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

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ABSTRACT

The aim of this study was to evaluate the effect of the QS molecule farnesol on single and mixed species biofilms formed by *Candida albicans* and *Streptococcus mutans*. The anti-biofilm effect of farnesol was assessed through total biomass quantification, counting of colony forming units (CFUs) and evaluation of metabolic activity. Biofilms were also analyzed by scanning electron microscopy (SEM). It was observed that farnesol reduced 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, farnesol treatment promoted significant log reductions in the number of CFUs, ie 1.3–4.2 $\log_{10}$ and 0.67–5.32 $\log_{10}$, respectively, for single and mixed species biofilms. SEM images confirmed these results, showing decreases in the number of cells in all biofilms. In conclusion, these findings highlight the role of farnesol as an alternative agent with the potential to reduce the formation of pathogenic biofilms.

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Biofilms; *Candida albicans*; quorum sensing; *Streptococcus mutans*

Introduction

Dental caries is considered to be the most common disease in humans, affecting 80–90% of the population in underdeveloped countries (Petersen 2004). This pathology is characterized by physical and chemical processes of demineralization and remineralization on the tooth surface (Metwalli et al. 2013; Struzycka 2014) which are related to 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 interactions and diet among other factors (Struzycka 2014). *Streptococcus mutans*, a Gram-positive bacterium, is known as the principal agent of dental caries (Broadbent et al. 2103). This microorganism lives in the human oral cavity and survives in environments with low pH (Metwalli et al. 2013). Moreover, acid production is the major virulence factor of this species (Metwalli et al. 2013).

Another important type of oral pathology is denture stomatitis (DS) which may affect up to 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).

Interestingly, *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). These microorganisms are found together in the oral cavity within biofilms and their association can result in more pathogenic biofilms. 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 the diseases associated with them. Quorum sensing (QS) molecules, for example, produced by *Candida* species have been used to combat the formation of pathogenic biofilms (Semighini et al. 2006). QS is a form of chemical communication among microorganisms

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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 in *C. albicans,* blocking the transformation of yeast cells to hyphae (Ramage et al. 2002). Furthermore, it is mainly secreted by *C. albicans* and *C. dubliniensis* (Weber et al. 2008). Although the literature indicates farnesol has a role as an anti-biofilm agent, to the authors' knowledge, its effect on mixed species biofilms formed by *C. albicans* and *S. mutans* remains unclear. Thus, the aim of this study was to evaluate the effects of different concentrations of farnesol on single and mixed species biofilms of *C. albicans* and *S. mutans* through quantification of the total biomass and cultivable cells, and assessment of the metabolic activity of the biofilm cells. The hypothesis tested was that farnesol has an inhibitory effect on single and mixed species biofilms formed by *C. albicans* and *S. mutans*.

Materials and methods

Artificial saliva medium

Artificial saliva (AS) medium used in this study was prepared according to Lamfon et al. (2003). Its composition per 1 l of deionized water is: 2 g of yeast extract, 5 g of peptone, 2 g of glucose, 1 g of mucin, 0.35 g of NaCl, 0.2 g of $CaCl_2$ and 0.2 g of KCl, all from Sigma-Aldrich (St Louis, MO, USA) as described by Monteiro et al. (2011). The pH was adjusted with NaOH to 6.8.

Strains and culture conditions

Two reference strains from the American Type Culture Collection (ATCC) were used in this study: *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175. *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). *Candida* colonies were then subcultured on Sabouraud dextrose agar medium (SDA; Difco) for 24 h. Next, yeast cells were harvested by centrifugation (8,000 rpm, 5 min), washed twice in phosphate buffered saline (PBS; pH 7, 0.1 M) and the cell concentration was adjusted to 1×10^7 cells ml^{-1} in AS using an improved Neubauer chamber. *S. mutans* ATCC 25175 was subcultured 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 by centrifugation (8,000 rpm, 5 min) after the incubation period, washed twice in PBS and adjusted spectrophotometrically (640 nm) to 1×10^8 cells ml^{-1} in AS.

