



Farnesol Anti-biofilm Activity against *Candida albicans* Reference and Mutant Strains

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AFPC and SD designed the study. Author BHDP managed the literature search. Authors AFPC, SFFG and PVS performed the experiments and collected data. Author MNJ performed the statistical analysis. Results interpretation and discussion were performed by all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Farnesol is known as a quorum sensing (QS) molecule that has a role as an anti-biofilm agent. It is produced by *C. albicans* and blocks the morphological transition from yeasts to hyphae. The hyphal development is important for the formation of substantial biofilm biomass. Mutant strains lacking the filamentation genes EFG1 and TEC1 are less virulent than their reference strains.

Aims: To determine the role of the transcription factors EFG1 and TEC1 by using knockout strains

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(Δ/Δ *efg1* and Δ/Δ *tec 1*) on farnesol's mechanism of action regarding dry weight and colony count of *Candida albicans* biofilms.

Study Design: *tt*-farnesol solutions at a concentration of 12.5 mM were prepared. Biofilms (n=6) of the strains *C. albicans* SN 425 (reference strain), *C. albicans* C.JN 2330 (Δ/Δ *tec1*) and *C. albicans* C.JN 2302 (Δ/Δ *efg1*) were treated twice daily with *tt*-farnesol or a vehicle control for 1 min during biofilm formation (48 h). Biofilms were also treated with 0.2% chlorhexidine (1 min) as a positive control and 0.89% NaCl (1 min) as a negative control. After treatments, biofilms were evaluated by dry weight (μ g) and colony forming units per milliliters (CFU/mL).

Results: Data were analyzed by two-way ANOVA and *post-hoc* t-tests ($\alpha=0.05$). Relative to the control, farnesol significantly reduced the CFU/mL of all the *C. albicans* strains and the biomass of the mutant strain C.JN 2330.

Conclusion: Twice daily treatment with *tt*-farnesol at a concentration of 12.5 mM exhibited anti-biofilm activity against *C. albicans*. Analysis of strain differences suggests that the presence of the transcription factor TEC1 protects the biofilm against *tt*-farnesol mechanism of action.

Keywords: *Candida*; biofilms; farnesol; *EFG 1*; *TEC 1*; antimicrobials; candidiasis; oral.

1. INTRODUCTION

Denture stomatitis is a common inflammatory reaction in denture-wearing patients, characterized by an erythematous inflammation of mucosal areas covered by dentures. Although this clinical entity is multifactorial, *Candida albicans* is the major etiological agent [1]. *Candida* infections are related mainly to the morphology transition of yeast to hyphae, because hyphae can adhere to and penetrate the host tissues [2]. This morphologic transition can be influenced by the quorum sensing system (QS), which is a complex cross-talking system where microorganisms communicate with each other in response to cell density via QS molecules [3].

Farnesol is a sesquiterpene alcohol (3,7,11-trimethyl-2,6,10-dodecatrien-1-ol) found in propolis and citrus fruits [4]. It has been found to be a natural anti-biofilm agent [5]. Topical applications of *tt*-farnesol for 1 min twice-daily reduced the exopolysaccharides amounts and development of single-species *Streptococcus mutans* biofilms on saliva-coated hydroxyapatite surfaces [5]. Additionally, *tt*-farnesol treatment in concentrations equal to or greater than 12.5 mM resulted in significant reductions in total biomass, CFUs and metabolic activity of a multispecies biofilm formed by *S. mutans* and *C. albicans*, and these reductions were also observed for the single species biofilms [6].

Farnesol has been recognized as a QS molecule involved in the coordination of activities among groups of many single-celled organisms [7]. It is produced by *C. albicans* and blocks the morphological transition from yeasts to hyphae

[8] via inhibition of MAP kinase cascades [9]. The literature indicates farnesol has a role as an anti-biofilm agent [6]. The hyphal development pathway is critical for the establishment of significant biofilm mass [10]. Mutants defective in the enhanced filamentous growth transcriptional factor (*EFG 1*), a major activator of hyphal development, does not form a bulky and regular biofilm on polystyrene surfaces [10]. *TEC 1* is a transcription factor required for hyphal development [11]. Mutant strains defective in these filamentation genes are less virulent than their reference strains and show lower levels of infectivity of endothelial cells and plasma-coated catheters [12-13].

The transcriptional network controlling *C. albicans* biofilm formation was previously investigated comparing the reference strain used in this study (SN425) to six gene knockout mutants of this diploid organism, including those with absence of the genes *EFG 1* (Δ/Δ *efg1*) and *TEC 1* (Δ/Δ *tec 1*) in both chromosomes [14]. In the same study, confocal images showed that the reference strain formed a biofilm with typical architecture and thickness of 250 μ m in depth, containing both round budding yeast-form cells adjacent to the substrate and hyphal cells extending throughout the biofilm. But the mutants formed rudimentary biofilms of 20–80 μ m in depth. Moreover, Δ/Δ *efg1* mutant strain did not form hyphae [14].

