biofilms, there was a reduction of 1.0 log of *C. albicans* with a statistically significant difference ( $p = 0.001$ ). *B. subtilis* was not affected in the mixed biofilm compared to monotypic ( $p = 0.7002$ ). The results, expressed in mean and standard deviation, for CFU/mL counts of *C. albicans* in monotypic biofilms were  $5.38 \pm 0.149$  log and  $4.37 \pm 0.183$  in mixed biofilms. For *B. subtilis*, the means were  $5.33 \pm 0.202$  log in monotypic biofilms and  $5.11 \pm 0.179$  in mixed biofilms.

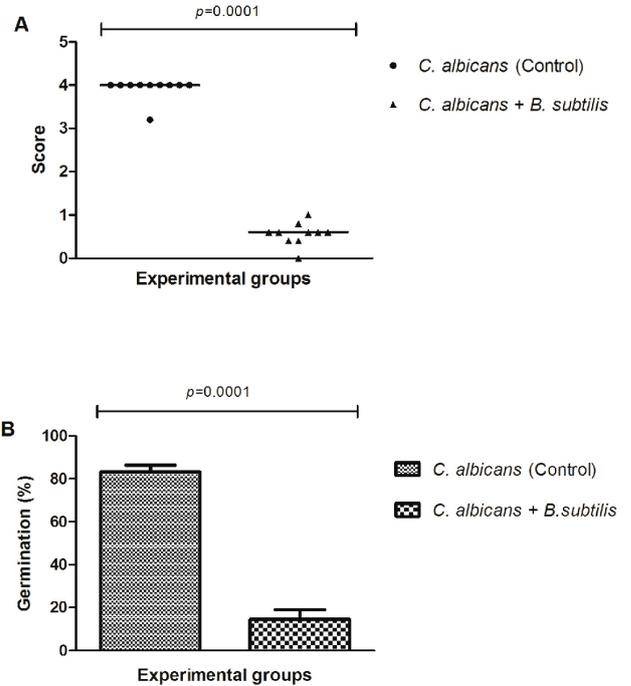
In relation to the filamentation test, the results showed reduction of morphological transition (yeast/hyphae) for *C. albicans* in

the presence of *B. subtilis* (*C. albicans* and *B. subtilis* interaction), with a statistically significant reduction ( $p=0.0001$ ) compared to the control group with PBS (Figure 2).

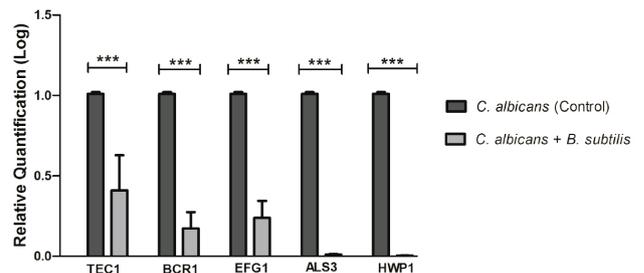
To elucidate the inhibitory mechanisms of *B. subtilis* on *C. albicans* biofilms, this study was extended to analyze *C. albicans* gene expression, concentrating on genes involved in biofilm formation and filamentation. The expression levels of the adhesion genes (*ALS3* and *HWP1*) and transcriptional regulatory genes (*TEC1*, *BCR1* and *EFG1*) were quantified in *C. albicans* cells from monotypic and mixed biofilms using qPCR. In *C. albicans* and *B. subtilis* association, *ALS3*, *HWP1*, *BCR1*, *TEC1* and *EFG1* genes were downregulated when compared to the *C. albicans* control group with statistically significant differences ( $p=0.0001$ ) (Figure 3). Among all of the analyzed genes, the *ALS3* and *HWP1* genes were the most affected, achieving 111.1- and 333.3-fold decreases in the *C. albicans* biofilms associated with *B. subtilis*, respectively. These results agreed with those obtained for the CFU/mL counts and filamentation assay and suggest that *B. subtilis* exerts inhibitory effects on *C. albicans* by interfering with yeast adherence during biofilm formation and reducing its development during filamentation.



**Figure 1** - Quantitative analysis of *in vitro* biofilm formation by CFU/mL count: Means and standard deviations of *C. albicans* ATCC18804 and *B. subtilis* ATCC19659 CFU/mL (Log) values in the following groups: monotypic, formed by *C. albicans* (Control group); mixed, formed by *C. albicans* + *B. subtilis*; monotypic, formed by *B. subtilis*; mixed, formed by *C. albicans* + *B. subtilis*. Student's *t*-test,  $p \leq 0.05$ .