Preparation of farnesol

Farnesol (trans, trans-farnesol; Sigma-Aldrich) 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. The minimum inhibitory concentration (MIC) for methanol was performed and 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 minimum inhibitory concentrations (MICs) for farnesol, 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. Then, 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 ($0.5–2.5 \times 10^3$ CFU ml^{-1} for *C. albicans*, and 1×10^5 CFU ml^{-1} for *S. mutans*), diluted (1:5) in saline solution, and then subsequently diluted (1:20) in RPMI 1640 (for *C. albicans* ATCC 10231) or BHI broth (for *S. mutans* ATCC 25175). One hundred microliters of each microbial suspension were added into each well of 96-well microtiter plates (Costar, Tewksbury, MA, USA) pre-filled with 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 included as a positive control. The MIC assay was repeated using three independent assays.

Single and mixed species biofilms formed in the presence of farnesol

Single and mixed species biofilms of *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 were formed in the wells of 96-well microtiter plates (Costar). An aliquot of 200 µl of the standardized cell suspension (1×10^7 and 1×10^8 cells ml^{-1} in AS for *C. albicans* and *S. mutans*, respectively) was added to the wells for single biofilms or 100 µl of each suspension (2×10^7 cells ml^{-1} of *C. albicans* plus 2×10^8 cells ml^{-1} of *S. mutans*) for mixed species biofilms. The plates

were statically incubated in 5% CO_2 at 37°C for 2 h to promote cell adhesion. Then, the AS medium was aspirated, and each well was washed once with 200 µl of PBS to remove non-adherent cells. Farnesol was diluted in AS to obtain final concentrations of 1.56, 3.12, 6.25, 12.5, 25, 50, 70, 150 and 300 mM. These concentrations were based on the MIC value of *S. mutans* (1/4 MIC, 1/2 MIC, MIC, 2 × MIC, 4 × MIC and 8 × MIC, corresponding to 1.56, 3.12, 6.25, 12.5, 25 and 50 mM respectively) and on the MIC value of *C. albicans* (~ 1/2 MIC, MIC and 2 × MIC, corresponding to 70, 150 and 300 mM, respectively). Then, 200 µl of each dilution were inoculated into the wells and the plates were incubated for 48 h at 37°C in 5% CO_2. The AS medium was renewed after 24 h. After the 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 × the MIC of *Candida*) was used as a positive control whilst the wells inoculated with AS devoid of farnesol were used as negative controls. All assays were performed independently and in triplicate.

Quantification of total biofilm biomass

The total biomass of single and mixed species biofilms exposed to farnesol was analyzed using the crystal violet (CV) staining method (Monteiro et al. 2011). The resulting biofilms were fixed with 200 µl of 99% methanol (Sigma-Aldrich). After contact for 15 min, 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 to the wells which were then incubated for 5 min. The 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 added to each well to solubilize the stain. Absorbance was read at 570 nm using a microtiter plate reader (Eon Microplate Spectrophotometer; Bio Tek, Winooski, VT, USA) and standardized in relation to the area of the wells (Abs cm^{-2}).

Quantification of cultivable biofilm 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 the single species biofilm of *C. albicans* ATCC 10231) and BHI agar (for the single species biofilm of *S. mutans* ATCC 25175). For mixed species biofilms, the serial dilutions were plated on CHROMagar *Candida* (Difco) and BHI agar supplemented with 7 µg ml^{-1} of 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 each well 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, 1998) was used to determine the metabolic activity of single and mixed species biofilms of *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175. After the biofilm formation period, the wells were washed once with PBS and incubated with 200 µl of a solution containing 150 mg XTT l^{-1} and 10 mg of phenazine methosulphate l^{-1} (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 each well (absorbance cm^{-2}). Wells containing AS without biofilms were used as blanks to measure background levels.

