RENAN APARECIDO FERNANDES

Efeito do farnesol sobre fatores de virulência de *Candida albicans* e *Streptococcus mutans*

Araçatuba – SP

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Dissertação apresentada à Faculdade de Odontologia do Campus de Araçatuba – Universidade Estadual Paulista "Júlio de Mesquita Filho"- UNESP, para obtenção do Título de MESTRE EM CIÊNCIA ODONTOLÓGICA (Área de concentração em Biomateriais).

Orientadora: Profª. Ass. Drª. Debora de Barros Barbosa

Co-Orientador: Prof. Dr. Douglas Roberto Monteiro

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Fernandes RA. **Efeito do farnesol sobre fatores de virulência de *Candida albicans* e *Streptococcus mutans*** [dissertação]. Araçatuba: Universidade Estadual Paulista; 2015.

RESUMO GERAL

O objetivo deste estudo foi avaliar a atividade da molécula de quorum sensing farnesol na formação de biofilmes simples e misto de *Candida albicans* ATCC 10231 e *Streptococcus mutans* ATCC 25175. Inicialmente o farnesol foi diluído em metanol e posteriormente em saliva artificial (SA). Os testes de concentração inibitória mínima (CIM) do farnesol contra as células de *Candida albicans* ATCC 10231 e *Streptococcus mutans* ATCC 25175 em suspensão foram baseados no método da microdiluição. O farnesol diluído nas concentrações de 1,56, 3,125, 6,25, 12,5, 25, 50, 70, 150 e 300 mM foi aplicado sobre os biofilmes de *Candida albicans* e *Streptococcus mutans* (formação) e após 48 horas de contato sua atividade antibiofilme foi determinada por meio da quantificação da biomassa total (coloração com violeta cristal (CV)), enumeração das unidades formadoras de colônias (UFCs) e atividade metabólica (XTT).

Foi ainda realizada uma curva de tempo de morte celular para os dois microrganismos estudados. Após o tratamento com o farnesol, a matriz dos biofilmes foi extraída e analisada em termos de concentração de proteínas e carboidratos, além do teste de pH para *S. mutans* e verificação das atividades enzimáticas de proteinase, fosfolipase e hemolítica para *Candida albicans,* ainda a estrutura dos biofilmes foi analisada por meio da microscopia eletrônica de varredura. Os resultados de CIM foram de 150 mM para *C. albicans* e de 6.25 mM para *S. mutans*. O farnesol diminuiu a formação de biofilmes simples e mistos, com reduções significativas de 37-90% e 64-96%, respectivamente para a biomassa total e atividade metabólica. Para os biofilmes simples, concentrações de farnesol iguais ou maiores que 3,125 mM promoveram reduções significativas (1,3-4,2 $\log_{10}$; $p < 0,05$) nas UFCs, enquanto que para os biofilmes mistos reduções (0,67-5,32 $\log_{10}$; $p < 0,05$) foram notadas a partir da concentração de 1,56 mM. Para a curva de morte celular com uma hora de contato entre os microrganismos e o farnesol houve uma redução de cerca de 1 $\log_{10}$ para *C.*

albicans e 3 $\log_{10}$ para *S. mutans*. Para a quantificação de proteínas e carboidratos, de forma geral as proteínas sofreram reduções para todas as concentrações de farnesol testadas, exceto para *S. mutans* na concentração de 12.5 mM, enquanto que para a quantidade de carboidratos não foi observado nenhuma redução. Na análise do teste de pH para *S. mutans* ambas as concentrações (0,78 mM e 1,56 mM) de farnesol foram capazes de manter o pH estável, enquanto que para a verificação de atividade enzimática para *C. albicans* não foi notada nenhuma redução na atividade de proteinase, fosfolipase e hemolítica. Finalmente imagens de microscopia eletrônica de varredura mostraram uma redução no número de células nos tratamentos com farnesol, além da diminuição de hifas nos biofilmes mistos. Todos esses achados incentivam a busca por novos estudos e formulações utilizando o farnesol como potencial antibiofilme.

Palavras-chave: Biofilmes**,** *Candida albicans*, *Streptococcus mutans*, Farnesol, fatores de virulência.

Fernandes RA. **Effect of farnesol on virulence factors of *Candida albicans* and *Streptococcus* mutans** [thesis]. Araçatuba: UNESP - São Paulo State University; 2015.

GENERAL ABSTRACT

The aim of this study was to evaluate the activity of a quorum sensing molecule, farnesol, in biofilm formation of *Candida albicans* ATCC 10231 and *Streptococcus mutans* ATCC 25175. Initially farnesol was diluted in methanol and then in artificial saliva (AS). Minimum inhibitory concentration tests (MIC) of farnesol against *Candida albicans* ATCC 10231 cells and *Streptococcus mutans* ATCC 25175 suspension were based on the microdilution method. Farnesol was prepared at concentrations of 1.56, 3.125, 6.25, 12.5, 25, 50, 70, 150 and 300 mM, and placed in contact with biofilms in formation of *Candida albicans* and *Streptococcus mutans* for 48 hours. Its antibiofilm activity was determined by quantifying the total biomass (stained with crystal violet (CV)), enumeration of colony forming units (CFUs) and metabolic activity (XTT). It was further carried out a cell death time curve for both microorganisms studied. After the treatment with farnesol, the matrix of the biofilm was extracted and analyzed in terms of protein and carbohydrates, in addition to the pH test for *S. mutans* and examination of enzymatic activities of proteinase, fosfolipase and hemolytic for *Candida albicans*. The structure of biofilms was analyzed by scanning electron microscopy. The MIC values were 150 mM for *C. albicans* and 6.25 mM for *S. mutans*. Farnesol decreased the formation of single and mixed biofilms, with significant reductions of 37-90% and 64-96% respectively for the total biomass and metabolic activity. For single biofilms, farnesol concentrations equal to or

higher than 3.125 mM promoted significant reductions (1,3-4,2log10; $p < 0.05$) in the CFU for single biofilms, while in mixed biofilms the reductions (0,67-5,32log10; $p < 0.05$) were noted at concentrations of 1.56 mM. For cell death curve after an hour in contact with those microorganisms, farnesol reduced 1 log_{10} for *C. albicans* and 3 log_{10} for *S. mutans*. For quantification of proteins and carbohydrates, proteins generally were reduced by farnesol at all concentrations tested except for *S. mutans* at 12.5 mM, whereas was not observed any reduction for the amount of carbohydrate. In the analysis of pH the concentrations of 0.78 and 1.56 mM were able to maintain the pH stable for *S. mutans* biofilm. For enzymatic activity for *C. albicans* were not noticed any reduction in the proteinase and phospholipase activity and hemolytic. Finally, the scanning electron microscopy suggested a reduction in the number of cells in all biofilms treated with farnesol, besides decreasing the hyphae emergence in mixed biofilms. All these findings encourage the search for new studies and future formulations using farnesol as an antibiofilm treatment.

Keywords: Biofilms, *Candida albicans*, *Streptococcus mutans*, Farnesol, virulence factors.

LISTA DE FIGURAS

LISTA DE TABELAS

SUMÁRIO

INTRODUÇÃO GERAL

1. INTRODUÇÃO GERAL

No corpo humano existem inúmeras áreas que permitem a colonização de microrganismos e o desenvolvimento de biofilmes. Entre elas, a cavidade bucal é uma das áreas que apresenta condições bastante favoráveis para a formação de biofilmes patogênicos (Jakubovics and Kolenbrander 2010). Os biofilmes são um conjunto de microrganismos organizados, envoltos em uma matriz extracelular e aderidos a superfícies bióticas ou abióticas em um ambiente aquoso (Costerton et al. 1999; Ramage et al. 2005). O surgimento de doenças possui relação direta com a presença de biofilmes, que podem expressar fatores de virulência diversos. Dentre as patologias bucais relacionadas diretamente com biofilmes, destacam-se a candidíase bucal e a cárie dentária (Jakubovics and Kolenbrander 2010; Ramage et al. 2005).

A *Candida albicans* é o fungo mais comumente encontrado em superfícies mucosas em humanos (Falsetta et al. 2014). Em indivíduos idosos, pacientes imunocomprometidos e usuários de próteses totais, esta espécie é a principal responsável pelo desenvolvimento de candidíase oral como a estomatite protética (Odds 1997; Coco et al. 2008).

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A expressão de fatores de virulência de *Candida*, tais como a formação de biofilmes e sua capacidade de produzir enzimas hidrolíticas (proteinase e fosfolipase) (Ghannoum. 2000; Schaller et al. 2005) são a chave para invasão dos tecidos humanos.

Além da *C. albicans*, diversas bactérias colonizam a cavidade bucal, em especial a bactéria gram positiva *Streptococcus mutans,* principal responsável pela cárie dental (Marsh 1999). Esta espécie apresenta propriedades acidogênicas e acidúricas, além da capacidade de sintetizar polissacarídeos extracelulares (Gregoire et al. 2011; Loesche 1986; Marsh and Bradshaw. 1995).

