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Original Article

Farnesol inhibits development of caries by augmenting oxygen sensitivity and suppressing virulence-associated gene expression in *Streptococcus mutans*

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Abstract

Streptococcus mutans is a primary etiological agent of dental caries. Farnesol, as a potential antimicrobial agent, inhibits the development of *S. mutans* biofilm. In this study, we hypothesized that farnesol inhibits caries development *in vitro* and interferes with biofilm formation by regulating virulence-associated gene expression. The inhibitory effects of farnesol to *S. mutans* biofilms on enamel surfaces were investigated by determining micro-hardness and calcium measurements. Additionally, the morphological changes of *S. mutans* biofilms were compared using field emission scanning electron microscopy and confocal laser scanning microscopy, and the vitality and oxygen sensitivity of *S. mutans* biofilms were compared using MTT assays. To investigate the molecular mechanisms of farnesol's effects, expressions of possible target genes *luxS*, *brpA*, *ffh*, *recA*, *nth*, and *smx* were analyzed using reverse-transcription polymerase chain reaction (PCR) and quantitative PCR. Farnesol-treated groups exhibited significantly higher micro-hardness on the enamel surface and lower calcium concentration of the supernatants as compared to the-untreated control. Microscopy revealed that a thinner film with less extracellular matrix formed in the farnesol-treated groups. As compared to the-untreated control, farnesol inhibited biofilm formation by 26.4% with 500 μmol/L and by 37.1% with 1,000 μmol/L ($P < 0.05$). Last, decreased transcription levels of *luxS*, *brpA*, *ffh*, *recA*, *nth*, and *smx* genes were expressed in farnesol-treated biofilms. *In vitro* farnesol inhibits caries development and *S. mutans* biofilm formation. The regulation of *luxS*, *brpA*, *ffh*, *recA*, *nth*, and *smx* genes may contribute to the inhibitory effects of farnesol.

Keywords: *Streptococcus mutans*, biofilm, farnesol, caries, virulent genes

Introduction

Dental caries, a biofilm-related oral infectious disease, continues to be a significant public health problem in many parts of the world. *Streptococcus mutans* is an important element of the dental plaque biofilm, and plays a crucial role in the development of dental caries[1]. *S. mutans* in biofilms is physically and functionally distinct from planktonic cells and displays an enhanced resistance to antibiotics[2]. Within biofilms,

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S. mutans metabolizes many sugars and produces various organic acids which subsequently lowers the pH of the surrounding environment and eventually leads to the dissolution or de-mineralization of enamel over time[3]. Furthermore, *S. mutans* synthesizes extracellular polysaccharides (EPS) from dietary sucrose. EPS enhances the virulence of dental biofilms by promoting the attachment and accumulation of cariogenic microorganisms or by maintaining the bulk and structural stability of the biofilm. Therefore, developing antibacterial agents with the capacities to weaken acid generation and to decrease the EPS content of *S. mutans* biofilms will help to prevent dental caries.

The investigation of natural products and their derivatives has been one of the most attractive strategies for developing new medicines[4]. Farnesol, a sesquiterpene alcohol (3,7,11-trimethyl-2,6,10-dodecatrien-1-ol) and the first quorum-sensing molecule discovered in eukaryotes, can be found in some essential oils[5], propolis (a resinous beehive product), and citrus fruits[6]. Studies have shown that this molecule has inhibitory effects against such microbes as *Paracoccidioides brasiliensis*[7], *Fusarium graminearum*[8], *Staphylococcus epidermidis*[9], and *Burkholderia pseudomallei*[10]. Topical application of farnesol could interrupt the accumulation of biofilms, reduce the polysaccharide content of biofilms, and weaken the ability of *S. mutans* to generate and tolerate acid. To date, very few studies have evaluated caries development *in vitro*, oxygen sensitivity and morphological changes of *S. mutans* biofilms exposed to farnesol, and little is known about the associated molecular mechanisms[11].