Structural analysis of biofilms

SEM was performed to visualize changes in the structure of biofilms exposed to farnesol. Single and mixed species biofilms of *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 were formed in the wells of 24-well plates, initially adjusted to the cell concentration described above. After the cell adhesion period (2 h), farnesol was added to the wells at concentrations of 3.12 and 12.5 mM. In general, significant reductions in the number of CFUs were noted for biofilms exposed to 3.12 mM farnesol. Further, 12.5 mM farnesol behaved similarly to CHG. After biofilm formation (48 h), the wells were gently washed with PBS and the biofilms were dehydrated using an ethanol concentration series (70% for 10 min, 95% for 10 min and 100% for 20 min), followed by air drying for 20 min (Silva et al. 2013). The bottom of the each well containing a biofilm was then cut with a flame sterilized scalpel blade (number 11, Solidor, Lamedid Commercial and Services Ltda, Barueri, Brazil). The biofilms were then coated with gold and SEM analysis was performed (S-360 microscope, Leo, Cambridge, MA, USA).

Statistical analyses

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

Results

MIC determination

Table 1 shows the values of MIC and MFC/MBC for the tested strains. *S. mutans* ATCC 25175 (MIC = 6.25 mM;

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

		Farnesol (mM)		CHG (mM)	
Species	Strain	MIC	MFC/MBC	MIC	MFC/ MBC
C. albicans	ATCC 10231	150	300	0.0074	0.0296
S. mutans	ATCC 25175	6.25	6.25	0.0018	0.0074

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 800–20,000 times lower than those for farnesol.

Quantification of total biofilm biomass

The results of total biomass quantification of single and mixed species biofilms are shown in Figure 1. For single species biofilms of *C. albicans* ATCC 10231, farnesol at concentrations equal to or greater than 3.12 mM produced significant reductions in the total biomass, ranging from 58.03 to 66.41%, compared to the negative controls. For *S. mutans* ATCC 25175 biofilms, all treatments resulted in significant reductions in total biomass compared to the negative controls, ranging from 80.48 to 90.24%. For the mixed species biofilms, treatment with farnesol at 1.56, 3.12, 6.25, 12.5, 25 and 50 mM resulted in 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

Treatment of *C. albicans* ATCC 10231 biofilms 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 (Figure 2). Treatment with concentrations of farnesol equal to or higher than 12.5 mM were similar to CHG. However, for *C. albicans* ATCC 10231 in mixed species biofilms, the effect of farnesol in the reduction of CFUs was dose-dependent, with the greatest decrease at 150 mM farnesol (5.27 $\log_{10}$; $p < 0.001$) compared to the positive controls. Interestingly, 150 mM farnesol was more effective against *C. albicans* in mixed species biofilms than in single species biofilms.

Treatment of *S. mutans* ATCC 25175 biofilms with farnesol at 3.12, 6.25 and 12.5 resulted in 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 with farnesol treatment at 25 mM or above. For *S. mutans* in mixed species biofilms, 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 treatment using 70 mM farnesol. There was no growth for the group treated with farnesol at 300 mM.

Quantification of biofilm metabolic activity

The results of the evaluation of metabolic activity are shown in Figure 3. For single *C. albicans* ATCC 10231 biofilms, 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 at concentrations equal to or greater than 6.25 mM. Furthermore, the greatest decrease (99.20%) in metabolic activity was observed for the group treated with CHG. Similar results were found for the mixed species biofilms. Exposure to farnesol at 1.56, 3.12, 6.25, 12.5, 25 and 50 mM resulted in significant decreases in metabolic activity of 80.15, 92.06, 91.26, 94.4, 93.65 and 94.4%, respectively. Farnesol had no effect on *S. mutans* metabolic activity in single species biofilms.

Structural analysis of biofilms

Figure 4 shows that both farnesol at 12.5 mM and CHG changed the cell morphology of *C. albicans* ATCC 10231 and reduced the number of cells for both microorganisms in single and mixed species biofilms. The micrographs suggest 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. It was also noted that there were fewer hyphae in the mixed species biofilms treated with farnesol compared to the negative control.

