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ORIGINAL ARTICLE

Influences of trans-trans farnesol, a membrane-targeting sesquiterpenoid, on *Streptococcus mutans* physiology and survival within mixed-species oral biofilms

Jae-Gyu Jeon[1,2], Santosh Pandit[1,2], Jin Xiao[3,4,5], Stacy Gregoire[4], Megan L. Falsetta[4], Marlise I. Klein[3,4], Hyun Koo[3,4]*

[1]*Department of Preventive Dentistry, School of Dentistry, Chonbuk National University, Jeonju 561756, Republic of Korea;* [2]*Institute of Oral Bioscience and BK 21 program, Chonbuk National University, Jeonju 561756, Republic of Korea;* [3]*Center for Oral Biology, University of Rochester Medical Center, Rochester, New York 14620, USA;* [4]*Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, New York 14620, USA;* [5]*State Key Laboratory of Oral Diseases, Sichuan University, Chengdu 610041, China*

Trans-trans farnesol (*tt*-farnesol) is a bioactive sesquiterpene alcohol commonly found in propolis (a beehive product) and citrus fruits, which disrupts the ability of *Streptococcus mutans* (*S. mutans*) to form virulent biofilms. In this study, we investigated whether *tt*-farnesol affects cell-membrane function, acid production and/or acid tolerance by planktonic cells and biofilms of *S. mutans* UA159. Furthermore, the influence of the agent on *S. mutans* gene expression and ability to form biofilms in the presence of other oral bacteria (*Streptococcus oralis* (*S. oralis*) 35037 and *Actinomyces naeslundii* (*A. naeslundii*) 12104) was also examined. In general, *tt*-farnesol (1 mmol·L^{-1}) significantly increased the membrane proton permeability and reduced glycolytic activity of *S. mutans* in the planktonic state and in biofilms (P<0.05). Moreover, topical applications of 1 mmol·L^{-1} *tt*-farnesol twice daily (1 min exposure/treatment) reduced biomass accumulation and prevented ecological shifts towards *S. mutans* dominance within mixed-species biofilms after introduction of 1% sucrose. *S. oralis* (a non-cariogenic organism) became the major species after treatments with *tt*-farnesol, whereas vehicle-treated biofilms contained mostly *S. mutans* (>90% of total bacterial population). However, the agent did not affect significantly the expression of *S. mutans* genes involved in acidogenicity, acid tolerance or polysaccharide synthesis in the treated biofilms. Our data indicate that *tt*-farnesol may affect the competitiveness of *S. mutans* in a mixed-species environment by primarily disrupting the membrane function and physiology of this bacterium. This naturally occurring terpenoid could be a potentially useful adjunctive agent to the current anti-biofilm/anti-caries chemotherapeutic strategies.

***Keywords*:** trans-trans farnesol; acid production; acid tolerance; biofilms; proton permeability; *Streptococcus mutans*

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Introduction

Dental caries is a transmissible and costly biofilm-related oral infectious disease [1-2], which continues to afflict the majority of the world's population [3]. This ubiquitous disease results from the interaction of specific bacteria with constituents of the diet within a biofilm formed on the tooth surface clinically known as dental plaque [4]. *Streptococcus mutans* (*S. mutans*), a member of the oral microbiome, is a particularly virulent orga-

*Correspondence: Hyun Koo
E-mail: Hyun_Koo@urmc.rochester.edu
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nism that (1) efficiently utilizes dietary sucrose to synthesize large amounts of extracellular polysaccharides (EPS) through glucosyl-/fructosyl-transferases, (2) adheres tightly to glucan-coated surfaces, and (3) is also highly acidogenic-acid tolerant [3, 5-6]. This unique combination of virulence attributes allows *S. mutans* to effectively colonize the tooth surface and modulate the transition of nonpathogenic to highly cariogenic dental biofilms.

If the biofilm is allowed to remain on tooth surfaces with a frequent consumption of a high carbohydrate diet (especially sucrose), *S. mutans*, as a constituent of the biofilm community, will continue to synthesize exopolysaccharides and metabolize the sugars to organic acids. The elevated amounts of EPS increase the bulk and structural stability of the biofilm [5]. In addition, the ability of *S. mutans* to utilize some EPS and intracellular polysaccharides (IPS) as short-term storage compounds offers an additional ecological benefit, which simultaneously increases the amount of acid production and the extent of acidification within the biofilm [4, 6-7]. The persistence of this aciduric environment leads to selection and dominance of highly acid tolerant (and acidogenic) microorganisms, such as *S. mutans* [3, 6, 8]. The low pH environment within the matrix results in dissolution of enamel, thus initiating the pathogenesis of dental caries. Clearly, therapeutic approaches aimed at disrupting the ability of *S. mutans* to utilize sucrose to produce EPS, organic acids and/or adapt aciduric environment, would be more precise than traditional antimicrobial therapies based on use of broad spectrum microbiocides [9].