**Figure 2** - *Bacillus subtilis* reduce *C. albicans* filamentation. (A): Median scores obtained by determining the number of hyphae in the *in vitro* *Candida*, filamentation assay for the following groups: *C. albicans* control group, interaction group with *B. subtilis*. A significant hyphal reduction was observed in the interaction group compared to the control group (Mann-Whitney test,  $p \leq 0.05$ ). (B) Percentage germination, expressed as the mean values of hyphae, in the viability of *C. albicans* when associated with *B. subtilis*.



**Figure 3** - *Bacillus subtilis* decreased the expression of *C. albicans* genes involved in biofilm formation. Relative quantification (Log) of *TEC1*, *BCR1*, *EFG1*, *ALS3* and *HWP1* genes in monotypic and mixed biofilms formed by *C. albicans*. The units in the Y-axis were calculated based on the  $2^{-DDCT}$  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 *RIP1*. Student's *t*-test was used to compare gene expression between the groups ( $p \leq 0.05$ ). \*\*\*indicates  $p \leq 0.0001$ .

## DISCUSSION

Biofilm growth is a main model of microbial life and up to 80% of all microorganisms are sessile attached to the surface of biofilm communities. Within this group of microorganisms, 65 to 80% can cause microbial infections in humans from pathogenic biofilm formation [20]. The capability of biofilm formation and morphological transformation from yeast to hyphae are important to *C. albicans* pathogenicity [21]. Biofilms create an area of higher antimicrobial resistance for the residing microorganisms [20]. Thus, the interactions between bacteria and fungi can influence survival and proliferation, which can be beneficial for microorganisms because it increases resistance to antimicrobials or antagonistic that lead to death [22].

In this study, the results demonstrated that *C. albicans* was reduced in association with *B. subtilis* during biofilm formation. The quantification of CFU/mL showed a reduction of 87.44% in *C. albicans* cells when compared to the control group. Thus, it demonstrated that in mixed biofilm the microorganisms interacted competitively and antagonistic, suggesting an inhibitory effect of *B. subtilis* on *C. albicans*. A possible explanation for the reduction of biofilm formation by *C. albicans* during the microbial interaction is the fact that *Bacillus spp.* secrete antimicrobial agents, such as bacteriocins, which have effects for suppressing the growth of competitive and opportunistic microorganisms [8,9]. One example is *Subtilisin A*, which is a bacteriocin isolated from *B. subtilis* [23]. *B. subtilis* in mixed biofilms did not present a significant reduction in relation to the monotypic biofilm. According to López et al. [24] *B. subtilis* uses a mechanism called cannibalism to slow its sporulation, and these cannibal cells secrete two toxins, *skf* and *sdp*, in which they can interfere with the growth of other microorganisms, constituting as a defense mechanism of *B. subtilis*.

Furthermore, we analyzed hyphae formation in *C. albicans*, and the results showed

a significant reduction in the number of hyphae when compared to the control group of *C. albicans*, which confirms the results previously obtained by the inhibitory effect exerted by *B. subtilis* on *C. albicans* biofilm. Ability of *Candida* to reversibly switch between budding yeast and filamentous form is crucial for establishing infection. This phenotypic plasticity is tightly regulated by several environmental cues and internal signaling pathways [25]. The hyphal formation is related to gene expression that encodes virulence factors, such as the hyphae wall proteins *HWP1* and *ALS3* [25].

To confirm the results obtained in the CFU/mL and filamentation assays, we analyzed the expression of *ALS3*, *HWP1*, *BCR1*, *EFG1* and *TEC1*, which are *C. albicans* virulence genes responsible for biofilm formation and morphogenesis of yeast, by the relative quantification method qPCR [21,25]. All of these genes were significantly downregulated compared to monotypic biofilms, demonstrating the reduction effect of *B. subtilis* on morphogenesis and biofilm formation in *C. albicans*. *HWP1* and *ALS3* were the most downregulated genes in the presence of *B. subtilis*, and this result can be associated with the decrease in the CFU counts, the hyphae formation interfering in biofilm formation. Our findings are in agreement with Rautela et al. [8] that evaluated the influence of cyclic lipopeptides, fengycin and iturin, produced by *Bacillus amyloliquefaciens* strain AR2 in *C. albicans* biofilms. They showed that the fungicidal activity was concentration dependent and sub-minimal concentrations of the lipopeptide showed a decrease in the hydrophobicity of the cell surface, formation of germinative tubes and reduced of the hyphae specific genes, such as *HWP1* and *ALS3*.