Discussion

C. albicans and *S. mutans* are important oral pathogens able to form biofilms on different surfaces which may favor the development of diseases such as caries and DS (Falsetta et al. 2014). Considering the resistance of these biofilms to conventional agents, anti-biofilm therapies based on QS molecules are being examined. In this context, the major question in this study was whether the QS

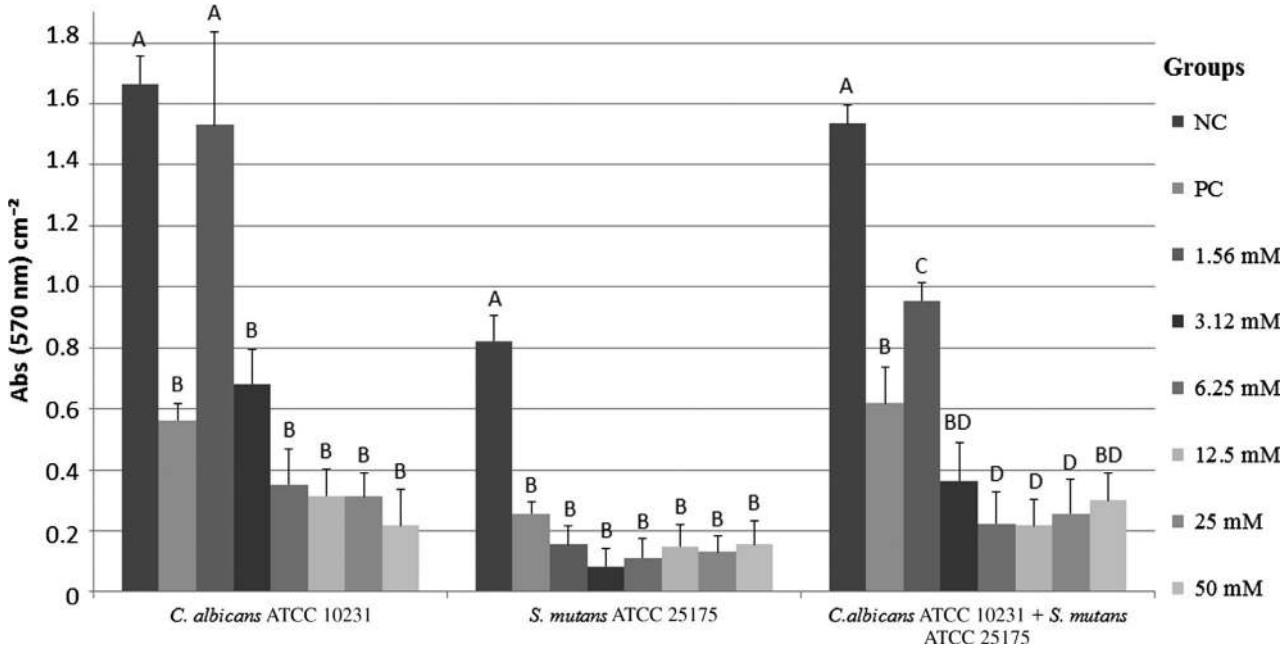

Figure 1. Average absorbances per cm^2 obtained with the CV assay for total biomass of single and mixed *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 biofilms (48 h) developed in the presence of farnesol at 1.56, 3.12, 6.25, 12.5, 25 and 50 mM. NC = negative control (*C. albicans* and *S. mutans* biofilms without farnesol). PC = positive control (chlorhexidine gluconate at 0.37 mM). Error bars display SDs of the means. Different capital letters denote significant differences ($p < 0.05$; one-way ANOVA followed by the *post hoc* Holm–Sidak test) among the treatments for each isolated biofilm.