Interações entre fungos e bactérias são comuns e tem recebido especial atenção nos últimos anos. Neste sentido, a associação entre o fungo *C. albicans* e a bactéria *S. mutans* foi avaliada e comprovou-se o desenvolvimento de cáries mais agressivas do que as produzidas por biofilmes simples (Falsetta et al. 2014). Além de serem mais virulentos estes biofilmes tornam-se mais resistentes aos mecanismos de defesa do hospedeiro e aos agentes antimicrobianos convencionais utilizados na prática clínica (Lewis 2001). Assim, a busca de alternativas de tratamentos para controlar a formação de biofilmes orais patogênicos e de reduzir sua resistência antimicrobiana é necessária.

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As moléculas de quorum sensing tem recebido atenção na literatura atual (Zhang and Dong 2004). O farnesol é uma dessas moléculas e é caracterizado como um álcool acíclico isolado de espécies de *Candida*, em especial a *Candida albicans* e a *Candida dublinenses* (Martins et al. 2007; Weber et al. 2008). Pensando dessa forma, o objetivo geral desta pesquisa foi avaliar o efeito antimicrobiano do farnesol, sobre a formação de biofilmes simples e misto de *C. albicans* ATCC 10231 e *S. mutans* ATCC 25175.

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CAPÍTULO 1

Biofilm formation by *Candida albicans* and *Streptococcus mutans* in the presence of farnesol: quantitative evaluation *

*Artigo enviadopara o periódico Biofouling

Biofilm formation by *Candida albicans* and *Streptococcus mutans* in the presence of farnesol: quantitative evaluation

2.1. Abstract

The aim of this study was to evaluate the effect of QS molecule farnesol on single and mixed biofilm formation of *Candida albicans* ATCC 10231 and *Streptococcus mutans* ATCC 25175. The antibiofilm effect of farnesol was assessed through total biomass quantification, counting of colony forming units (CFUs) and evaluation of metabolic activity. Chlorhexidine gluconate was used as positive control. It was observed that farnesol decreased the formation of single and mixed biofilms, with significant reductions of 37 % to 90 % and 64 % to 96%, respectively for total biomass and metabolic activity. Regarding cell viability, the treatments with farnesol promoted significant decrease in the number of CFUs ranging from 1.3-4.2 log10 and 0.67-5.32log10, respectively for single and mixed biofilms. In conclusion, these findings highlight the role of farnesol as an alternative agent with potential to reduce the formation of pathogenic biofilms.

Keywords: Biofilms, *Candida albicans,* Quorum sensing, *Streptococcus mutans,* farnesol

2.2. Introduction

Dental caries are considered the most common disease in humans, affecting 80-90% of the underdeveloped countries population (Petersen 2004). This pathology is characterized by physical and chemical processes of demineralization and remineralization on the tooth surface (Struzycka 2014; Metwalli et al. 2013), which are related with the capacity of bacteria to produce acids (Sookkhee et al. 2001). However, several factors are involved in the emergence and development of the disease, including microbial genetic, immunological, environmental interaction, diet, among others (Struzycka 2014). *Streptococcus mutans*, a Gram-positive bacterium, is known as the principal agent of dental caries (Broadbent et al. 2013). This microorganism lives in the human oral cavity and survives in environment with low pH (Metwalli et al. 2013). Moreover, the acid production is the major virulence factor of that species (Metwalli et al. 2013).

Another important oral pathology is denture stomatitis (DS), which may affect 50-70 % of complete denture wearers (Budtz-Jorgensen et al. 1996; Al-Dwairi 2008). It is an inflammatory condition of the oral mucosal areas covered by dentures and has been associated with deficient denture hygiene, trauma, wearing dentures during nocturnal sleep, immunosuppression and microbial colonization of dentures (Karaagaclioglu et al. 2008). The polymorphic fungus *Candida albicans* is the principal pathogen isolated in cases of DS (Sato et al. 1997). However, *S. mutans* and other bacterial species may colonize denture surfaces and contribute to the development of DS (Karaagaclioglu et al. 2008). In

addition, *C. albicans* may mediate the progression of carious lesions through interspecies interactions (Barbieri et al. 2007; Jarosz et. al 2009)

C. albicans and *S. mutans* are found together in the oral cavity forming biofilms and play a fundamental role in the development of dental caries and DS. Biofilms are organized microbial communities adhered to live or inert surfaces and surrounded by an extracellular matrix produced by the cells (Costerton et al. 1999). Normally, these biofilms are resistant to conventional therapies (Talbot et al. 2006), and this fact has stimulated the search for alternative antimicrobial agents that may prevent biofilm formation and diseases associated with them. In this sense, quorum sensing (QS) molecules produced by *Candida* species have been used to combat the formation of pathogenic biofilms (Semighini et al. 2006). QS is a form of communication among microorganisms of the same or different species, controlled by different chemical signals (Hense et al. 2007) and essential for the development of biofilms (Bandara et al. 2012). QS is responsible for mediating a wide variety of biofilm features, such as virulence, maturation, population density and antibiotic resistance (Bandara et al. 2012).

The first QS molecule isolated from *C. albicans* was the acyclic alcohol farnesol (Hornby et al. 2001). This molecule participates in the control of morphogenesis of *C. albicans* blocking the transformation of yeast to hyphae (Ramage et al. 2002). Furthermore, it is mainly secreted by *C. albicans* and *C. dubliniensis* and has antibiofilm activity (Weber et al. 2008). Although the literature shows the role of farnesol as an antibiofilm agent, to the authors‟ knowledge, its activity on single and mixed biofilm formation of *C. albicans* and *S. mutans* remains unknown. Thus, the aim of this study was to evaluate the effect

of different concentrations of farnesol on single and mixed biofilm formation of *C. albicans* and *S. mutans* through quantification of the total biomass and cultivable cells, and assessment of metabolic activity of biofilm cells. The hypothesis tested was that farnesol has an inhibitory effect on single and mixed biofilm of *C. albicans* and *S. mutans*.

2.3. Materials and methods

Artificial saliva medium

Artificial saliva (AS) medium used in this study was prepared according to Lamfon *et al*. (2003) and its composition is detailed elsewhere (Monteiro et al. 2011).

Strains and culture conditions

C. albicans (n° 10231) and *S. mutans* (n° 25175) were purchased from the American Type Culture Collection (ATCC). *C. albicans* ATCC 10231 was grown under agitation (120 rpm) at 37 °C for 18 (± 2) h in 10 mL of Sabouraud dextrose broth (SDB; Difco, Le Pont de Claix, France) employing *Candida* colonies subcultured on Sabouraud dextrose agar medium (SDA; Difco) for 24 h. Next, yeast cells were harvested by centrifugation (8000 rpm, 5 min), washed twice in phosphate buffered saline (PBS; pH 7, 0.1 M) and the cellular concentration was adjusted to 1 x 10^7 cells/mL in AS, using an improved Neubauer chamber. On the other hand, *S. mutans* ATCC 25175 was subcultered on Brain Heart Infusion agar medium (BHI; Difco) at 37 °C for 24 h in 5 % CO_2. Then, the bacterial cells were inoculated in 10 mL of BHI broth medium (Difco), incubated under static conditions at 37 °C for 18 h in 5 % CO_2, harvested (8000 rpm, 5 min) after the incubation period, washed twice in PBS and adjusted spectrophotometrically (640 nm) to 1 x 10^8 cells/mL in AS.

Preparation of farnesol

Farnesol (trans,trans-farnesol; Sigma-Adrich, St. Louis, USA) was prepared in 7.5 % methanol (v/v), and diluted in RPMI 1640 (Sigma-Aldrich) medium, BHI broth or AS to achieve the desired concentrations for each assay. It was found that 7.5 % methanol did not alter the growth of the strains tested.

MIC determination

The broth microdilution method was used to determine the farnesol MICs, according to the Clinical and Laboratory Standards Institute M27-A2 document. Briefly, a stock solution of farnesol (300 mM) was geometrically diluted (2 to 1024 times) in deionized water. After, each farnesol concentration was diluted (1:5) in RPMI 1640 and BHI broth, respectively for *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175. Inocula of each strain were adjusted to the 0.5 McFarland standard in 0.85 % saline solution, diluted (1:5) in saline solution, and after diluted (1:20) in RPMI 1640 (for *C. albicans* ATCC 10231) and BHI broth (for *S. mutans* ATCC 25175). One hundred microliters of each microbial suspension was added into 96-well microtiter plates (Costar, Tewksbury, EUA) containing 100 µL of each farnesol concentration and the plates were incubated at 37 °C in 5 % CO_2. After 48 h, the MICs were visually determined. Chlorhexidine gluconate (CHG; Periogard, Colgate Palmolive Industrial Ltda, São Paulo, Brazil) was also include as positive control. It was performed three independent assays in triplicate.