Several chemical agents have been employed for cleaning dentures to prevent cross-infection. However, previous studies have demonstrated changes in characteristics of denture base resin due to immersion in chemical cleaners, such as sodium-hypochlorite (NaOCl) and alkaline

peroxides that may affect the lifetime of dentures [15]. These agents can compromise the mechanical properties and color stability of the dentures. Also, sodium- hypochlorite (NaOCl) has oxidative properties in metal framework [16]. Moreover, denture stomatitis is related to the growth of *Candida* biofilms on the prosthesis and not on the palate mucosa, so the ideal treatment to this disease should be directed to the prosthesis [17]. Thus, new disinfectants are necessary, considering an optimal denture disinfectant is not available. Farnesol is a potential anti-biofilm agent [5-6], however, its exact mechanism of action still unknown. In the present study we used knockout strains (Δ/Δ *efg1* and Δ/Δ *tec 1*) to determine the role of the transcription factors EFG1 and TEC1 on farnesol's mechanism of action regarding dry weight and colony count (CFU/mL) of *C. albicans* biofilms.

2. METHODOLOGY

2.1 Inoculum and Biofilm Model

2.1.1 Biofilm inoculums

Three strains of *Candida*- *C. albicans* SN 425, CJN 2302 and CJN2330 [14]-, maintained as frozen stocks at -80°C , were reactivated on Sabouraud dextrose agar plates (SDA) plates. To prepare the inoculum, colonies of *Candida* were inoculated into 10 mL of Yeast Nitrogen Base (YNB) supplemented with 100 mM of glucose and incubated at 37°C for 16 h. After 16 h of incubation, the inoculums were diluted into fresh YNB medium until the three strains reached the mid-log growth phase. Then, the inoculums were adjusted to 10^7 cells/mL at the OD of 540 nm [18].

2.1.2 Biofilm formation

One (1) mL of the inoculums was transferred to the wells of a 24-well polystyrene plate. The biofilms were incubated for 90 minutes at 37°C for the adhesion of cells to the bottom of the wells. After 90 min, the cells that were not adhered were removed by 2 washes with 1 mL of 0.89% NaCl. After washings, 1 mL of fresh RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) was added to each sample. The biofilms were then incubated at 37°C and treatments were applied twice-daily. After 48-hour incubation, the biofilms were processed analyzed.

2.2 Preparation of Treatment Solutions

The tested solutions were prepared at the time of use from a commercial solution of *tt*- farnesol (277541-1G Trans, trans-Farnesol, Sigma-Aldrich) using a solution containing 20% ethanol and 2% tween 80 as the vehicle. A solution of 12.5 mM of *tt*-farnesol was prepared for the assays [6]. A vehicle solution containing 20% ethanol and 2% tween 80 was also used as a control. As positive control, 0.2% chlorhexidine (CHX) was applied. As a negative control, 0.89% NaCl was used. The biofilms were treated with *tt*-farnesol or control solutions (1 min) twice-daily until complete 48 h of the experimental period.

2.3 Biofilm Analysis

After the last treatment, 1 mL of 0.89% NaCl was added to the biofilms and the biofilms were removed from the bottom of each well by scratching with a pipette tip. Then, the biofilms removed with 1 mL of NaCl were individually transferred to sterile centrifuge tubes. Additional 1 mL of 0.89% NaCl was added to these tubes, resulting in 2 mL of biofilm suspension. From this suspension, 100 μL was serially diluted and plated in SDA plates for the quantification of colony forming units (CFU/mL). For the dry weight (biomass), 100 μL of biofilm solution [18] was transferred to microcentrifuge tubes previously weighed. One (1) mL of ice-cold 99% ethanol was added to the tubes. Before, tubes were centrifuged (10,000 $\times g$, 10 min, 4°C) and the supernatant of each tube was discarded. The *pellet* was washed twice with ice-cold 75% ethanol and air-dried. The tubes were then weighed again, and the dry weight was determined in micrograms (μg).

2.4 Statistical Analyses

Tests were performed in three separate occasions in duplicate ($n=6$). IBM SPSS v. 22 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis with a confidence level of 95%. CFU/mL data were Log_{10} transformed prior to analysis and were further rank transformed for analysis of variance (ANOVA). For biomass, ANOVA was performed with data in micrograms (μg). Group differences were compared using a two-way ANOVA with factors of treatment and strain, and given a significant omnibus test, *post-hoc* t-tests were pursued using a pooled estimate of the standard error.