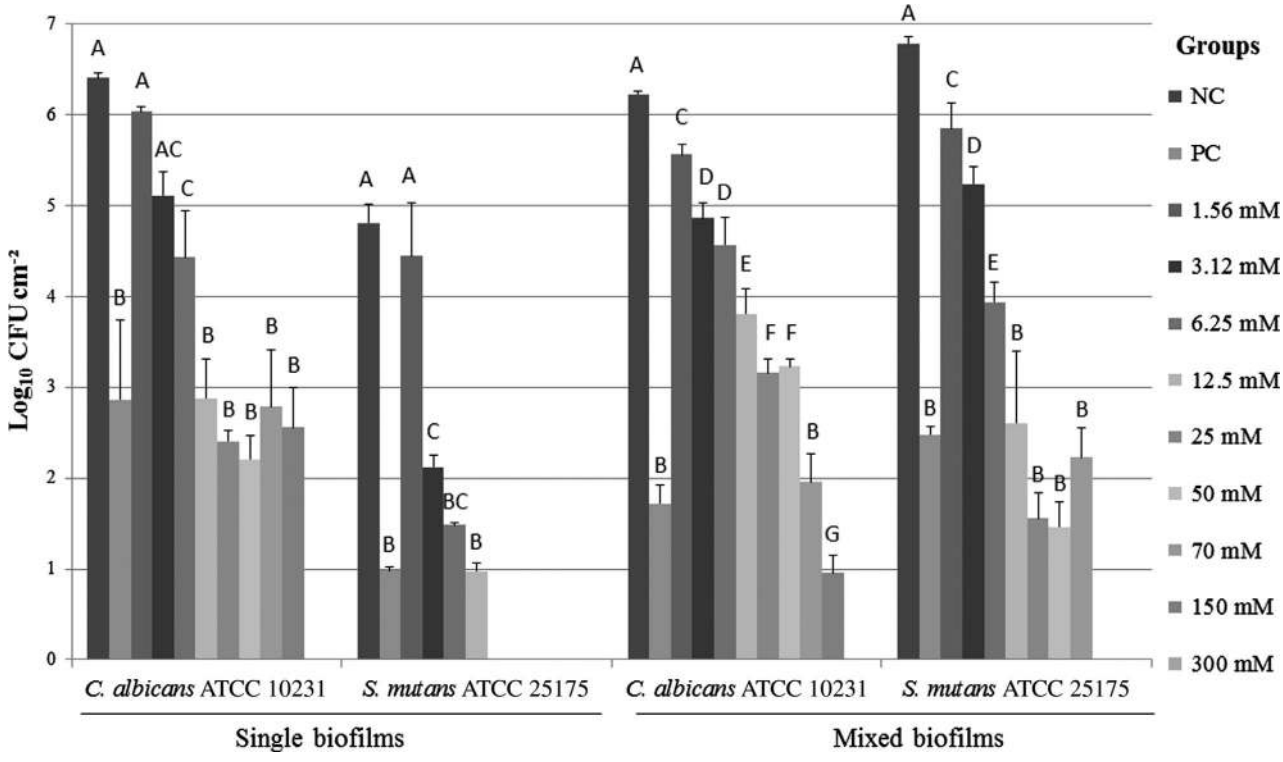

Figure 2. Mean values of the logarithm of colony forming units per cm^2 ($\log_{10}$ CFU cm^{-2}) for single and mixed *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 biofilms (48 h) developed in the presence of farnesol at 1.56, 3.12, 6.25, 12.5, 25, 50, 70, 150 and 300 mM. NC = negative control (*C. albicans* and *S. mutans* biofilms without farnesol). PC = positive control (chlorhexidine gluconate at 0.37 mM). Error bars display SDs of the means. Different capital letters denote significant differences ($p < 0.05$; one-way ANOVA followed by the *post hoc* Holm–Sidak test) among the treatments for each isolated biofilm.