Recently, we have identified *tt*-farnesol, a sesquiterpene alcohol (3,7,11-trimethyl-2,6,10-dodecatrien-1-ol) commonly found in propolis (a resinous beehive product) and citrus fruits, as a novel naturally occurring anti-caries/anti-biofilm agent [10-11]. Topical applications of *tt*-farnesol (1 min exposure, twice daily) diminished the EPS content and development of single-species *S. mutans* biofilms on saliva-coated hydroxyapatite (sHA) surfaces, and reduced the severity of smooth-surface caries in rats [10]. However, treatment is not lethal to *S. mutans* biofilms *in vitro*, did not inhibit glucosyltransferases activity, and *tt*-farnesol displayed minimal effects on the viability of the oral flora populations *in vivo* [10, 12-13]. It is possible that *tt*-farnesol may cause acid sensitization and affect intracellular metabolism of *S. mutans* by disrupting the bacterial membrane function due to its fatty acid-like structure and lipophilic characteristics [10, 12]; consistent with reduction of IPS accumulation in *S. mutans* biofilms treated with *tt*-farnesol [11,13]. We hypothesize that *tt*-farnesol can directly affect *S. mutans* physiology by damaging the cell-membrane, and thereby disrupt its ability to compete and become dominant in a mixed-species biofilm environment.

Therefore, the aim of this study was to evaluate the influence of *tt*-farnesol on proton permeability, acid production and acid tolerance of *S. mutans* in the planktonic state and within biofilms. Furthermore, we investigated whether this naturally occurring sesquiterpene affects the development of biofilms and gene expression by *S. mutans* grown on sHA surfaces in the presence of sucrose and other oral bacteria.

Materials and Methods

Test compounds

tt-Farnesol was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). The test agent was dissolved in 15% ethanol containing 2.5% dimethyl sulphoxide (DMSO) just prior to carrying out the assays. For this study we tested 0.25, 0.5 and 1 mmol·L^{-1} *tt*-farnesol.

Bacterial strains and culture condition

S. mutans UA159 (serotype c), a proven virulent cariogenic dental pathogen and the strain selected for genomic sequencing, was used for proton permeability and glycolytic pH-drop assays. *S. mutans* UA159, *Actinomyces naeslundii* (*A. naeslundii*) ATCC 12104 and *Streptococcus oralis* (*S. oralis*) ATCC 35037 were used for the mixed-species biofilm experiment [14]. The cultures were stored at –80 ℃ in tryptic soy broth containing 20% glycerol. All the assays were done with bacterial cells grown in filtered (10 kDa molecular-weight cut-off membrane; Prep/Scale, Millipore, MA, USA) buffered tryptone-yeast extract broth (UFTYE; 2.5% tryptone and 1.5% yeast extract, pH 7.0) supplemented with 1% (*W*/*V*) glucose (UFTYE-G; for planktonic cells) or 1% sucrose (UFTYE-S; for biofilms).

Proton permeability assay

The effects of *tt*-farnesol on proton permeability of planktonic and biofilm cells of *S. mutans* were assessed using standard procedures described by Phan *et al*. [15]. For planktonic cells, cultures of *S. mutans* in UFTYE-G were harvested at late-exponential phase by centrifugation (10 000 *g*, 10 min, 4 ℃). For biofilm-cells, *S. mutans* biofilms were formed on saliva-coated hydroxyapatite (sHA) discs (2.93 cm^2; Clarkson Chromatography Products, Inc., South Williamsport, PA, USA) placed in a vertical position in 24-well plates containing UFTYE-S until 75 h (mature-phase) (Figure 1) [13-14]. Briefly, cells in suspensions (2 mg cell dry weight per mL) or *S. mutans* biofilms (5 mg biomass dry-weight per mL of which ~2 mg cell-weight) were initially washed with salt

solution (50 mmol·L^{-1} KCl plus 1 mmol·L^{-1} $MgCl_2$). Subsequently, they were incubated in salt solution at a constant pH value of approximately 4.6. Then, HCl was added to drop pH values by about 0.4 units followed by addition of *tt*-farnesol (0.25, 0.5 and 1 mmol·L^{-1}) or vehicle control (15% ethanol + 2.5% DMSO, *V*/*V*). The subsequent rise in pH associated with movements of protons across the cell membrane into the cytoplasm was monitored with a glass electrode. The initial rate of proton entry was also estimated because changes in initial rates of proton uptake give the best measure of disruptive effects. The proton entry rate was calculated using the pH changes of 0–0.5 min (planktonic cells) or 0–10 min (biofilms) incubation period. Butanol (final concentration: 10%, *V*/*V*) was added to the suspensions at 25 (planktonic cells) or 80 min (biofilms) to damage the cell membrane [15].