Single and mixed biofilm formation in the presence of farnesol

Single and mixed biofilms of *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 were formed within the 96-well microtiter plates (Costar, Tewksbury, EUA). An aliquot of 200 µL of the standardized cell suspension (1 x 10^7 and 1 x 10^8 cells/mL in AS for *C. albicans* and *S. mutans*, respectively) was added to wells for single biofilms or 100 µL of each suspension (2 x 10^7 cells/mL *C. albicans* plus 2 x 10^8 cells/mL *S. mutans*) for mixed biofilms. The plates were statically incubated in 5 % CO_2 at 37 °C during 2 h for promoting cell adhesion. Afterwards, the AS medium was aspirated, and each well washed once with 200 µL of PBS to remove non-adherent cells. Farnesol was diluted in AS to obtain final concentrations of 1.56, 3.125, 6.25, 12.5, 25, 50, 70, 150 and 300 mM. Then, 200 µL of each dilution were inoculated into wells and the plates incubated for 48 h at 37 °C in 5 % CO_2. AS medium was renewed after 24 h. After biofilm formation period (48 h), the medium was removed and the wells were rinsed once with 200 µL of PBS to remove the planktonic cells. CHG at 0.37 mM (50 x *Candida* MIC) was used as positive control, while the wells inoculated with AS devoid of farnesol were used as negative controls. All assays were performed independently and in triplicate, at least three times.

Quantification of total biofilm biomass

The total biomass of single and mixed biofilms exposed to farnesol was analyzed through the crystal violet (CV) staining method (Monteiro et al. 2011). The resulting biofilms were fixed with 200 µL of 99 % methanol (Sigma-Aldrich). After 15 min of contact, the methanol was removed, the wells were dried at room temperature, and then 200 µL of CV stain (1%, v/v) (Sigma-Aldrich) were added

into wells and incubated for 5 min. CV was withdrawn and the wells were washed once with 200 µL of deionized water. Lastly, 200 µL of acetic acid (33 %, v/v) (Sigma-Aldrich) were inserted into wells to detach the stain, and the obtained absorbance was read in a microtiter plate reader (Eon Microplate Spectrophotometer; Bio Tek, Winooski, USA) at 570 nm and standardized in relation to the area of wells (Abs/cm^2).

Quantification of biofilm cultivable cells

The wells containing biofilms were scraped with PBS and vigorously vortexed for 1 min to disaggregate biofilm cells Each biofilm cell suspension was serially diluted in PBS and plated on SDA (for single biofilm of *C. albicans* ATCC 10231) and BHI agar (for single biofilm of *S. mutans* ATCC 25175). For mixed biofilms, the serial dilutions were plated on CHROMagar *Candida* (Difco) and BHI agar supplemented with 7 µg/mL amphotericin B (Sigma-Aldrich). After incubation at 37 °C for 24-48 h, the total number of colony-forming units (CFUs) per unit area (Log_{10} CFU/cm^2) of wells was quantified.

Quantification of biofilm metabolic activity

The XTT (2,3-(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) (Sigma-Aldrich) reduction assay (Hawser 1996; Hawser 1998) was used to determine the metabolic activity of single and mixed biofilm cells of *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175. After biofilm formation period, the wells were washed once with PBS and incubated with 200 µL of a solution containing 150 mg XTT/L and 10 mg of phenazine

methosulphate/L (Sigma-Aldrich) for 3 h in the dark, at 37 °C under agitation (120 rpm). Absorbance values of XTT-formazan were measured at 490 nm and standardized per unit area of wells (absorbance/cm^2). Wells containing AS without biofilms were considered as blanks to measure background levels.

Statistical analyses

SigmaPlot 12.0 software (Systat Software Inc., San Jose, USA) was employed for the statistical analysis with a confidence level of 95 %. Assays passed on normality test (Shapiro-Wilk) and then parametric statistical analyses were conducted with one-way ANOVA followed by post-hoc Holm-Sidak test.

2.4. Results

MIC determination

Table 1 shows the values of MIC and MFC/MBC for the tested strains. It was possible to observe that *S. mutans* ATCC 25175 (MIC = 6.25 mM; MBC = 6.25 mM) was more susceptible to farnesol than *C. albicans* ATCC 10231 (MIC = 150 mM; MFC = 300 mM). Moreover, the MIC and MFC/MBC values for CHG were about 844-20270 times lower than those found for the farnesol.

Quantification of total biofilm biomass

The results of total biomass quantification of single and mixed biofilms are expressed in the Figure 1. For single biofilm of *C. albicans* ATCC 10231, it was possible to note that farnesol in concentrations equal to or greater than 3.125 mM produced significant reductions in the total biomass, ranging from 58.03 to 66.41 %, when compared to the negative control. In comparasion to CHG there were no differences among the groups exposed to farnesol, and these groups behaved similarly to CHG, except for the biofilm formed in the presence of farnesol at 1.56 mM. For *S. mutans* ATCC 25175 in single biofilm, all treatments promoted significant reductions in total biomass compared to negative control, ranging of 80.48 to 90.24 %, and there were no differences among them. For the mixed biofilm, the exposures to farnesol at 1.56, 3.12, 6.25, 12.5, 25 and 50 mM promoted significant reductions in total biomass of 37.90, 76.47, 85.62, 85.62, 83.33 and 80.76 %, respectively. The treatments with farnesol at 6.25, 12.5 and 25 mM were more effective in decreasing total biomass than the treatment with CHG.

Quantification of biofilm cultivable cells

According to the Figure 2, it can be noted that, for single *C. albicans* ATCC 10231 biofilm, the treatment with farnesol at 6.25 mM or above significantly reduced (1.98-4.2$\log_{10}$) the number of CFUs, compared with the negative control. In addition, concentrations equal to or higher than 12.5 mM behaved similarly to CHG. However, for *C. albicans* ATCC 10231 in mixed biofilm, the effect of farnesol in the reduction of CFUs was dose-dependent, and the highest decrease was verified for the group exposed to 150 mM farnesol (5.27-$\log_{10}$; $p < 0.001$), differing also from the positive control. Interestingly, 150 mM farnesol was more effective against *C. albicans* in mixed biofilm than in single biofilm.

For single biofilm of *S. mutans* ATCC 25175, it was found that the treatments with farnesol at 3.12 mM, 6.25 mM and 12.5 mM promoted significant reductions in the number of CFUs of 2.69-$\log_{10}$ ($p < 0.001$), 3.33-$\log_{10}$ ($p < 0.001$) and 3.84-$\log_{10}$ ($p < 0.001$), respectively, compared to the negative control group. There was no growth of colonies for farnesol at 25 mM or above. For *S. mutans* in mixed biofilm, all farnesol concentrations significantly reduced the number of CFUs. Concentrations equal to or greater than 12.5 mM behaved similarly to CHG, and the highest reduction (5.32-$\log_{10}$; $p < 0.001$) was noted for the group 70 mM farnesol. There was no growth of colonies for the group treated with farnesol at 300 mM.

Quantification of biofilm metabolic activity

The results of evaluation of metabolic activity are shown in the Figure 3. For single *C. albicans* ATCC 10231 biofilm, farnesol in concentrations equal to or greater than 1.56 mM significantly reduced (64-96 %; $p < 0.001$) the metabolic activity of biofilm cells, when compared to the negative control group. Interestingly, there were no differences among the groups exposed to farnesol in concentrations equal to or greater than 6.25 mM. Furthermore, the highest decrease (99.20 %) in the metabolic activity was observed for the group treated with CHG, with significant differences among this group and all others. Similar results were found for the mixed biofilms. Exposures to farnesol at 1.56, 3.12, 6.25, 12.5, 25 and 50 mM induced significant decreases of 80.15, 92.06, 91.26, 94.4, 93.65 and 94.4 %, respectively. On the other hand, all treatments were not able to reduce the metabolic activity of single *S. mutans* ATCC 25175 biofilm.

2.5. Discussion

C. albicans and *S. mutans* are important oral pathogens able to form biofilms in different surfaces, which may favour the development of diseases like caries and DS (Falsetta et al. 2014). Considering the resistance of these biofilms to conventional agents, antibiofilm therapies based on QS molecules have been used. In this context, the major question of this study was whether the QS molecule farnesol would be able to reduce the single and mixed biofilm formation by *C. albicans* and *S. mutans*. The study hypothesis was partially accepted because the farnesol displayed an inhibitory effect on single and mixed biofilm formation, except for the *S. mutans* metabolic activity in single culture (Fig. 3).

The MIC results (Table 1) showed that *S. mutans* ATCC 25175 was more susceptible to farnesol than *C. albicans* ATCC 10231. Probably, this finding is related to differences in the cell structure of the microorganisms tested. The presence of a thicker cell wall in *C. albicans* ATCC 10231 may have hindered the farnesol action. Furthermore, the MIC values for farnesol in the current study are higher than those found in previous studies. Cordeiro et al. (2013) found values ranging from 18.75 to 150 μM for *C. albicans*, while in the study of Koo et al. (2012) the MIC value for *S. mutans* was 125 μM. These differences may be due to the different dilution methods used. Moreover, physiological differences between the tested strains may help explain the discrepancies between the MIC values found in the various studies.

On the other hand, the main objective of the present study was to assess the effect of farnesol on single and mixed biofilm formation by *C. albicans* and *S. mutans*. For this, the quantifications of total biomass, number of cultivable cells

and metabolic activity were performed. These methods are considered complementary, with specific advantages and disadvantages for each one. The crystal violet assay comprises a simple and effective method to quantify the total biomass (cells and extracellular matrix), however, its limitation is that both living and dead cells are stained (Monteiro et al. 2015). Although the CFU quantification is an easy and inexpensive method, some viable cells are not culturable in agar medium (Monteiro et al. 2015). In turn, the XTT reduction assay is a sensitive method that eliminates the use of radioactive materials. However, the results from this assay do not always show correspondence with cell death (Monteiro et al. 2015).