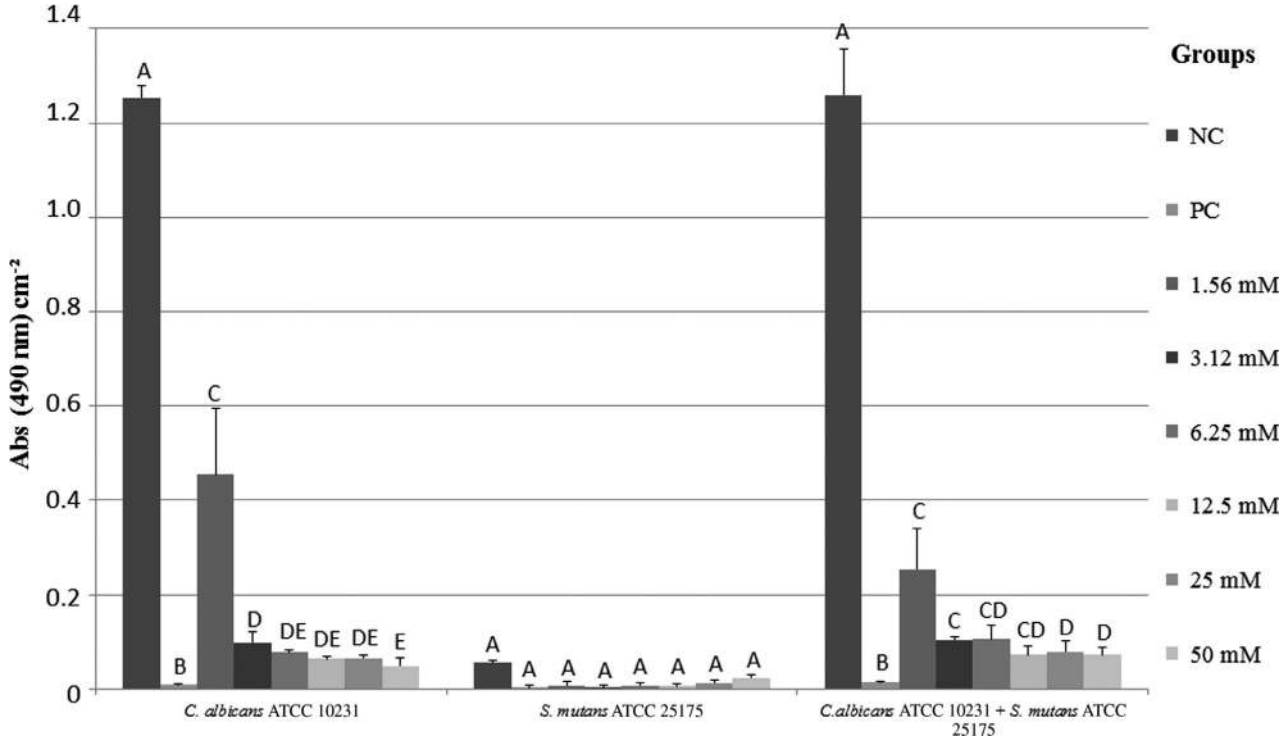

Figure 3. Average absorbances per cm^2 obtained with the XTT reduction assay for single and mixed *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 biofilms (48 h) developed in the presence of farnesol at 1.56, 3.12, 6.25, 12.5, 25 and 50 mM. NC = negative control (*C. albicans* and *S. mutans* biofilms without farnesol). PC = positive control (chlorhexidine gluconate at 0.37 mM). Error bars display SDs of the means. Different capital letters denote significant differences ($p < 0.05$; one-way ANOVA followed by the *post hoc* Holm–Sidak test) among the treatments for each isolated biofilm.