Figure 1 Saliva-coated hydroxyapatite (sHA) biofilm model. EPS-extracellular polysaccharides (adapted from [14]).

Glycolytic pH-drop assay

The effects of *tt*-farnesol on acid production by planktonic and biofilm cells of *S. mutans* were measured by glycolytic pH-drop assay [16]. Briefly, cells in suspensions (2 mg cell dry weight per mL) or *S. mutans* biofilms (5 mg biomass dry-weight per ml of which ~2 mg cell-weight) were washed with salt solution (50 mmol·L^{-1} KCl plus 1 mmol·L^{-1} $MgCl_2$, pH 7.0) and resuspended (or placed, for biofilms) in a salt solution containing the test agent (0.25, 0.5 and 1 mmol·L^{-1}) or vehicle control (15% ethanol + 2.5% DMSO, *V*/*V*). The pH was adjusted to 7.2 with 0.01 mol·L^{-1} KOH solution, glucose was added in the mixture to give a final concentration of 1% (*W*/*V*), and the decrease in pH was assessed by means of a glass electrode over a period of 120 min (Futura Micro Combination pH electrode, 5 mm diameter, Beckman Coulter Inc., CA, USA). The rate of pH drop was calculated using the pH values in the linear portion.

Preparation and treatment of mixed-species biofilms

Mixed-species biofilms were formed on sHA discs placed in a vertical position in 24-well plates (Figure 1) [14], and was designed to mimic the formation of biofilms according to "ecological plaque-biofilm" concept. We selected *S. oralis* 35037 and *A. naeslundii* 12104, in addition to *S. mutans* UA159, to generate mixed-species biofilms; these three organisms are found in supragingival plaque in humans [3]. *S. oralis* is one of the most commonly detected early colonizers of the tooth surface [17]; *A. naeslundii* is also found in the early stages of plaque formation and may be also associated with development of root caries [3]; the strain 12104 is acidogenic and produces EPS, including fructans [18].

S. mutans UA159, *A. naeslundii* 12104 and *S. oralis* 35037 cells were grown in UFTYE-G at 37 ℃ and 5% CO_2 to mid-exponential phase (OD_{600nm} 0.5 for streptococci and OD_{600nm} 0.75 for *A. naeslundii*). Each of bacterial suspensions were mixed to provide an inoculum with a defined microbial population of *S. mutans* (10^2 CFU·mL^{-1}), *A. naeslundii* (10^6 CFU·mL^{-1}), and *S. oralis* (10^7 CFU·mL^{-1}). The preparation of a defined mixture of bacterial species is critical for repeatability and reproducibility of our biofilm model [14]. The mixed population of bacterial cells were inoculated into 2.8 mL of UFTYE with 0.12% sucrose, and incubated at 37 ℃ and 5% CO_2. During the first 19 hours, the organisms were grown undisturbed to allow initial biofilm formation. Then, after 19 hours, the culture medium was replaced, and the biofilms were grown until 29 hours to establish a mixed-species community. After 29 hours of biofilm growth, the biofilms were transferred to UFTYE broth containing either 0.12% sucrose (sucrose-limited condition) or 1% sucrose (sucrose-excess; to induce environmental changes to simulate a cariogenic challenge).

The culture medium was then changed twice daily (8 am and 6 pm) until the end of the experimental period (91 h). The biofilms were treated twice daily (one-minute exposure, at 10 am and 4 pm) with 1 mmol·L^{-1} *tt*-farnesol or vehicle control from 45 h to the end of the experimental period (91 h). The biofilms were exposed to the treatments, dip-rinsed two times in sterile saline solution (to remove excess of agents or vehicle-control) and transferred to fresh culture medium. Each biofilm was exposed to the respective treatment a total of four times. Brief exposure (twice-daily) to vehicle solution allowed the continued formation of biofilms, and did not affect the biochemical composition and cell viability compared to biofilms treated with saline solution. The overview of the experimental design is shown in the diagram (Figure 2).