3. RESULTS AND DISCUSSION

C. albicans is the major etiological agent of denture stomatitis [1] and the capacity to undergo a reversible yeast-hypha transformation is linked to its virulence [13]. When infecting humans and animals, *C. albicans* hyphae predominate at the primary site of infiltration of epithelial cell layers and tissues, while yeast cells are normally found either on the epithelial cell surface or emerging from penetrating hyphae that are infiltrating tissues [19]. Thus, reducing hyphae is important to reduce the penetration of *C. albicans* into the host tissues. Considering that the ideal treatment to denture stomatitis should be directed to the prosthesis [17], treating denture with farnesol might be a good alternative to the classic treatments. Farnesol effects on *C. albicans* biofilm were previously demonstrated [6, 20-22]; however, its exact mechanism of action is still unknown. The major question in this study was to investigate if the transcription factors EFG1 and TEC1 were involved in farnesol's mechanism of action in *C. albicans*' biofilm biomass and colony count.

In the present study, *tt*-farnesol was applied at a concentration of 12.5 mM, based on previous

results [6]. Farnesol is known to act mainly in the morphological transition from yeasts to hyphae, which happens during the maturation of the biofilm [23]. Therefore, we performed the treatments during biofilm development, starting after the adhesion phase, twice a day, trying to simulate the denture cleaning that could be performed at home by the patient. Until we are aware, this is the first time *tt*-farnesol is tested against *C. albicans* mutant strains.

The results of CFU/mL are summarized in Fig. 1. Two-way ANOVA showed an interaction between "strain" and "treatment" factors ($p < .001$). Post-hoc testing then showed that *tt*-farnesol at 12.5 mM was associated with lesser colonies than the negative control group (NaCl) for all strains, but more colonies than the positive control, CHX.

Comparing strains for *tt*-farnesol 12.5 mM treatment, the reference strain (SN 425) exhibited the fewest colonies (Fig. 1). Moreover, the treatment with *tt*-farnesol caused a significant reduction in the CFU counts of all strains compared to the treatment with 0.89% NaCl. According to previous studies, in addition to the fact that farnesol blocks the conversion of yeast cells to hyphae in *C. albicans* [8],

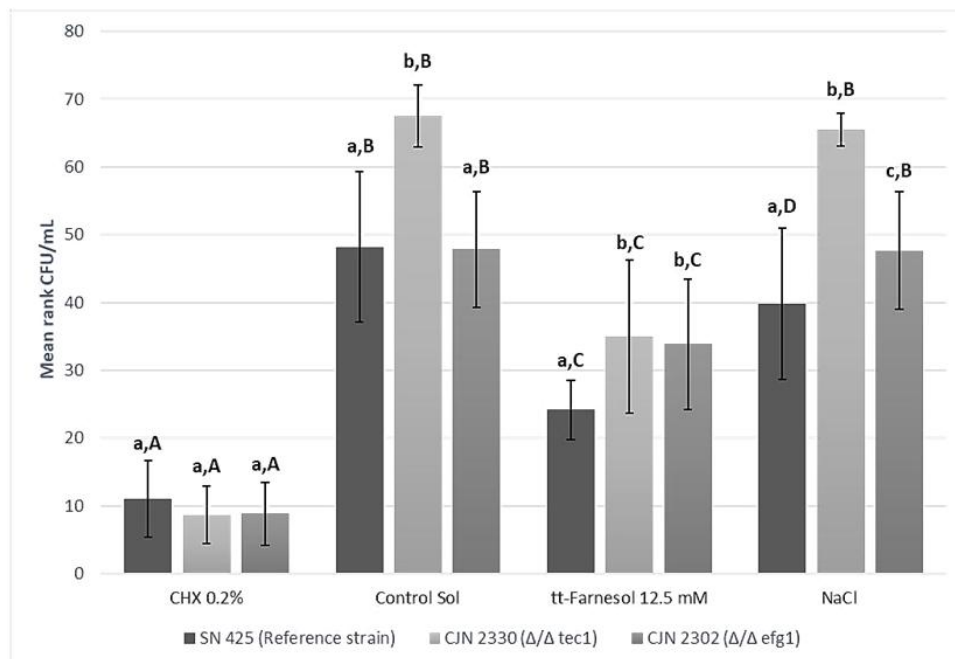


Fig. 1. Mean rank and standard deviations of rank CFU/mL. Means followed by the different letters are significantly different ($p < .05$)
Lowercase letters represent differences between strains within each treatment. Uppercase letters represent differences between treatments for each strain.