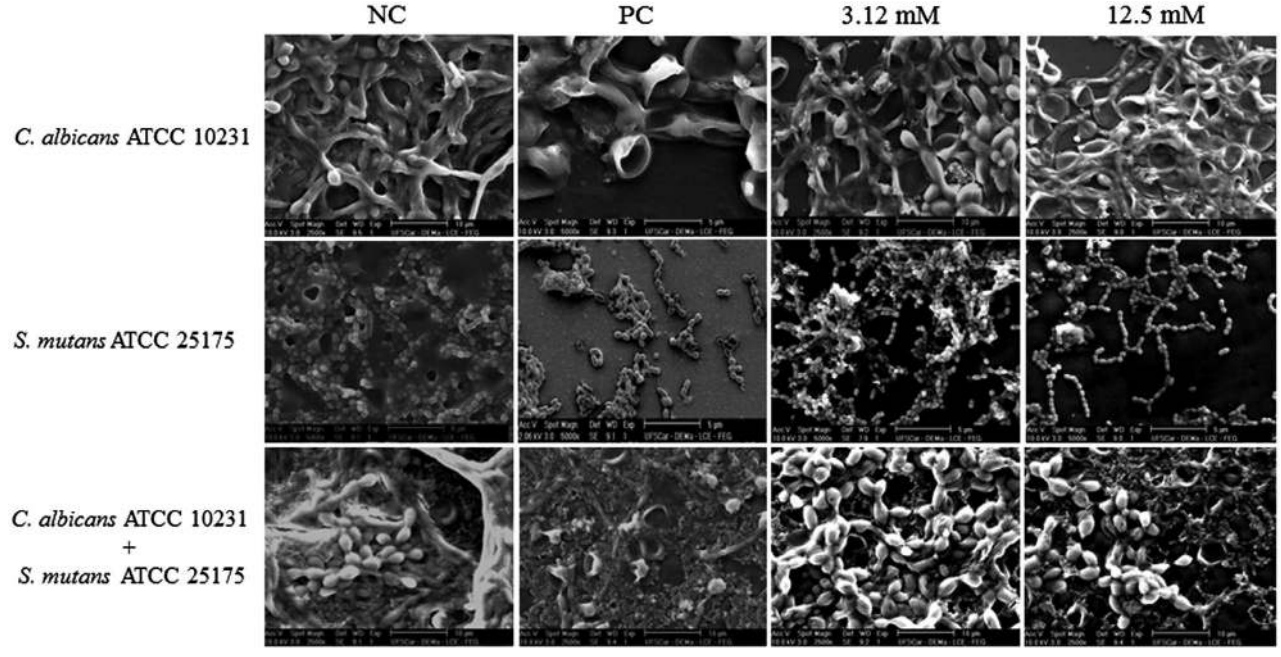

Figure 4. SEM images of single and mixed *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 biofilms (48 h) developed in the presence of farnesol at 3.12 mM, NC = negative control (biofilms without farnesol). PC = positive control (chlorhexidine gluconate at 0.37 mM). Magnification: × 2,500 and × 5,000. Bars, 5 µm and 10 µm.

molecule farnesol would be able to reduce both single and mixed species biofilm formation by *C. albicans* and *S. mutans*. The study hypothesis was partially confirmed because treatment with farnesol resulted in an inhibitory effect on single and mixed species biofilm formation, except for the metabolic activity of *S. mutans* in single species culture (Figure 3).

The MIC results (Table 1) showed that *S. mutans* ATCC 25175 was more susceptible to farnesol than *C. albicans* ATCC 10231. This finding is probably 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 action of farnesol. 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. (2002) the MIC value for *S. mutans* was 125 μM. These differences may be due to the different dilution protocols used. In the studies of Cordeiro et al. (2013) and Koo et al. (2002), farnesol was diluted in dimethyl sulfoxide (DMSO)-ethanol and 30% DMSO, respectively. The effect of farnesol diluted in these compounds could have been enhanced against the microorganisms tested by these authors. Also, 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 species biofilm formation by *C. albicans* and *S. mutans* using assays for the quantification of total biomass, the number of cultivable cells and metabolic activity. These methods are considered complementary, with specific advantages and disadvantages for each one. The CV assay comprises a simple and effective method to quantify the total biomass (cells and extracellular matrix). However, its limitation is that besides staining the extracellular matrix, the CV assay also stains both living and dead cells (Monteiro et al. 2015). So, it cannot be used alone as a susceptibility test of the response of biofilm cells to antimicrobials. Although 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 indicated that, in general, farnesol concentrations equal to or greater than 1.56 mM produced significant reductions in total biomass (Figure 1), the number of viable cells (Figure 2) and metabolic activity (Figure 3) in single and mixed species biofilms, except for the metabolic activity of *S. mutans* ATCC 25175 single species biofilms. Depending on the strain, XTT may not be absorbed and/or metabolized and, consequently, the stains are not detected by a spectrophotometer. This might have happened with *S. mutans* biofilms since, in single species culture, the values were very low and almost at the limit of detection Also, in mixed species biofilms, the values were almost the same as those for *C. albicans,* indicating that *S. mutans* cells may not have absorbed and/or metabolized XTT (Kuhn et al. 2003; Gobor et al. 2011), making comparison of these results difficult.