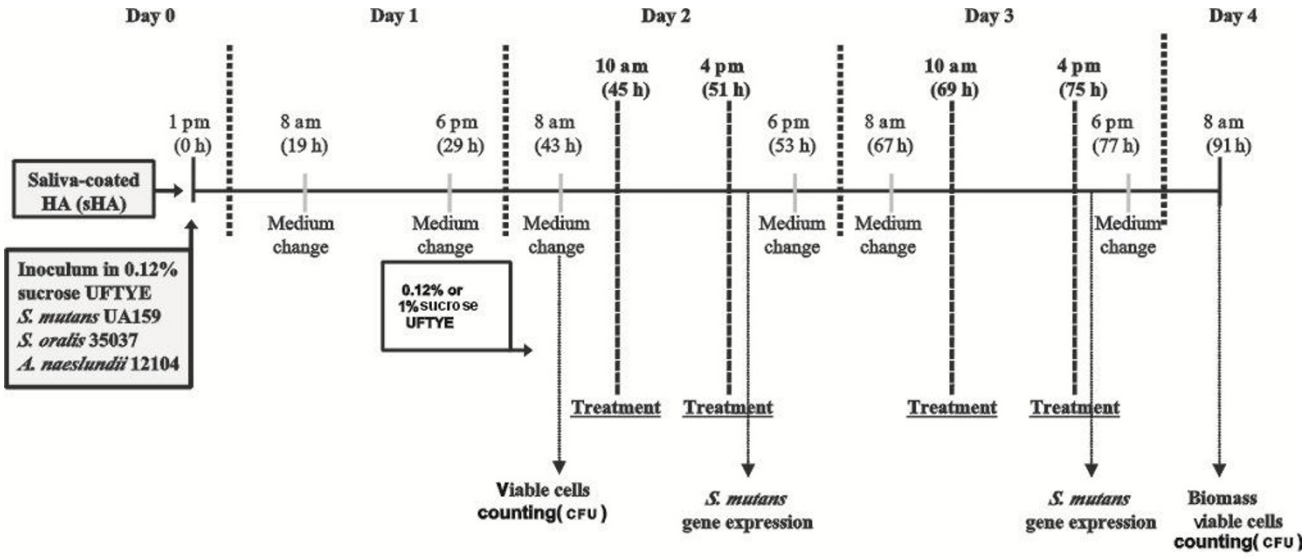

Figure 2 Mixed-species biofilm preparation and the experimental design. This study examined the effects of *tt*-farnesol on accumulation (biomass), *S. mutans* proportion and gene expression in mixed-species biofilms subjected to environmental perturbance (caused by sucrose challenge). The biofilms were twice daily treated with *tt*-farnesol (1 min exposure/treatment) a total of four times during biofilm formation.

Determination of biomass and viable bacterial cells population

Biofilms at 43 h (before treatments) and at 91 h (after four treatments) were removed and subjected to sonication using three 30 s pulses at an output of 7 W (Branson Sonifier 150, Branson Ultrasonics, Danbury, CT, USA) [10]. The homogenized suspension was used for determination of biomass (dry-weight), number of viable cells, and composition of microbial cells population. The biofilm suspension was serially diluted and cultivated on blood agar using an automated spiral plater, and incubated for 72 h at 37 ℃ under 5% CO_2. After incubation, the plates were retrieved and the total colony forming units per biofilm was determined. Differentiation of the three species was achieved by observation of colonial morphology with the aid of a stereomicroscope in conjunction with microscopic examination of cells from selected colonies [19].

Gene expression by S. mutans within mixed-species biofilms

The RNA from mixed-species biofilms (after two or four treatments) was extracted and purified as described elsewhere [20]. All the purified RNA samples showed RIN > 9 as determined by Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). These samples served as templates for cDNA synthesis using the Bio-Rad iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Samples in which no reverse transcriptase was added to the reaction were used as negative controls to check for DNA contamination during qPCR assays. cDNAs were amplified

with specific primers using a MyiQ real-time PCR detection system with iQ SYBR Green supermix (Bio-Rad). The following genes were amplified in qPCR assays with specific primer sets that were used in previous studies: *gtfB, gtfC*, *gtfD* (associated with EPS synthesis), *pdhA* (associated with glycolysis), *atpD* (acid tolerance), 16S rRNA (reference gene) [21-23]. Primers for *fabM* (acid tolerance) (5'-ACTGATTAATGCCAATG GGAAAGTC-3' and 5'-TGCGAACAAGAGATTGTAC ATCATC-3'), and *glgP* (intracellular sugar metabolism) (5'-GACTTTAAAGACACTCTGCATGAAG-3' and 5'-A CGAACAACCTTAGCCAAAGAAG-3') were designed using Beacon Designer 2.0 software (Premier Biosoft International, Palo Alto, CA, USA). Standard curves were used to determine the relative number of cDNA molecules, which were normalized to the relative number of 16S rRNA cDNA molecules in each sample, as previously described [23]. These values were used to determine the fold of change between each treated sample and the vehicle control.