The *luxS*, *brpA*, *ffh*, *recA*, *nth*, and *smx* genes have been found to be related to *S. mutans* biofilm virulence. The *luxS* gene has a primary role in regulating autoinducer-2 production, which is an important quorum-sensing molecule in bacteria, and is able to elicit a specific response across species barriers[12]. The *brpA* gene encodes a predicted surface-associated protein associated with biofilm formation, autolysis, and cell division[13]. The *ffh* gene encodes a homolog of the 54 kDa subunit of a signal recognition particle involved in resistance to acid stress[14]. The *recA* gene influences glucosyltransferase (GTF) expression, and GTFs are essential for cellular adhesion[15]. The *nth* gene encodes a predicted EndoIII-related endonuclease involved in DNA replication, recombination, and repair[16]. The *smx* gene encodes a class II-like AP endonuclease that regulates the exonucleolytic activity and the acid-adaptive response of an organism[17]. In brief, *luxS* and *brpA* are involved in the regulation of *S. mutans* biofilm formation associated with EPS-production and responses to oxidative stress, while *ffh*, *recA*, *nth*, and *smx* play a role in the acid tolerance of *S. mutans*[12–18].

In this study, we hypothesized that farnesol, a chemical compound with antimicrobial properties, may inhibit the development of caries, and interfere with the formation of *S. mutans* biofilms by regulating virulence-associated gene expression. To test this, the inhibitory effects of farnesol to *S. mutans* biofilms on enamel surfaces were investigated. Furthermore, the morphological changes and vitality of the biofilm were compared among farnesol-treated and untreated control groups. Additionally, the virulence-associated genes underlying the inhibitory effects of farnesol were assessed.

Materials and methods

Bacterial strains and growth conditions

S. mutans UA159 was kindly provided by the Institute of Microbiology, Chinese Academy of Sciences. This strain is a proven virulent cariogenic dental pathogen with a known genomic sequence[19].

Freshly grown *S. mutans* cells from Mitis Salivarius–bacitracin agar (Rishui Biotechnology Inc., Qingdao, China) were propagated in fresh brain–heart infusion (BHI) broth (Oxoid Ltd., Cambridge, UK) at 37°C in a 95% air and 5% CO_2 (v/v) environment for 24 hours. The cells were collected by centrifugation (3,100 × g, 10 minutes), washed in sterile phosphate-buffered saline (PBS; Sigma Co., St. Louis, MO), and then suspended in BHI broth containing 1% (w/v) sucrose. The optical density was 0.5 at 600 nm (OD_{600}) on an ultraviolet spectrophotometer (Amersham Bioscience, Buckinghamshire, UK). The cells were then diluted 1:10 in BHI media containing 1% (w/v) sucrose for all experiments.

Test compounds

Stock solutions (100 mmol/L) of farnesol (*E*,*E*-farnesol; Sigma) were dissolved in 100% (vol/vol) methanol and frozen at −70°C until use[20]. Farnesol was diluted to a working concentration in BHI broth for all experiments. Three concentrations, 250, 500, and 1,000 μmol/L, of farnesol were chosen for the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (KeyGen Bio Co., Nanjing, China) assay of biofilm formation, based on a previous study[11]. The same series of concentrations were also used in determining micro-hardness of the enamel blocks and measuring calcium in the supernatants while 1,000 μmol/L farnesol was chosen for morphological obser-

vations, the oxygen sensitivity assay and transcription level study.

The experiments were performed mainly in two groups: farnesol-treated and untreated control. The farnesol-treated group was *S. mutans* biofilm treated with farnesol diluted in BHI broth. The untreated control groups were *S. mutans* biofilm treated with an equal volume of deionized water, instead of farnesol, in BHI solution.

Biofilm preparation and farnesol treatments

Biofilms of *S. mutans* were formed on the surface of enamel blocks or flat-bottomed culture flasks in BHI broth containing 1% (w/v) sucrose[21]. Briefly, the standardized suspension was dispensed into flat-bottom plates, and the organisms were grown undisturbed for 24 hours to allow initial biofilm formation. After a 24-hour-incubation of the suspension, non-adherent cells were removed by thoroughly washing the biofilms three times with PBS (pH 7). At this point, the culture medium was replenished, and equal volumes of farnesol or deionized water were added to the farnesol-treated and untreated control groups, respectively. For this treatment, the biofilms were exposed to farnesol or deionized water for 1 minute, rinsed three times in a sterile saline solution, and transferred to fresh culture medium. The medium was replaced twice daily, which means that biofilms in each group were treated a total of five times, at 24, 36, 48, 60, and 72 hours after the cells were added to the flat-bottom plates. All experiments were performed in triplicate on three separate occasions.