It is well known that farnesol blocks the conversion of yeast cells to hyphae in *C. albicans* (Langford et al. 2013; Piispanen et al. 2013), prevents cell adhesion and promotes detachment of biofilms from some surfaces (Cerca et al. 2012; Nagy et al. 2014). These effects may explain the significant reductions in biomass found for *C. albicans* ATCC 10231 in single and mixed species biofilms. As hyphae are larger structures which absorb greater amount of CV than yeast cells, the treatments with farnesol might have resulted in biofilms with lower numbers of hyphae than the untreated biofilms, therefore resulting in lower absorbance values (Figure 1). This was confirmed using SEM analysis (Figure 4). Moreover, these findings are consistent with those found for XTT reduction (Figure 3), suggesting that the decreases in cell metabolism might have contributed to reducing the production of extracellular matrix material. It is reported in the literature that for *C. albicans*, farnesol treatment may induce apoptosis and disordered mitochondria due to the presence of reactive oxygen species (Zhu et al. 2011) (Langford et al. 2009), change cell development (Uppuluri et al. 2007) and result in necrosis (Dumitru et al. 2007). These mechanisms of action might justify the significant reductions observed in the number of viable cells (Figure 2) and metabolic activity (Figure 3) for *Candida* biofilms treated with farnesol in this study.

SEM images also indicated that the treatments with farnesol (mainly at 12.5 mM) reduced biofilm formation in both species in single and mixed species cultures. SEM images of single species biofilms of *S. mutans* showed a reduction in the number of cells when treated with farnesol, while for mixed species biofilms this reduction is not as apparent, suggesting a decreased susceptibility to farnesol when *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 are grown together.

For *S. mutans* ATCC 25175 in single and mixed species biofilms, in general, all treatments with farnesol promoted significant reductions in total biomass (Figure 1) and the number of viable cells (Figure 2). Although there was no significant reduction in metabolic activity (Figure 3), farnesol may have increased cell permeability in *S. mutans* and acted directly on the enzyme F-ATPase, 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 from the present study was that, for *C. albicans* ATCC 10231 in dual species biofilms, farnesol showed a more marked dose-dependent effect on reducing the number of cells than in single species biofilms (Figure 2). Indeed, farnesol at 150 mM showed the highest reduction in the number of CFUs for *C. albicans* ATCC 10231 in mixed species biofilms. Clinically, this result is very important since *C. albicans* is found in polymicrobial biofilms in the oral cavity. However, for *S. mutans* ATCC 25175, both farnesol and CHG were more effective against single species biofilms (Figure 2). According to the negative control groups, the number of viable *S. mutans* ATCC 25175 cells in mixed species biofilm was significantly higher than in single species biofilms, indicating that the presence of *C. albicans* ATCC 10231 favored 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 biofilm acidification (Klinke et al. 2009), creating an environment that favors the growth of *S. mutans*. In addition, *S. mutans* cells adhere to the cells and hyphae of *C. albicans*, secrete more EPS and acquire additional protection from antimicrobials (Metwalli et al. 2013). Thus, this synergistic relationship limits the competition between those species and favors mixed species biofilm formation in different oral niches.

Farnesol treatment in concentrations equal to or greater than 12.5 mM resulted in significant reductions in total biomass, the number of CFUs and metabolic activity. Furthermore, these decreases were similar to those found for CHG (positive controls). From a clinical point of view, these findings demonstrate that the use of very high concentrations of farnesol is not required to achieve a beneficial effect on inhibition of biofilm formation, and the use of low farnesol concentrations may prevent toxic effects on human cells. Additionally, compared to CHG, farnesol seems to have similar effects at 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 antifungal drugs should be encouraged.

Finally, despite using only one strain of each species in this study, it may be concluded that farnesol has an inhibitory effect on single and mixed species biofilms formed by *C. albicans* and *S. mutans*, and this compound in concentrations above 12.5 mM behaved similarly to CHG. An *in vivo* study has shown good results in treatments using farnesol with fluoride on dental caries (Falsetta et al. 2012). 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 such as candidiasis and tooth decay.

Disclosure statement

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