Statistical analyses

All experiments were performed in triplicate and each experiment was repeated at least four times. Data are presented as means ± SD. Intergroup differences were estimated by one-way analysis of variance (ANOVA), followed by a post hoc multiple comparisons, Tukey test, for the comparison of multiple means. Values were considered statistically significant when P value was less than 0.05. Statistical analyses were performed using SPSS 12 software (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Natural products and their derivatives are still major sources of innovative therapeutic agents to treat human diseases [24]. About 70% of all anti-infective agents introduced between 1981 and 2002 were derived from natural products [25]. The structures of naturally occurring compounds have high chemical diversity, biochemical specificity, and other molecular properties that make them favourable as lead structures for drug discovery [24]. Nevertheless, studies using natural products to prevent or treat oral diseases such as dental caries have received, comparatively to other fields in medicine, limited attention.

Previous studies have shown that *tt*-farnesol, a commonly found natural sesquiterpenoid, may change the permeability and fluidity of the cell membrane of other microbial species in a planktonic state (*e.g. Staphylococcus aureus*) because of its lipophilic properties; which favour localization in the membrane, possibly causing structural damage [26-27]. Thus, we first investigated whether *tt*-farnesol disrupts the integrity of the cell-membrane of *S. mutans* in planktonic phase using a well-established proton permeability assay [15]; the results are shown in Figure 3A. *tt*-Farnesol increased the initial rate of proton entry of *S. mutans* planktonic cells, especially at 0.5 and 1 mmol·L^{-1}, compared to the vehicle control (P<0.05). In general, protons from the extracellular environment diffuse inward across the cell membrane after acidification of the suspension but then can be extruded by the membrane associated F-ATPase enzyme [28]. The proton-translocating F-ATPase protects *S. mutans* against environmental acid stress by regulating pH homeostasis, which is critical for the optimum function of glycolysis in *S. mutans* [29]. However, we have shown previously that *tt*-farnesol, at the concentrations tested in this study, is devoid of direct inhibitory effect against enzymatic activity of isolated F-ATPase. Thus, it is apparent that the increase in proton permeability caused by *tt*-farnesol is likely due to direct damage of the membrane barrier function (although disruption of membrane structural integrity could affect the optimal function of membrane-embedded enzymes, such as F-ATPase).

To investigate the influence of *tt*-farnesol on acid production of *S. mutans* planktonic cells, glycolytic pH-drop assays were performed. Acid sensitization can be rapidly seen in glycolytic pH-drop experiment in which cells are given excess glucose. *S. mutans* cells rapidly degrade glucose and lower the pH value of the suspension until they can no longer maintain a cytoplasmatic pH compatible with activity of glycolytic enzymes. Thus, the rate of pH drop reflects acid production of the cells, while final pH values of the suspensions reflect acid tolerance. As shown in Figure 3B, *tt*-farnesol reduced the rate of the pH drop of *S. mutans* planktonic cells at 0.25, 0.5 and 1 mmol·L^{-1} (P <0.05). Furthermore, the test agent dramatically sensitized the cells to acidification to the point that the final pH values were significantly higher (1.5 to 2 units) than those in the presence of vehicle control (P<0.05), without displaying bactericidal activity. These effects could be related, in part, to disruption of proton permeability of *S. mutans* cell membrane caused by *tt*-farnesol (Figure 3A), which would affect the pH gradient (ΔpH) across the membrane, leading to inhibition of the overall intracellular metabolism, including acid production. Whether, *tt*-farnesol can inhibit the glycolytic enzymes directly awaits further evaluation. Despite the lack of killing activity of *tt*-farnesol in our experimental conditions, it is possible that such disruptive effects could also alter the growth rate of *S. mutans*, especially at higher concentrations (*e.g.*