Our results show the potential antifungal effect of *B. subtilis*, since this species interferes in the transcriptional analysis of *C. albicans*. Recently, Subramenium et al. [26] evaluated the antibiofilm effect of *B. subtilis*, a marine bacterial isolate from Palk Bay against *C. albicans*. This bacterium was able to reduce the biofilm of *C. albicans* by up to 90%, the filamentation and

production of hydrolytic enzymes. In addition, the genes *ERG11*, *FLU1*, *CDR2* and *CDR1* (involved in drug resistance mechanisms) were downregulated when associated with *B. subtilis*.

The interactions between bacteria and fungi are highly complex and many factors need to be analyzed together, such as microorganism virulence associated with environmental factors and interactions between different species of microorganisms present in certain niches, requiring proper development of *in vitro* and *in vivo* models to characterize these interactions. The results from the experimental conditions described in this study demonstrated that *B. subtilis* inhibited *C. albicans*, suggesting that this bacterium produced compounds and/or metabolites with antifungal activity.

## CONCLUSION

In conclusion, this study demonstrated that *B. subtilis* reduced the biofilm formation and filamentation of *C. albicans* by negatively regulating the *ALS3*, *HWP1*, *BCR1*, *EFG1* and *TEC1* genes that are essential for the production of biofilm and hyphae.

## ACKNOWLEDGEMENTS

This study was supported by the São Paulo Council of Research - FAPESP, Brazil (Grants 2012/15250-0, 2013/25181-8 and 2015/09770-9) for supporting this research.

## REFERENCES

- Ganguly S, Mitchell AP. Mucosal biofilms of *Candida albicans*. *Curr Opin Microbiol*.2011;14:380-385. doi:10.1016/j.mib.2011.06.001.
- Ramage G, Mowat E, Jones B, Williams C, Lopez-Ribot J. Our current understanding of fungal biofilms. *Crit Rev Microbiol*.2009;35:340-355. doi:10.3109/10408410903241436.
- Chevalier M, Ranque S, Precheur I. Oral fungal-bacterial biofilm models *in vitro*: a review. *Med Mycol*.2018;56:653-667.doi:10.1093/mmy/myx111.
- Bink A, Govaert G, Vandenbosch D, Kucharikova S, Coenye T, Nelis H, Thevissen K. Transcription factor Efg1 contributes to the tolerance of *Candida albicans* biofilms against antifungal agents *in vitro* and *in vivo*. *J Med Microbiol*.2012;61:813-819.doi: 10.1099/jmm.0.041020-0.
- Lohse MB, Gulati M, Johnson AD, Nobile CJ. Development and regulation of single- and multi-species *Candida albicans* biofilms. *Nat Rev Microbiol*.2018;16:19-31.doi: 10.1038/nrmicro.2017.107
- Onishi J, Meinz M, Thompson J, Curotto J, Dreikorn S, Rosenbach M, et al. Discovery of novel antifungal (1,3)-beta-D-glucan synthase inhibitors. *Antimicrob Agents Chemother*. 2000;44:368-377. PMC89685.
- Zhang X, Jacob MR, Rao RR, Wang YH, Agarwal AK, Newman DJ, Khan IA, Clark AM, Li XC. Antifungal cyclic peptides from the marine sponge. *Res Rep Med Chem*.2012;2:7-14.doi:10.2147/RRMC.S30895.
- Rautela R, Singh AK, Shukla A, Cameotra SS. Lipopeptides from *Bacillus* strain AR2 inhibits biofilm formation by *Candida albicans*. *Antonie Van Leeuwenhoek*.2014;105:809-821.doi:10.1007/s10482-014-0135-2.
- Sella SR, Vandenberghe LP, Socol CR. *Bacillus atrophaeus*: main characteristics and biotechnological applications - a review. *Crit Rev Biotechnol*.2015;35:533-45.doi:10.3109/07388551.2014.922915.
- Pinchuk IV, Bressollier P, Verneuil B, Fenet B, Sorokulova IB, Megraud F, Urdaci MC. *In vitro* anti-*Helicobacter pylori* activity of the probiotic strain *Bacillus subtilis* 3 is due to secretion of antibiotics. *Antimicrob Agents Chemother*. 2001;45:3156-3161.doi:10.1128/AAC.45.11.3156-3161.2001.
- Zheng G, Slavik MF. Isolation, partial purification and characterization of a bacteriocin produced by a newly isolated *Bacillus subtilis* strain. *Lett Appl Microbiol*.1999;28:363-7. PMID:10347890.
- Cladera-Olivera F, Caron GR, Brandelli A. Bacteriocin like substance production by *Bacillus licheniformis* strain P40. *Lett Appl Microbiol*. 2004;38:251-6. PMID:15214721.
- Mayer FL, Kronstad JW. Breaking the bad: *Bacillus* blocks fungal virulence factors. *Microb Cell*.2017;30:384-6. doi: 10.15698/mic2017.11.599.
- Seneviratne CJ, Silva WJ, Jin LJ, Samaranyake YH, Samaranyake LP. Architectural analysis, viability assessment and growth kinetics of *Candida albicans* and *Candida glabrata* biofilms. *Arch Oral Biol*. 2009;54:1052-1060.doi:10.1016/j.archoralbio.2009.08.002.
- Bridier A, Sanchez-Vizuetel Mdel P, Le Coq D, Aymerich S, Meylheuc T, Maillard JY, Briandet R. Biofilms of a *Bacillus subtilis* hospital isolate protect *Staphylococcus aureus* from biocide action. *PLoS One*.2012;7:e44506.doi: 10.1371/journal.pone.0044506.
- Ribeiro FC, de Barros PP, Rossoni RD, Junqueira JC, Jorge AOC. *Lactobacillus rhamnosus* inhibits *Candida albicans* virulence factors *in vitro* and modulates immune system in *Galleria mellonella*. *J Appl Microbiol*. 2017;122:201-211.doi: 10.1111/jam.13324.
- de Barros PP, Rossoni RD, Freire F, Ribeiro FC, Lopes L, Junqueira JC, Jorge AOC. *Candida tropicalis* affects the virulence profile of *Candida albicans*: an *in vitro* and *in vivo* study. *Pathog Dis*. 2018;76:2 doi:10.1093/femspd/fty014.
- Nailis H, Kucharikova S, Ricicova M, Van Dijck P, Deforce D, Nelis H, Coenye T. Real-time PCR expression profiling of genes encoding potential virulence factors in *Candida albicans* biofilms: identification of model-dependent and -independent gene expression. *BMC Microbiol*. 2010;10:114. doi:10.1186/1471-2180-10-114.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-</sup>(Delta Delta C(T)) Method. *Methods*.2001;25:402-408.doi:10.1006/meth.2001.1262.