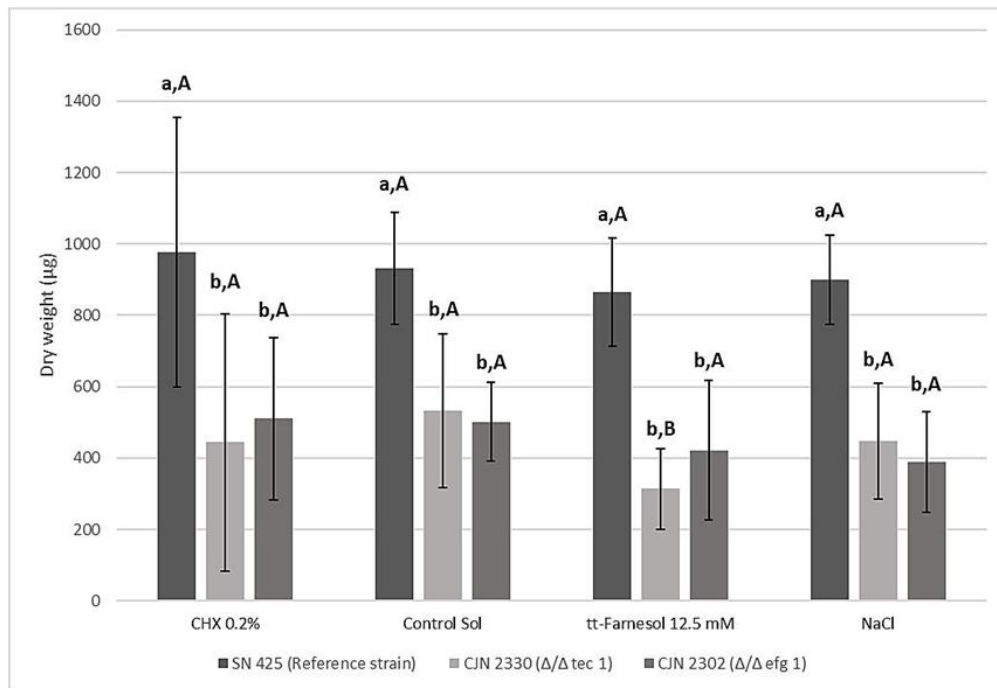


Fig. 2. Mean and standard deviations of dry weight (µg). Means followed by the different letters are significantly different ($p < .05$)

Lowercase letters represent differences between strains within each treatment. Uppercase letters represent differences between treatments for each strain.

it has also been shown to prevent cell adhesion and promote detachment of biofilms from different surfaces [24]. In contrast, *tt*-farnesol did not exhibit similar results of CFU/mL to the positive control (CHX). Although CHX is considered a gold standard antibacterial disinfectant [25], it has disadvantages including color changing in auto polymerizing relene resins for denture [26] and in acrylic denture teeth [27]. So, an ideal disinfectant is not available, and the positive results obtained with farnesol can open doors for new trials with this compound for denture disinfection.

For biomass (dry weight), results showed again an interaction between the factors “strain” and “treatment” ($p < .001$) (Fig. 2). While *tt*-farnesol treatment was associated with reduced biomass in the CJN 2330 strain, it had no effect on SN 425 or CJN 2302 strains.

The reference strain (SN 425) presented a higher biomass than the mutant strains for all treatments (Fig. 2), confirming the mutant strains have deficiency in biofilm formation. Interestingly, *tt*-farnesol significantly reduced the biomass only of the mutant strain CJN 2330 (Fig.

2), not only regarding Control and NaCl treatments, but also lower than CHX. The biomass comprises cells in the biofilm and the extracellular matrix produced by the biofilms, which is composed of exopolysaccharides, proteins and extracellular DNA [18]. Therefore, *tt*-farnesol acted reducing the colony counting of all strains but might not have reduced components of the extracellular matrix of CJN 2302 and SN 425. On the other hand, *tt*-farnesol might have reduced some component(s) of the extracellular matrix of CJN 2330 and might have acted to reduce the transition from yeast to hyphae, resulting in lower biomass. This outcome suggests that the presence of the transcription factor TEC1 protects the biofilm against *tt*-farnesol mechanism of action.

4. CONCLUSION

The aim of the study was to determine the role of the transcription factors EFG1 and TEC1 on farnesol's mechanism of action in *C. albicans*' biofilm biomass and colony count. Within the limitations of this *in vitro* study, we conclude that the presence of the transcription factor TEC1 might protect the biofilm against *tt*-farnesol

mechanism of action and that twice-daily treatment with 12.5 mM *tt*-farnesol showed anti-biofilm effects against both *C. albicans* mutant and reference strains. However, the biological significance of these findings would need further studies.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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