The results of biofilm quantification showed that, in general, farnesol concentrations equal to or greater than 1.56 mM produced significant reductions in total biomass (Fig. 1), number of CFUs (Fig. 2) and metabolic activity (Fig. 3) of single and mixed biofilms, except for the metabolic activity of *S. mutans* ATCC 25175 single biofilms. These findings indicate that the farnesol acts by reducing the extracellular matrix production and cell metabolism, as well as by promoting cell death.

It is well known that farnesol blocks the conversion of yeasts to hyphae in *C. albicans* (Langford et al. 2013; Piispanen et al. 2013); prevents cell adhesion and promotes detachment of biofilms from some surfaces (Nagy et al 2014; Cerca et al. 2012). These effects may explain the significant reductions in biomass found for *C. albicans* ATCC 10231 in single and mixed biofilms. As hyphae are larger structures, which absorb greater amount of crystal violet than yeasts, the treatments with farnesol might have resulted in biofilms with lower amount of

hyphae than the untreated biofilms, therefore resulting in lower absorbance values (Fig. 1). Moreover, these findings are consistent with those found for XTT reduction (Fig. 3), suggesting that the decreases in cell metabolism might have contributed to reduce the production of extracellular matrix. Literature also shows that, for *C. albicans* cells, farnesol may induce apoptosis and oxidative stress (Zhu et al. 2011), and this fact might justify the significant reductions observed in the number of cultivable cells (Fig. 2) for *Candida* biofilms.

For *S. mutans* ATCC 25175 in single and mixed biofilms, in general, all treatments with farnesol promoted significant reductions in total biomass (Fig. 1) and number of cultivable cells (Fig. 2). Although there was no significant reduction in metabolic activity (Fig. 3), farnesol may have increased the *S. mutans* cell permeability and acted directly on the F-ATPase enzyme, reducing the production of exopolysaccharides (EPS) and intracellular polysaccharides (Koo et al. 2003; Jeon et al. 2011), as well as the number of cells.

Another interesting observation of the present study was that, for *C. albicans* ATCC 10231 in dual species biofilms, farnesol showed a more marked dose-dependent effect on reducing number of cells than in single biofilms (Fig. 2). Indeed, farnesol at 150 mM showed the highest reduction in the number of CFUs for *C. albicans* ATCC 10231 in mixed biofilm. Clinically, this result is very important since *C. albicans* is found in the oral cavity forming polymicrobial biofilms. However, for *S. mutans* ATCC 25175, it was observed better effect of farnesol and CHG against single biofilms (Fig. 2). According to the negative control groups, the number of *S. mutans* ATCC 25175 cultivable cells in mixed biofilm was signifcantly higher than in single biofilm, indicating that the presence

of *C. albicans* ATCC 10231 favored the biofilm formation by *S. mutans* ATCC 25175. Studies have shown a symbiotic relationship between *C. albicans* and *S. mutans* (Metwalli et al. 2013; Falsetta et al. 2014). *C. albicans* excretes pyruvic and acetic acids which contribute to the biofilm acidification (Klinke et al. 2009), creating an environment that favors the growth of *S. mutans*. In addition, *S. mutans* cells adhere to the yeasts and hyphae of *C. albicans*, secret more EPS and acquire an additional protection from antimicrobials (Metwalli et al. 2013). Thus, this synergistic relationship limits the competition between those species and favors the mixed biofilm formation on different oral niches.

With regard to biofilm quantification assays, farnesol in concentrations equal to or greater than 12.5 mM promoted significant reductions in total biomass, number of CFUs and metabolic activity. Furthermore, these decreases were similar to those found for CHG (positive control). From a clinical point of view, these findings demonstrate that the use of very high concentrations of farnesol is not required to achieve a good effect on the biofilm formation inhibition, and the use of low farnesol concentrations may prevent toxic effects to human cells. Additionally, compared to CHG, farnesol seems to have similar effects with higher concentrations. Despite this, it should be noted that CHG and other conventional antimicrobial agents present some disadvantages and side-effects (Ernst et al. 1998). Therefore, the search for new agents (like farnesol) that could potentially be used as substitutes to or in combination with traditional antifungal drugs should be encouraged.

Finally, despite the small number of strains used in this study, it may be concluded that farnesol has an inhibitory effect on single and mixed biofilm

formation of *C. albicans* and *S. mutans*, and this compound in concentrations above 12.5 mM behaved similarly to CHG. An *in vivo* study has shown a good performance of farnesol associated with fluoride (Falsetta et al. 2012) on dental caries.

2.6. Conclusions

Thus, the results of the present study should stimulate the development of new antimicrobials or biomaterials containing farnesol in order to prevent oral diseases associated with biofilm formation, like candidiasis and tooth decay.

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Table 1. Minimum inhibitory concentrations (MIC), minimum fungicidal concentrations (MFC) and minimum bactericidal concentrations (MBC) of farnesol and chlorhexidine gluconate (CHG) against *Candida albicans* and *Streptococcus mutans*.

		Farnesol (mM)		**CHG (mM)**	
Species	**Strain**	**MIC**	**MFC/MBC**	**MIC**	**MFC/ MBC**
Candida albicans	ATCC 10231	150	300	0.0074	0.0296
Streptococcus mutans	ATCC 25175	6.25	6.25	0.0018	0.0074

Figure 1. Average absorbances per cm^2 obtained with crystal violet assay for total biomass of single and mixed *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 biofilms (48 h) developed in the presence of farnesol at 1.56 mM (1.56F), 3.125 mM (3.125F), 6.25 mM (6.25F), 12.5 mM (12.5F), 25 mM (25F) and 50 mM (50F). NC = negative control (*C. albicans* and *S. mutans* biofilms without farnesol). PC = positive control (chlorhexidine gluconate at 0.37 mM). Error bars display standard deviations of the means. Different capital letters denote significant differences ($p < 0.05$; one-way ANOVA followed by post-hoc Holm-Sidak test) among the groups.

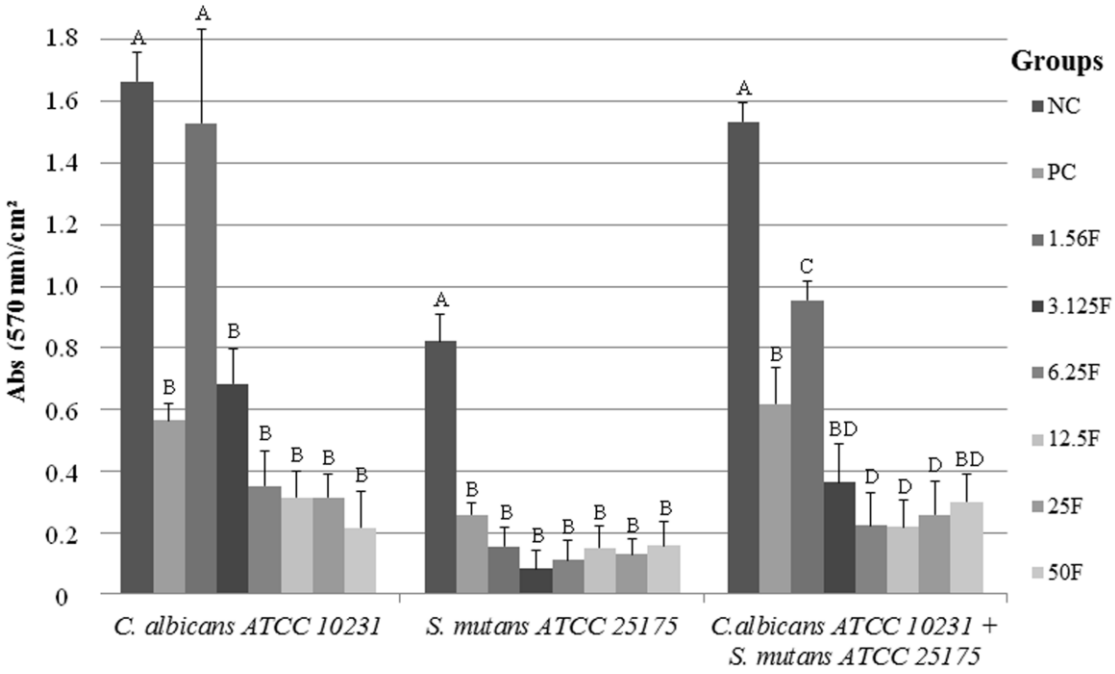


Figure 2. Mean values of the logarithm of colony forming units per cm^2 (log_{10} CFU/cm^2) for single and mixed *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 biofilms (48 h) developed in the presence of farnesol at 1.56 mM (1.56F), 3.125 mM (3.125F), 6.25 mM (6.25F), 12.5 mM (12.5F), 25 mM (25F), 50 mM (50F), 70 mM (70F), 150 mM (150F) and 300 mM (300F). NC = negative control (*C. albicans* and *S. mutans* biofims without farnesol). PC = positive control (chlorhexidine gluconate at 0.37 mM). Error bars display standard deviations of the means. Different capital letters denote significant differences ($p < 0.05$; one-way ANOVA followed by post-hoc Holm-Sidak test) among the groups.