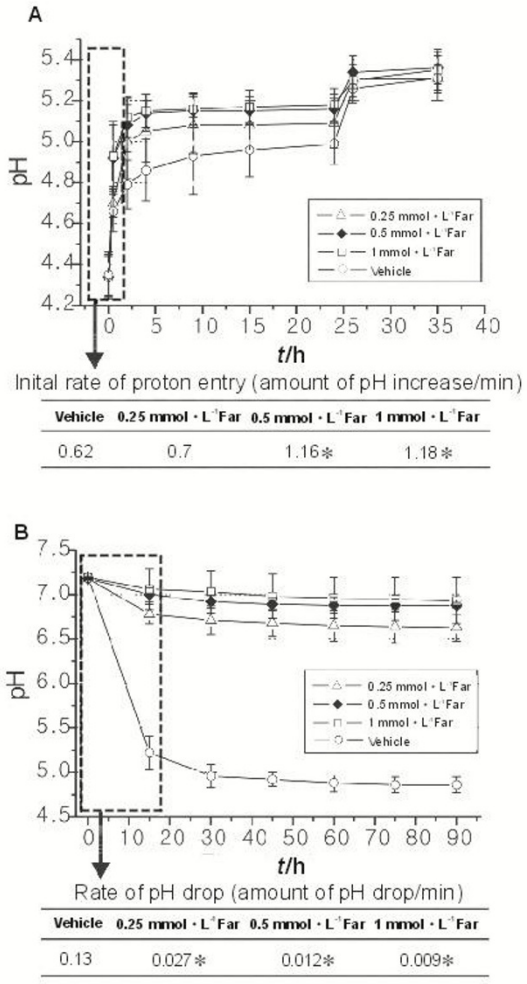

Figure 3 Influence of *tt*-farnesol (Far) on proton permeability (**A**) and glycolytic pH-drop (**B**) by planktonic cells of *S. mutans* UA159. Data represent mean ± standard deviation. **P*<0.05: significantly different from the vehicle control (Vehicle, 15% ethanol + 2.5% DMSO, *V*/*V*). Butanol (10%, *V*/*V*) was added at 25 min. to disrupt cell membranes.

1 mmol·L^{-1}). Next, we examined whether the test agent can affect *S. mutans* physiology within biofilms, which is the mode of growth associated with virulence in the oral cavity.

Biofilms are typically characterized by dense, highly hydrated clusters of bacterial cells enmeshed in an extracellular matrix. Bacterial cells in biofilms are physiologically and functionally distinct from planktonic cells, displaying enhanced resistance to antibiotics [30-31]. Thus, we examined the effects of *tt*-farnesol on the proton permeability and acid production of *S. mutans* in biofilms. The results of the assays are shown in Figure 4 A and B. Overall, the biological activity of the test agent was reduced against biofilm-cells of *S. mutans* (compared to the effects observed against planktonic cells). Nevertheless, *tt*-farnesol was able to significantly increase the rate of proton entry (at 1 mmol·L^{-1}; Figure 4A) and reduce the rate of glycolytic pH drop (at 0.5 and 1 mmol·L^{-1}; Figure 4B) by *S. mutans* biofilms when compared to the vehicle control (P<0.05). The final pH values of biofilms treated with *tt*-farnesol (all concentrations) were significantly higher (0.5 to 1 units) than those in the presence of vehicle control (P<0.05). Overall, our data suggest that *tt*-farnesol can affect the

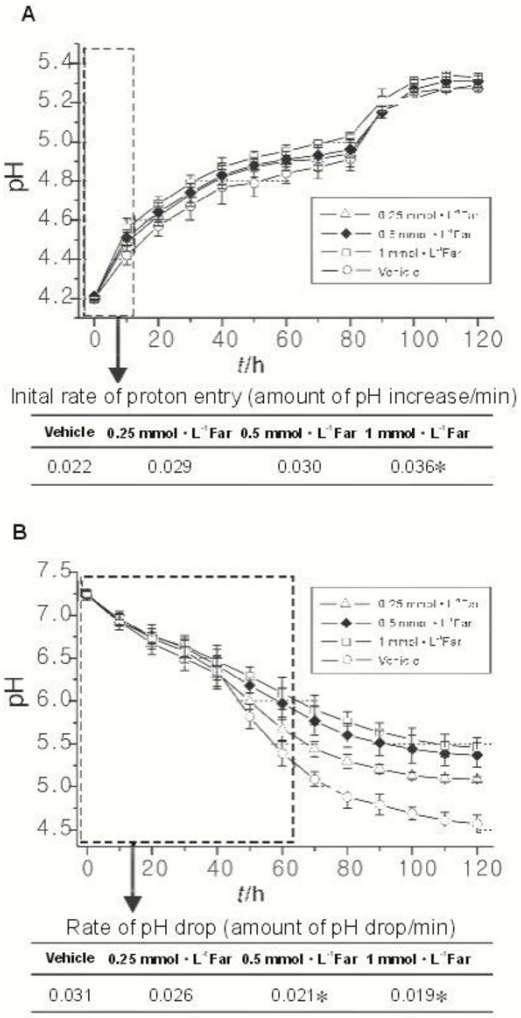

Figure 4 Influence of *tt*-farnesol (Far) on proton permeability (**A**) and glycolytic pH-drop (**B**) by biofilm cells of *S. mutans* UA159. Data represent mean ± standard deviation. **P*<0.05: significantly different from the vehicle control (Vehicle, 15% ethanol + 2.5% DMSO, *V*/*V*). Butanol (10%, *V*/*V*) was added at 80 min. to disrupt cell membranes.

acidogenicity and aciduricity of *S. mutans* both in planktonic phase and within biofilm by increasing the proton permeability of the membrane likely through disruption of the cell-membrane functional integrity.