20. Bonhomme J, d'Enfert C. *Candida albicans* biofilms: building a heterogeneous, drug-tolerant environment. *Curr Opin Microbiol.* 2013;16:398-403. doi: 10.1016/j.mib.2013.03.007.
21. Mayer FL, Wilson D, Hube B. *Candida albicans* pathogenicity mechanisms. *Virulence.* 2013;4:119-128. doi: 10.4161/viru.22913.
22. Peleg AY, Hogan DA, Mylonakis E. Medically important bacterial-fungal interactions. *Nat Rev Microbiol.* 2010;8:340-349. doi: 10.1038/nrmicro2313.
23. Sutyak KE, Wirawan RE, Aroutcheva AA, Chikindas ML. Isolation of the *Bacillus subtilis* antimicrobial peptide subtilisin from the dairy product-derived *Bacillus amyloliquefaciens*. *J Appl Microbiol.* 2008;104:1067-1074. doi:10.1111/j.1365-2672.2007.03626.x
24. Lopez D, Vlamakis H, Losick R, Kolter R. Cannibalism enhances biofilm development in *Bacillus subtilis*. *Mol Microbiol.* 2009;74:609-618. doi:10.1111/j.1365-2958.2009.06882.
25. Tsang PW, Bandara HM, Fong WP. Purpurin suppresses *Candida albicans* biofilm formation and hyphal development. *PLoS One.* 2012;7:e50866. doi: 10.1371/journal.pone.0050866.
26. Subramenium GA, Swetha TK, Iyer PM, Balamurugan K, Pandian SK. 5-hydroxymethyl-2-furaldehyde from marine bacterium *Bacillus subtilis* inhibits biofilm and virulence of *Candida albicans*. *Microbiol Res.* 2018;207:19-32. doi: 10.1016/j.micres.2017.11.002.

**Patrícia Pimentel de Barros  
(Corresponding address)**

Department of Biosciences and Oral Diagnosis  
Univ Estadual Paulista/UNESP  
Av. Eng Francisco José Longo 777, São Dimas, São José dos Campos  
CEP: 12245-000, SP, Brazil.  
E-mail: barrosdnapp@yahoo.com.br

Date submitted: 2018 Nov 13

Accept submission: 2019 Feb 26