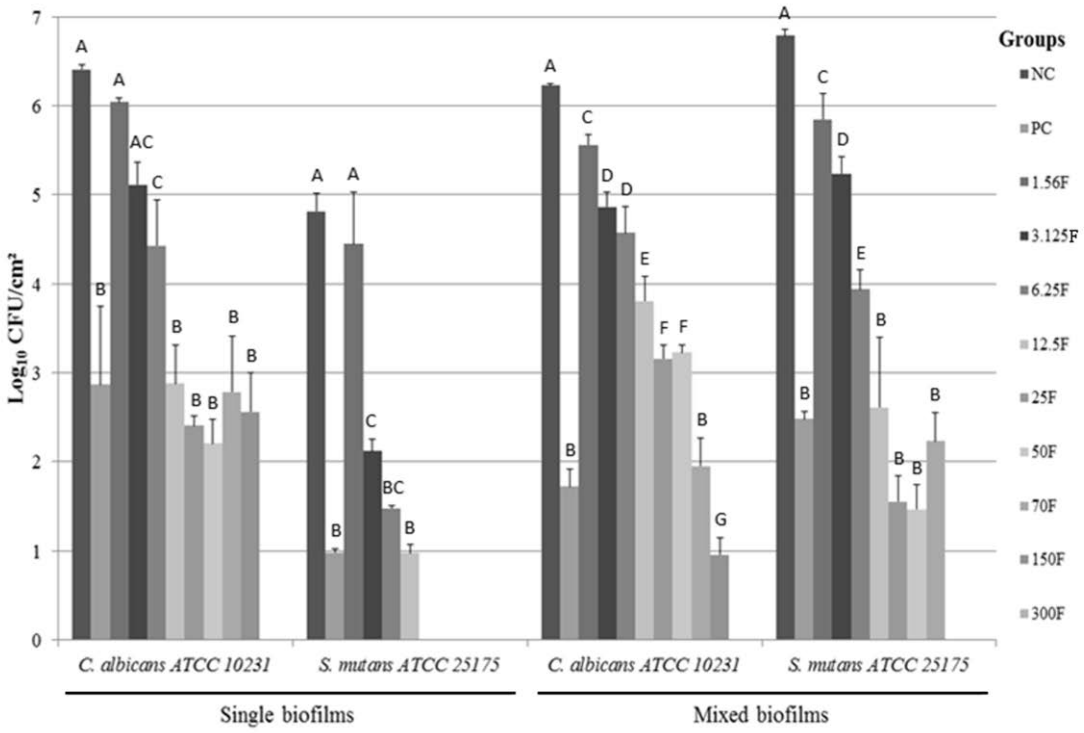
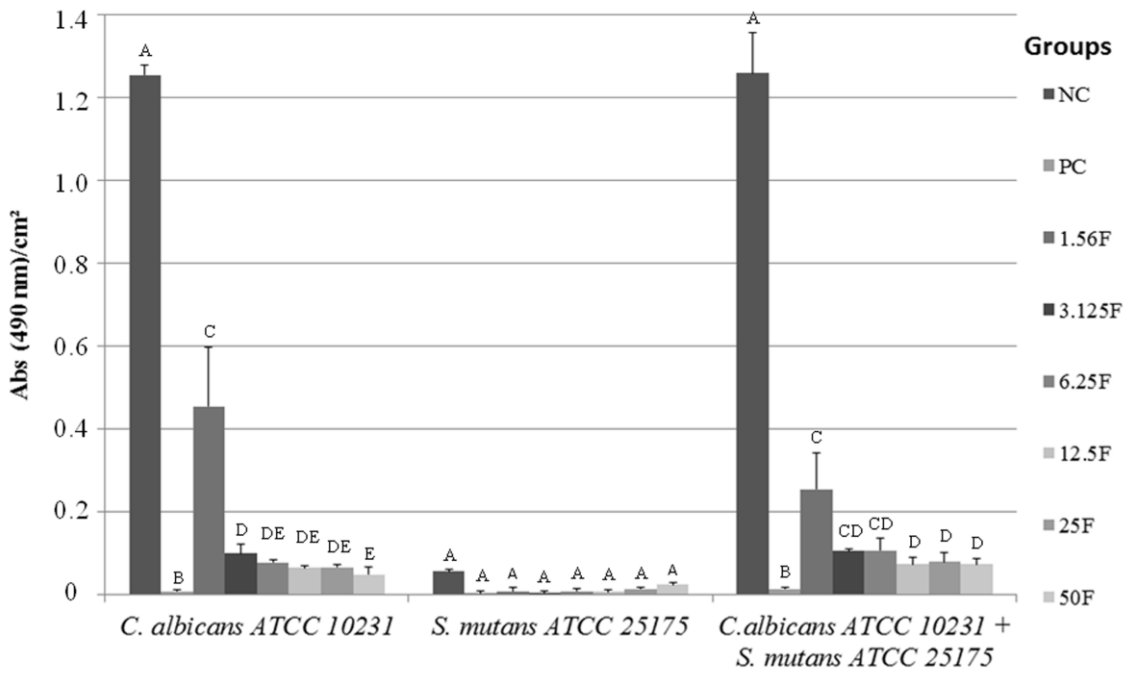


Figure 3. Average absorbances per cm^2 obtained with XTT reduction assay for single and mixed *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 biofilms (48 h) developed in the presence of farnesol at 1.56 mM (1.56F), 3.125 mM (3.125F), 6.25 mM (6.25F), 12.5 mM (12.5F), 25 mM (25F) and 50 mM (50F). NC = negative control (*C. albicans* and *S. mutans* biofilms without farnesol). PC = positive control (chlorhexidine gluconate at 0.37 mM). Error bars display standard deviations of the means. Different capital letters denote significant differences ($p < 0.05$; one-way ANOVA followed by post-hoc Holm-Sidak test) among the groups.



CAPÍTULO 2

Influence of Farnesol on virulence factors of biofilm of *Candida albicans* and *Streptococcus mutans* biofilms [*]

[*]*Artigo nas normas do periódico Biofouling.*

Influence of Farnesol on virulence factors of biofilm of *Candida albicans* and *Streptococcus mutans* biofilms

3.1. Abstract

This study evaluated the action of farnesol on virulence factors of biofilms of *Candida albicans* ATCC 10231 and *Streptococcus mutans* ATCC 25175. Time-kill curve assay, extracellular matrix composition for single and mixed biofilms of both species, pH assessment for *S. mutans* ATCC 25175 and enzymatic activity for *C. albicans* ATCC 10231 as well as analysis of single and mixed biofilms by scanning electron microscopy (SEM) were carried out. Chlorhexidine gluconate was the positive control in the pH assay and the composition of extracellular matrix. Farnesol had a fast effect on the decrease of colony forming units in time-kill curve assays for both microorganisms, reducing around 3 $\log_{10}$ and 1 $\log_{10}$ for *S. mutans* and *C. albicans*, respectively. For the composition of the extracellular matrix both concentrations tested of farnesol (3.12 and 12.5mM) decreased the amount of protein for single and mixed biofilms, except at 12.5 mM for the single biofilm of *S. mutans*. On the other hand, in general farnesol did not interfere in the amount of carbohydrates for both microorganisms. Also, at sub-inhibitory concentrations of 0.78 mM and 1.56 mM the pH was maintained stable in *S. mutans* biofilm, and the enzymatic activity of *C. albicans* ATCC10231 was not affect by any concentration of farnesol tested. SEM images suggested a decrease in the number of cells for both single biofilms of *S. mutans* ATCC25175 and *C. albicans* ATCC25175, as well as there was a reduction of hyphae cells of *C. albicans* ATCC 10231 present in single and mixed biofilms.

These results support the thought for further research with farnesol with the possibility of being used as an antibiofilm agent.

Keyword: Biofilms, *Candida albicans*, Quorum sensing, *Streptococcus mutans*, factors of virulence.

3.2. Introduction

The biofilms formed in the oral cavity have specific characteristics that make them virulent when interacting with external factors, as in the case of the bacteria *Streptococcus mutans* which uses sucrose from the host diet and in response produces extracellular polysaccharides in significant amount (Bowen et al. 1988). Biofilms formed by this microorganism have potential to develop diseases such as caries, a disease that is still characterized by a global health issue (Klein et al. 2015). *S. mutans* form biofilms able to produce extracellular matrix, one of the major constituents for the survival of biofilm and also one of the factors responsible for microorganisms resistance to treatments with conventional therapies (Klein et al. 2015). The exopolysaccharides (EPS) are one of the main constituents of the matrix that provide virulence of oral biofilms, besides being a protection against breakage and dispersion of these biofilms (Bowen & Koo 2011).

The production of EPS by *S. mutans* is strongly reported in the literature, by having multiple exoenzymes which make it one of the highest producers of EPS, beyond being aciduric and acidogenic (Paes et al. 2006). In a biofilm where other microorganisms can produce glicosiltranferases and glucans on their surface, *S. mutans* can adhere over those microorganisms (Hwang et al. 2015). With the accumulation of microorganisms acid production occurs in the

extracellular matrix, being *S. mutans* the main acid producer in the oral biofilm. Enamel dissolution starts on the tooth surface the resulting in the process of caries, being the acid production one of the major virulence factors assigned to *S. mutans* (Marsh et al. 2003).