S. mutans cells in the mouth form biofilms on tooth-enamel surfaces in the presence of other organisms. We designed a mixed-species biofilm model to mimic the formation of cariogenic biofilms according to the ecological plaque-biofilm concept [3]. Analyses of species composition in the healthy dental plaque have shown that the majority (47% to 90%) of cultivable bacteria are non-mutans streptococci, such as *S. oralis*, and that one-third of the remaining bacteria are *Actinomyces* sp., such as *A. naeslundii* [32]. The bacterial composition of the human dental plaque remains relatively stable when exposed to minor environmental changes [3, 33]. However, if environmental perturbations occur, such as increase of sucrose availability, the development of virulent-cariogenic biofilms would be induced by triggering EPS matrix formation, and the growth and dominance of cariogenic aciduric streptococci, such as *S. mutans* [3, 5]. Thus, we assessed whether the effects of *tt*-farnesol on *S. mutans* membrane physiology impacts the establishment of this pathogen in a mixed-species environment and further accumulation of the biofilms on saliva-coated apatitic surface. The regimen of one-minute exposure (and further removal of excess agent) and daily treatments was selected for this experiment to simulate the likely exposure of the test agents in the oral cavity. The test concentration of 1 mmol·L^{-1} was used because of higher biomass densities of biofilms, data showing that biofilms are less sensitive to *tt*-farnesol than cells in suspension, and lack of solubility of higher concentrations in the vehicle solution [10, 12].

Figure 5 shows the composition of the microbial populations in the biofilms before and after treatments. At 43 h (before treatment), the biofilms were mostly comprised of *S. oralis* 35307, followed by *A. naeslundii* 12104. However, *S. mutans* UA159 becomes the dominant species in the biofilms (at 91 h-old) growing in 1% sucrose even after treatments with vehicle-control; a similar bacterial population shift was observed with untreated biofilms (data not shown). In contrast, topical applications of *tt*-farnesol prevented the ecological shift towards *S. mutans* dominance within the mixed-species biofilms grown in 1% sucrose (versus vehicle-control treated biofilms, $P<0.05$). Table 1 shows that the proportion of *S. mutans* in biofilms treated with *tt*-farnesol was 22% of the total population, whereas the vehicle-treated biofilms displayed mostly *S. mutans* (>90% of the total population). *S. oralis* (a non-cariogenic organism) became the major species after treatments with *tt*-farnesol. Concomitantly, treatments with *tt*-farnesol significantly reduced the biomass of the biofilms grown in 1% sucrose (*vs*. vehicle-treated biofilms, $P<0.05$) as shown in Table 2. The bacterial populations and biomass of the biofilms grown in 0.12% sucrose were unaffected by *tt*-farnesol showing similar profile to those found in vehicle-treated biofilms. It is noteworthy that *S. oralis*, rather than *S. mutans*, was the major species in biofilms formed with 0.12% sucrose irrespective of whether the biofilms were treated with the test agent or vehicle-control.

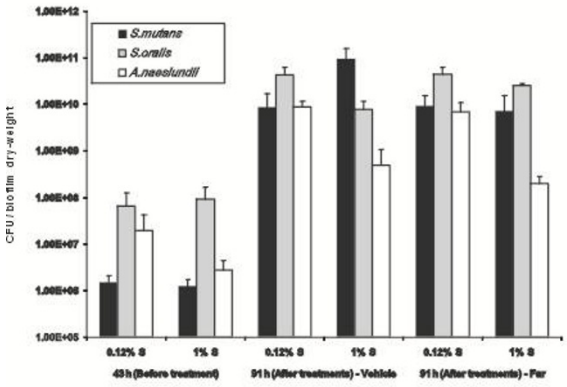

Figure 5 The number of viable cells and bacterial composition of the mixed-species biofilms before (43 h) and after treatments (91 h). Biofilms formed in UFTYE with 0.12% or 1% (*V*/*V*) sucrose (0.12% S and 1% S, respectively) were treated twice daily with 1 min exposure to either vehicle-control (15% ethanol + 2.5% DMSO, *V*/*V*) or test agent (1 mmol·L^{-1} *tt*-farnesol).