In this environment other microorganisms also inhabit such as fungi. *Candida albicans* is one of the most commonly fungi found in the oral cavity (Falsetta et al. 2014) which also has a biofilm with virulence capacity. It can colonize different surfaces like denture acrylic resins as well as medical devices leading, in this case, to a high mortality rate of hospitalized patients (Douglas 2003). Some studies reported that an extracellular carbohydrate present in *C. albicans* matrix, β- 1,3 glucan, appears to be involved in drug sequestration, thereby assisting in antimicrobial resistance (Nobile et al. 2009). The basic composition of the extracellular matrix of *Candida albicans* is 55% of protein, 25% of carbohydrates, 15% of lipids, and 5% of deoxyribonucleic acids and sugars such as arabinose, glucose and xylose (Zarnowski et al. 2014). Moreover, *Candida* species can produce hydrolytic enzymes (e g, phospholipase and proteinase) that contribute to their invasion of the host tissues by digestion or destruction of cell membrane (Schaller et al. 2005). These enzymes can also attack the cells and molecules of the host immune system (Schaller et al. 2005). The production of these hydrolytic enzymes and the capacity of forming biofilms (Douglas 2002) on oral tissues and implants are considered one of the major virulence factors related to *Candida* species (Schaller et al. 2005).

Although *S. mutans* was considered the main causative or the main etiological agent of dental caries (Marcenes et al. 2013), studies have shown that

the association of *C. albicans* (Raja et al. 2010) in dental biofilms causes more aggressive caries than when compared to a biofilm formed only by *S. mutans* (Falsetta et al. 2014), especially in the presence of sucrose (Peleg et al. 2010). *C. albicans* also presents interaction in multi-species biofilms (Diaz et al. 2012) highlighting their affinity for other species of *Streptococcus* like *S. viridans*, *S.gordonii* and *S. oralis* (Metwalli et al. 2013). Thinking on the affinity of *S. mutans* and *C. albicans* in virulent dental biofilms, as well as on the biofilm resistance to conventional drugs, alternatives to treat dental caries are stimulated. Accordingly, the use of some quorun sensings (QS) as antimicrobials have been reported (Zhang et al. 2004; Pacheco et al. 2009). These molecules work regulating gene expression, cellular differentiation and others functions (Zhang et al. 2004; Pacheco et al. 2009). Among the QS, farnesol, an acyclic alcohol secreted by *Candida* species such as *C. albicans* and *C. dubliniensis* (Martins et al. 2007) has received attention for presenting antibiofilm activity. Farnesol also influences other species of fungi and bacteria (Semighini et al. 2006).

This study evaluated the time-kill curve assay of *S. mutans* and *C. albicans* in the presence of farnesol, as well as their effect on the extracellular matrix composition for single and mixed biofilms of *S. mutans* and *C. albicans*, on the acid production by *S. mutans*, and on the enzymatic activity (proteinase, phospholipase and hemolytic) of *C. albicans*. Analysis of single and mixed biofilms by scanning electron microscopy (SEM) after being exposed to farnesol was also carried out.

3.3. Materials and methods

Artificial saliva medium

Artificial saliva (AS) medium was prepared according to Lamfon et al. (2003) and the composition is described in detail elsewhere (Monteiro et al. 2011).

Strains and culture conditions

The strains of *C. albicans* (10231) and *S. mutans* (25175) were purchased from the American Type Culture Colletion (ATCC). The culture condition of *C. albicans* was under agitation (120 rpm) at 37°C during 20 h in 10 ml of Sabourand dextrose broth (SDB; Difco, Le Pont de Claix, France) obtained from colonies subcultered on Sabourand dextrose agar medium (SDA; Difco) for 24 h at 37°C. The yeast cells were centrifuged (8000 rpm, 5 min) and harvested, in the sequence the cells were washed twice in phosphate buffered saline (PBS; pH 7,0 0.1M) and adjusted at 1 x 10^7 cells/mL in AS using an improved Neubauer chamber. For *S. mutans* ATCC 25175, cells were inoculated in 10 ml of BHI broth medium (Difco), incubated under static conditions at 37°C for 18 h in 5% CO_2, harvested by centrifugation (8000 rpm, 5 min), washed twice in PBS and then its concentration were adjusted by spectrophotometric (640 nm) to 1 x 10^8 cells/mL in AS. The colonies were obtained from the BHI agar medium (Difco) cultured 24 h before beginning the assays.

Preparation of farnesol

Farnesol was first diluted in 7.5% methanol (v/V) and the second dilution was prepared in AS. Methanol was tested and did not interfere in the cells viability of microorganisms studied.

Single and mixed biofilm formation in the presence of farnesol

For the formation of single and mixed biofilms of *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 96-well microliter plates (Costar, Tewksbury, EUA) were used. In each well was added 200 µL of cell suspension (1 x 10^7 and 1 x 10^8 cells/mL in AS for *C. albicans* and *S. mutans*, respectively) for single biofilms, while for mixed biofilms 100 µL of each suspension (2 x 10^7 cells/mL *C. albicans* plus 2 x 10^8 cells/mL *S. mutans*) were added in each well. Incubation of the plates was performed in static conditions in 5% CO_2 at 37 °C for 2 hours for cell adhesion of both microorganisms on the wells. Subsequently AS was removed and each well was washed once with PBS, to promote the removal of non-adherent cells. Then farnesol diluted in AS, in specific dosages for each test, was added in the wells. Following the plates were stored for 48 hours at 37 °C in 5% CO_2, and the media was renewed after 24 hours. Chlorhexidine gluconate (CHG) was used as positive control, while the negative control was composed of saliva without farnesol. All assays were performed independently and in triplicate, at least three times.

Time-kill curve assay

The method used in this study was based on Tong Z et. al (2014). First the cellular concentration of *C. albicans* and *S. mutans* were adjusted in AS medium, (1 x 10^7 and 1 x 10^8 cells/mL for *C. albicans* and *S. mutans*, respectively). Farnesol was added to each inoculum at concentration of 3.125 mM, which was chosen according to our previous study (unpublished data). Then they were incubated at 37ºC at 1, 2, 6, 8, 10, 12 and 24 hours, the content was diluted in PBS and plated on BHI agar and SDA media (Difco) respectively for *S. mutans* and *C. albicans*.

After 24 hours of incubation (37°C) for *C. albicans* and 48 hours (37°C 5% CO_2) for *S. mutans*, the cells were enumerated.

Effect on Acid Production

For this test the method was according to Hasan et al. (2012). *S. mutans* cells concentration were adjusted like described before, incubated during 2 h for adhesion cells, washed once with PBS and farnesol was added at concentrations of 0.78 and 1.56 mM. The initial values of pH were verified with a specific electrode and a pH metrer. The plates were incubated at 37°C for 24 h in 5% CO_2; after this period the medium was renovated and they were incubated for more 24 h. In the sequence the final pH values were verified.

Protein and carbohydrate quantification of the extracellular matrix

For the extraction of extracellular matrix primarily the number of *C. albicans* and S. *mutans* cells were adjusted as described anteriorly and added to the wells of 96 well plates. After the adhesion (2 h) the media were removed, cells were washed with PBS and farnesol at 3.12 and 12.5 mM was added to the wells. Incubation was for 24 h at 5% CO_2, the media were renewed and the plates were incubated for 24 h. The biofilms were then washed once with PBS, scraped and resuspended in PBS. Subsequently a sample of each biofilm was collected and vacuum filtered on cellulose membrane (0.45 uM), and the membrane was dried at 60° C for about 24 hours. The final dry weight and the weight at the initial process of these samples were recorded. The biofilms were sonicated for 30 seconds at 30 W and then vortexed for 2 minutes. Finally, the suspensions underwent centrifugation at 3000 g for 10 minutes and the supernatant filtered through nitrocellulose filters (0.22 uM; Orange Scientific, Braine I`Alleud, Belgium) (Monteiro et al. 2012).

For quantification of extracellular matrix proteins and carbohydrates it was used the method of bicinchoninic acid (BCA kit, Sigma-Aldrich) and the method described by Dubois et al. (1956) respectively for protein and carbohydrates content. For determination of protein, bovine serum albumin curve (Monteiro et al. 2012) was used as a standard. A volume of 25 uL of supernatant obtained after centrifugation was pipetted into the wells of 96 well plates containing 180 uL of the mixture of BCA kit reagents. Then the plates were incubated at 37 °C for 30 minutes and the absorbance was measured at a wavelength of 562 nm. As for the dosage of extracellular carbohydrate matrix, a glucose curve was used as a standard the glucose curve. Subsequently, 100 uL of the supernatant prepared before were pipetted inside glass tube, and mixed with 100 uL of phenol 9% and 500 uL of sulfuric acid (Sigma - Adrich). The solution was vortexed, rested for 15 minutes at room temperature, and the absorbance was read at 490 nm. The data of both proteins and carbohydrates content were expressed on the basis of the biofilm dry weight (mg / g dry weight), where the absorbance value was correlated with the concentration of proteins and carbohydrates.

Determination of *C. albicans* enzymatic activity (proteinase, phospholipase and hemolytic)

Farnesol was diluted in AS at concentrations of 0.78 mM and 1.56 mM (corresponding to 1/8 and 1/4 of the minimum inhibitory concentration (MIC) determined for planktonic cells). Each farnesol solution was then added to the wells containing the adhered cells. Plates were incubated at 37 ^{o}C in 5% CO_2 for 48 h. Farnesol-containing AS was renewed after 24 h. Positive and negative controls were chlorexidine gluconate (CHG) at 1.85 µM (1/4 of MIC) and AS

without farnesol, respectively. After 48 h the biofilms were scraped and pippeted in specific media for proteinase, phospholipase and hemolytic activity.

Proteinase activity was determined according to Aoki et al (1990). A solution (60 mL) composed of 0.04 g , $MgSO_4.7H_2O$, 1 g, NaCl, 0.5 g KH_2PO_4, 4 g glucose, 0.2 g yeast extract and 0.5 g bovine serum albumin (BSA) (all chemicals from Sigma-Aldrich) was prepared, the pH was adjusted to 3.5 with HCl and sterilized by filtration. This solution was mixed with 140 mL of melted agar (Difco) and poured onto petri dishes. Phospholipase activity was assessed according to the egg yolk agar method (Price et al. 1982). The agar was prepared by adding an egg yolk emulsion (10% v/v) to SDA (13 g) supplemented with NaCl (11.7 g) and $CaCl_2$ (0.11 g), in 184 mL of deionized water (Tsang et al. 2007). Hemolytic activity was evaluated by using SDA medium supplemented with 7% of fresh sheep blood and 3% of glucose (Sacristán et al. 2011).

For proteinase, phospholipase and hemolytic activity determination, biofilms were scraped from the wells (in PBS) and 10 µL of the resulting biofilm suspension were inoculated on the agar plates and incubated at 37 ^{o}C for 3 days (hemolytic activity) and 6 days (proteinase and phospholipase activity). Enzymatic activity was expressed according to Pr_z, Pz and H_z indexes, respectively for proteinase, phospholipase and hemolytic activity. These indexes represent the ratio between the diameter of the colony and the diameter of the translucent/precipitation zones. Enzymatic activity was graded as: high (indexes < 0.4), medium (indexes from 0.41 to 0.60), low (indexes from 0.61 and 0.99) and absent (indexes = 1) (Price et al. 1982; Williamson et al. 1986).

Structural analysis of biofilms

SEM was performed for the structural analysis of biofilms exposed to farnesol. Single and mixed biofilms of *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 were formed in wells of 24-well plates, which was initially adjusted to the cell concentration as described before. After the adhesion time (2 h) of both microorganisms, farnesol was added into the wells at concentrations of 3.12 and 12.5 mM. Chlorhexidine gluconate (CHG) at 0.37 mM (50 x *C. albicans* MIC) and AS without farnesol were used as positive and negative control, respectively. After 24 hours of incubation the medium was renewed and incubated for another 24 hours. The wells were then gently washed with PBS and the biofilms were dehydrated progressively in ethanol at 70% for 10 minutes, 95% for 10 minutes and 100% for 20 minutes, followed to air dry for 20 minutes (Koo et al. 2002). The bottom of the wells were cut with a scalpel blade (number 11, Solidor, Lamedid Commercial and Services Ltda, Barueri, Brazil) heated in flame. After the biofilms received gold bath, the SEM analysis was carried out (S-360 microscope, Leo, Cambridge, USA).

Statistical analyses

SigmaPlot 12.0 software (Systat Software Inc., San Jose, USA) was employed for the statistical analysis with a confidence level of 95 %. Assays passed on normality test (Shapiro-Wilk) and then parametric statistical analyses were conducted with one-way ANOVA followed by the post-hoc Holm-Sidak test.

3.4. Results

Time-kill curve assay

Farnesol started to be significantly effective after 8 hours in contact with *C. albicans* ATCC 10231 reducing approximately 2 log_{10}, and keeping similar reduction for 24 h of farnesol exposition (Figure 1A). Similarly to *C. albicans* the time for farnesol to produce significant cell death for *S. mutans* ATCC 25175 was 8 h (Figure 1B). Although the reduction of cells was approximately 4 $logs_{10}$, the time-kill curve for *S. mutans* ATCC 25175 showed an increased growth of about 2 log_{10} from 12 to 24 h.

Effect on Acid Production

Farnesol at 0.78 mM and 1.56 mM was able to maintain the *S. mutans* pH stable (Table 1). Also there was no significant difference between the pH values of the positive control (chlorhexidine gluconate at 0.45 μM (1/4 of MIC to *S. mutans* ATCC 10231)) and the negative control.

Proteins and carbohydrates quantification of the extracellular matrix

The amount of proteins was significantly reduced for both strains in simple and mixed biofilms compared to the negative control group, except for the concentration of 12.5 mM of farnesol for *S. mutans* ATCC 25175 (Table 2). However, farnesol treatment was not able to reduce the amount of carbohydrates in any biofilm studied.

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Determination of *C. albicans* enzymatic activity (proteinase, phospholipase and hemolytic)

Farnesol at sub inhibitory concentration could not reduce the enzymatic activity of ATCC 10231 (Table 3), showing medium, medium and high activity respectively for protease, phospholipase and hemolytic.

Structural analysis of biofilms

Figure 2 shows that farnesol at 12.5 mM and chlorhexidine gluconate could especially change the cell morphology of *C. albicans* ATCC 10231, and reduce the number of cells for both single and mixed biofilms. Figure 2 also shows that the quantity of *C. albicans* ATCC 10231 cells was higher when in the presence of S. mutans ATCC 25175, regardless of the farnesol concentration tested.

3.5. Discussion

Microorganisms are able to produce biofilm with virulence mechanisms which make them virulent, leading to an imbalance in the health of individuals (Klein et al. 2015). The virulence factors are variable and closely related to the ability of microorganisms to produce extracellular matrix. Gram positive bacterium *S. mutans* is reported as one of the biggest producers of EPS (Paes et al. 2006), in addition to produce and tolerate acids, causing dental caries. Another microorganism present in the oral cavity is the opportunistic fungus *C. albicans* which can produce enzymes able to attack human‟s tissues (Schaller et al. 2005). With the increase of their resistance to conventional antimicrobial agents, the quorum sensing molecules have received attention as potential antibiofilm drugs. Farnesol is one of the quorum sensing molecules produced by *C. albicans* ATCC10231, and its effect on some virulence factors of both *C. albicans* ATCC10231 and *S. mutans* ATCC 25175 was tested in this present study, as well as the time kill of cells of these microorganisms was carried out.

There was approximately 1 $\log_{10}$ of *C. albicans* ATCC10231 cells reduction after being an hour in contact with farnesol (Fig. 1A), and about 3 $\log_{10}$ reduction for *S. mutans* (Fig. 1B). It was a better initial reduction of *S. mutans* than that found by Koo et al. (2002) (1 $\log_{10}$). It could be explained by origin of farnesol tested in each study, synthetic and natural (obtained from propolis) respectively in the present study and in the study carried out by those authors. Also, for *S. mutans*, Melo et al. (2015) had the highest cells reduction after being 8 hours in contact with farnesol.

Interesting results were found for the pH assay, where the *S. mutans* biofilm pH stayed stable at both subinhibitory concentrations of farnesol (0.78 and 1.56 mM), which are in accordance with the study of Jeon et al (2011). A plausible explanation for this was given by the same authors (Jeon et al. 2011) whom cited that farnesol acts in the permeability of *S. mutans* protons membrane, which might affect the pH gradient across the membrane, inhibiting the cellular metabolism, and thereby the acids production. Nevertheless, the positive control used in the present study (clohrexidine) reduced significantly the pH from 7.0 to 5.3 after 48 hours. Probably the stress caused by clorhexidine to the cells stimulated the production of acids, resulting in a reduction of pH in the environment.

The dosage of protein and carbohydrates showed farnesol was able to reduce only proteins in the single and the mixed biofilms of *C. albicans* ATCC10231 and *S.mutans*, except for the single biofilm of *S. mutans* ATCC 25175 at the concentration of 12.5 mM. For *S. mutans* ATCC 25175, the explanation for the reduction of proteins at lower concentration of farnesol (3.125 mM) might be compatible with that mentioned before for the pH results founded at subinhibitory concentrations (0.78 and 1.56 mM), where farnesol might act on the membrane permeability of *S. mutans* and reduce its metabolism.

The values of proteinase and phospholipase and hemolytic activity for *C. albicans* ATCC 10231 were not affected by farnesol at subinhibitory concentrations of 0.78 mM and 1.56 mM. These results are contrary of those found by Singh et al. (2015) where farnesol obtained from extracts of a plant

(*Usnea longíssima*) was able to reduce all these factors of virulence expressed by a strain of *C. albicans* resistant to fluconazole.

The SEM images suggest that the treatments with farnesol induced the cellular apoptosis (Lu et al. 2014) especially for *C. albicans* found in both single and mixed biofilms. The presence of hyphae was lower in mixed biofilm treated with farnesol than the untreated, and it can be due to the capacity of farnesol to block the conversion of *C. albicans* from yeasts to hyphae (Lu et al. 2014). In the single biofilm of *S. mutans* the images showed a reduction of cells treated with both farnesol and clorhexidine, while in the mixed biofilm the reductions seemed to be lower suggesting more resistence of both microorganisms against farnesol.