Clearly, *tt*-farnesol can reduce the competitiveness of *S. mutans* in mixed-species environment when high sucrose challenge is present, which seems logical considering that excess of sucrose could be rapidly metabolized to EPS and acids affecting the biofilm homeostasis. As shown in Figure 3A and 4A, *tt*-farnesol can enhance the proton permeability of *S. mutans* cell membrane, which would cause cytoplasmic acidification and, consequently acid sensitization. Such effects may also trigger energy starvation of *S. mutans* and inhibit intracellular polysaccharide (IPS) synthesis and accumulation [21]. Furthermore, according to our previous studies, *tt*-farnesol affects the synthesis of EPS by *S. mutans* in biofilms, particularly insoluble glucans, without directly affecting the enzymatic activity of glucosyltransferases-Gtfs [10, 12]. Enzyme secretion by bacterial cells is generally coupled to Δp, the proton-motive force, across the cell membrane. Because *tt*-farnesol acts to diminish Δp by

Table 1 The proportion of different bacterial species in the mixed-species biofilms before (43 h) and after treatments (91 h)

Bacterial species	Before treatment		After treatment			
	43 h		91 h-vehicle		91 h-Far	
	0.12% S	1% S	0.12% S	1% S	0.12% S	1% S
S. mutans	1.7 %[1]	1.3 %[1]	14.3 %[1]	92.0 %[1]	15.2 %[1]	22.0 %[1]
S. oralis	75.9 %[2]	96.9 %[2]	70.9 %[2]	7.5 %[2]	73.3 %[2]	77.4 %[1]
A. naeslundii	22.4 %[3]	1.8 %[1]	14.8 %[1]	0.5 %[3]	11.5 %[1]	0.6 %[2]

Biofilms formed in UFTYE with 0.12% or 1% (*V*/*V*) sucrose (0.12% S and 1% S, respectively) were treated twice daily with 1 min exposure to either vehicle-control (15% ethanol + 2.5% DMSO, *V*/*V*) or test agent-Far (1 mmol·L^{-1} *tt*-farnesol). Values in the same column followed by the same superscript numbers are not significantly different from each other (P> 0.05, ANOVA, comparison for all pairs using Tukey test).

Table 2 Biomass (dry weight) of the mixed-species biofilms after treatments

Treatments	Biofilm formed in 0.12% S (91 h-old)		Biofilm formed in 1% S (91 h-old)	
	Vehicle-control	1 mmol·L^{-1} Farnesol	Vehicle-control	1 mmol·L^{-1} Farnesol
Dry weight /mg	0.46 (0.12) [1]	0.5 (0.15) [1]	1.52 (0.13) [2]	1.09 (0.02) [3]

Biofilms formed in UFTYE with 0.12% or 1% (*V*/*V*) sucrose (0.12% S and 1% S, respectively) were treated twice daily with 1 min exposure to either vehicle-control (15% ethanol + 2.5% DMSO, *V*/*V*) or test agent (1 mmol·L^{-1} *tt*-farnesol). Values followed by the same superscript numbers are not significantly different from each other (P>0.05, ANOVA, comparison for all pairs using Tukey test).

increasing proton permeability and discharge of ΔpH across the cell membrane, it is possible that the agent will affect the production and secretion of Gtfs and thereby diminish the synthesis of extracellular glucans. These observations correlate with earlier findings showing that this agent can reduce the EPS and IPS content of *S. mutans* biofilms [10].

We also examined the potential effects of *tt*-farnesol treatments on expression of specific *S. mutans* genes within mixed-species environment. Interestingly, topical treatments with 1 mmol·L^{-1} *tt*-farnesol did not affect the mRNA levels of genes associated with the EPS (*gtfBCD* and *dexA*), IPS (*glgP*) and acidogenicity-aciduricity (*atpD*, *fabM, pdhA*) when compared to vehicle-treated biofilms (data not shown). These results suggest that *tt*-farnesol does not have a direct effect on the transcription of these genes, likely because it may not reach the cytoplasm, as the lipophilic nature of the compound could allow it to become trapped in the cytosol-membrane barrier.

Collectively, the data indicate that the major mechanisms by which *tt*-farnesol influences the biomass and *S. mutans* survival in the mixed-species biofilms are associated with disturbances in the proton motive force, possibly through the interaction of a lipophilic moiety with bacterial membrane. By damaging the cell-membrane function, the ability of *S. mutans* to (1) produce acids, (2) tolerate acids, and (3) synthesize extra-/intracellular polysaccharides would be compromised [10, 12]. Our data indicate that treatment with *tt*-farnesol may subsequently disrupt the accumulation of mixed-species oral biofilms and also hinder deleterious ecological shifts in these biofilms when grown under environmental perturbations caused by sucrose challenge. *tt*-Farnesol, a natural sesquiterpene alcohol, may represent a potentially useful adjunctive agent to the current chemotherapeutic strategies to prevent formation of virulent biofilms related to dental caries disease. Future studies are warranted to elucidate the exact mechanisms by which *tt*-farnesol disrupts the architecture and functional integrity of the *S. mutans* cell-membrane.

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