In conclusion, *C. albicans* and *S. mutans* had a significant level of dead cells for both in about 8 hours in contact with farnesol. Farnesol controlled the factor of virulence for *S. mutans* (pH), and had no effect on the virulence of *C. albicans* (proteinase, phospholipase and hemolytic activity). Also, in general, proteins were significantly reduced in both single and mixed biofilms of *C. albicans* and *S. mutans*. Thus, these results suggest that the use of farnesol to control those biofilms especially considering the lower concentrations tested in this study, is promising as well as stimulate its use in combination with conventional antimicrobial agents to combat the pathogenicity of these microorganisms.

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Table 1 Mean (SD) pH values obtained with acid production assay by *Streptococcus mutans* ATCC 10231 biofilms formed in the presence of farnesol at 0.78 and 1.56 mM. NC: negative control (non-treated biofilms); PC: positive control (490 µM clorhexidine gluconate).

Treatments	Initial pH	Final pH
NC	7.0 (0.19)	5.1 (0.21)
PC	7.0 (0.10)	5.3 (0.22)
0.78F	6.8 (0.06)	6.6 (0.05)*
1.56F	6.9 (0.06)	6.6 (0.05)*

*Indicates significant difference between initial values and final values, as compared to the control group, using 2-way ANOVA and Holm-Sidak test, $p < 0.05$.

Table 2. Composition of extracellular matrix of single and mixed *Candida albicans* and *Streptococcus mutans* biofilms (48 h) grown in the presence of different concentrations of farnesol (0, 3.125 and 12.5 mM).

Matrix composition (mg/g of biofilm dry weight)	*C. albicans* ATCC 10231				*S. mutans* ATCC 25175				*C.albicans* ATCC 10231 + *S. mutans* ATCC 25175			
	Farnesol (mM)			chlorhexidine (mg/ml)	Farnesol (mM)			chlorhexidine (mg/ml)	Farnesol (mM)			chlorhexidine (mg/ml)
	0	3.12	12.5	250	0	3.12	12.5	250	0	3.12	12.5	250
Carbohydrate	40.27 ± 18.98	42.07 ± 13.92	26.22 ± 2.77	63.23 ± 28.61	30.55 ± 13.80	58.15 ± 16.63	62.81 ± 10.25	41.73 ± 11.65	32.84 ± 6.65	26.54 ± 1.99	52.49 ± 13.99	60.27 ± 14.41
Protein	7.81 ± 1.38	0.00 ± 0.00*	0.84 ± 0.61*	0.00 ± 0.00*	4.76 ± 2.44	0.05 ± 0.08*	5.80 ± 1.54	0.00 ± 0.00*	8.75 ± 1.07	0.00 ± 0.00*	0.17 ± 0.24*	0.14 ± 0.19*

*Indicates $P < 0.05$, as compared to the control group, using ANOVA with Holm-Sidak test

Table 3. Mean (SD) of Pr_z, P_z and H_z values obtained with the enzymatic activity assays for *Candida albicans* ATCC 10231 biofilms formed in the presence of farnesol at 0.78 and 1.56 mM. NC: negative control (non-treated biofilms); PC: positive control (490 µM clorexidine gluconate).

Treatments	Proteinase activity		Phospholipase activity		Hemolytic activity	
	Pr_z value		P_z value		H_z value	
NC	0.55 (0.05)°	Medium	0.55 (0.03)°	Medium	0.36 (0.02)°	High
PC	0.58 (0.02)°	Medium	0.55 (0.01)°	Medium	0.37 (0.02)°	High
0.78F	0.54 (0.06)°	Medium	0.54 (0.05)°	Medium	0.37 (0.02)°	High
1.56F	0.55 (0.05)°	Medium	0.53 (0.02)°	Medium	0.35 (0.00)°	High

No significant differences were observed among the treatments, (2-way ANOVA, $p > 0.05$)

Figure 1. Time kill curve at different time periods for *C. albicans* ATCC 10231(A) and *S. mutans* ATCC 25175 (B) in the presence of farnesol at 3.12 mM.

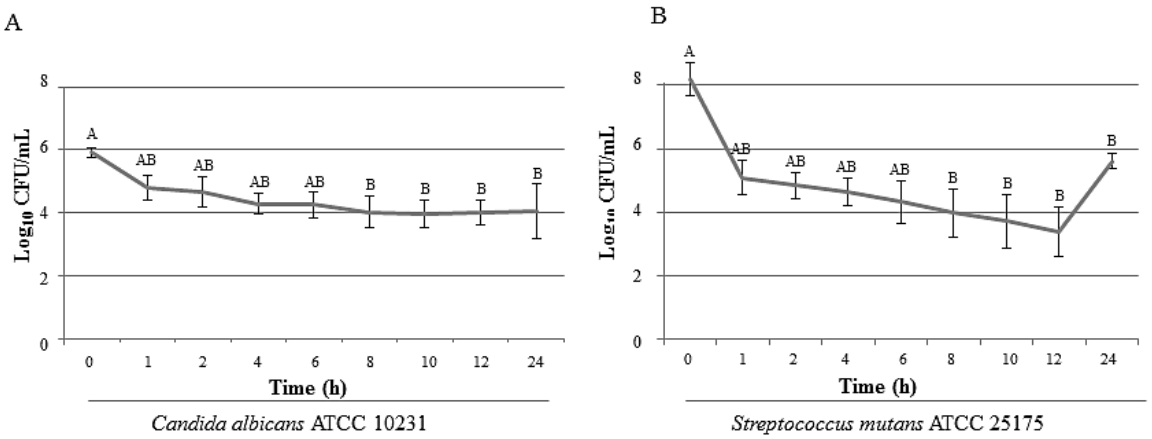
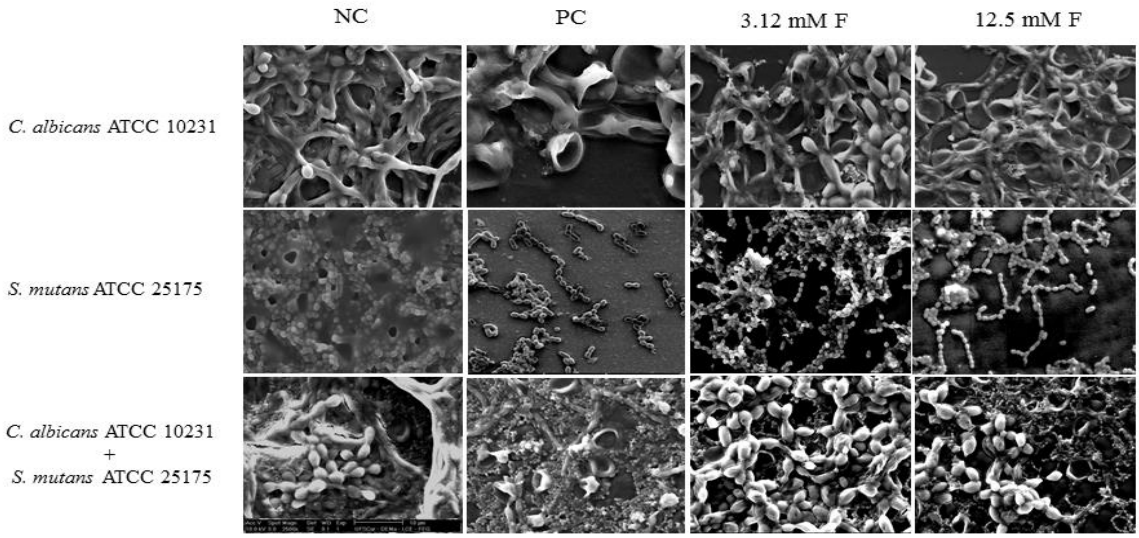


Figure 2. Images of scanning electron microscopy of single and mixed *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 biofilms (48 h) developed in the presence of farnesol at 3.125 mM (3.125 mM F) and 12.5 mM (12.5 mM F), NC = negative control (biofilms without farnesol). PC = positive control (chlorhexidine gluconate at 0.37 mM). All images are in the magnification of 2500 x, except for the image of positive control in single biofilm of *C.albicans*.



ANEXOS

Anexo A – Normas do periódico *Biofouling*

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Anexo B-
Metodologia proposta pela FAPESP

Foram realizados os MICs em diferentes condições de O_2 e variando se o meio de Cultura. Os resultados estão expressos na tabela abaixo:

Tabela 1.Valores de Concentração Inibitória Mínima (CIM), Concentração Fungicida Mínima (CFM) e Concentração Bactericida Mínima (CBM) de Farnesol em saliva artificial variando-se as condições de crescimento (maior ou menor concentração de O2).

		Farnesol (mM) 02		**Farnesol (mM) C02**	
Espécies	Cepas	MIC	MFC/MBC	MIC	MFC/MBC
Candida albicans	ATCC 10231	37,5	300	300	Não obtido
Streptococcus mutans	ATCC 25175	0,3	0,3	0,3	0,7