Cells from the same batch were used for the farnesol-treated and untreated control groups for the initial adherence step.

***In vitro* caries development assays**

The study and use of patients' teeth for this study were approved by the Ethics Committee of Dentistry Hospital, Nanjing Medical University (Institutional Review Board) (IRB; approval number PJ2011-019-03). The isolated teeth without caries or dysplasia were collected from the patients whose teeth had to be extracted for orthodontic reasons. Before the surgical extraction of teeth, the participants were informed of the objective of this study and the uses of the teeth *in vitro* experiments. All study participants provided written informed consent. After extraction, the teeth were washed with normal saline and stored at 4°C for 1 week. Stereomicroscopic observation showed that the enamel on the teeth was intact without white spots. Enamel blocks (6 mm × 6 mm × 2 mm in length, width, and thickness) were cut from the surface coronal region of premolars perpendicular to the tubule direction using low-speed diamond slicers. Then, the enamel blocks were prepared and sterilized by gamma radiation as previously described[22]. Subsequently, the blocks were covered with nail varnish, with only a 4 mm × 4 mm window exposed to bacterial culture. The enamel blocks were rehydrated in BHI overnight before each *in vitro* caries assay.

The 40 blocks were randomly and blindly divided equally into five groups: three farnesol-treated *S. mutans* biofilms (250, 500, and 1,000 μmol/L farnesol), one untreated control with *S. mutans* biofilm, and one untreated control without *S. mutans*.

The enamel blocks were placed into 12-well flat-bottom tissue culture plates (Cellstar Greiner Bio-One, Frickenhausen, Germany), and 1,000 μL of standardized *S. mutans* suspension or BHI broth without *S. mutans* was dispensed onto them. Biofilm preparation and farnesol treatments were the same as described above. The biofilms were then treated with deionized water or different concentrations of farnesol twice daily, followed by medium replenishment, until the end of the experiment (at 72 hours). Meanwhile, the supernatants from each well were collected for calcium content measurements.

The enamel blocks were gently removed from the wells, washed twice with PBS, and then put into sterile 2 mL snap-top vials containing 0.5 mL of PBS. The vials were placed in an ultrasonic water bath and sonicated for 15 minutes to remove the biofilm. The blocks were then transferred to fresh 2 mL tubes, dried at 37°C, and stored at 4°C.

The micro-hardness of the enamel blocks was measured by the Micro-Hardness Tester (DHV-100, Shanghai Technology Co., Shanghai, China) before and after the experiments. The blocks were fixed on metal discs using a very thin layer of cyanoacrylate (MDS Adhesive QX-4; MDS Products Inc., Laguna Hills, CA) for micro-hardness analyses. Each enamel block was statically loaded with 100 g for 15 seconds with a Vickers diamond pyramid indenter. Then, the diagonal length of the formed indentation was measured, and the Vickers micro-hardness values (Hv) were converted to micro-hardness values[23]. Six indentations were measured in the window and the average of these measurements was used[24]. Each experiment was repeated three times.

The mineral loss of the enamel blocks was analyzed by comparing the calcium concentrations in the collected supernatants between the farnesol-treated and untreated control groups. The calcium ions atomized by flame were in ground state indicated by

selective absorption on the spectrum. The calcium concentration was determined using an AA6300 atomic absorption spectrophotometer (Shimadzu, Japan) at a wavelength of 422.7 nm[25]. Concentrations were determined from a standard curve after calibrating the instrument with known concentration standards. Under the constant condition, the relationship between absorbance and concentration of calcium ion was applied to the Beer-Lambert law: A = KC (A is the measured absorbance, C is the analytic concentration, and K is a constant). The assays were performed in triplicate.

Comparison of biofilm organization by FE-SEM and CLSM

Biofilms in the farnesol-treated and untreated control groups were formed on the surface of glass-bottomed chamber slides (Nunc, Roskilde, Demark). Biofilm preparation and farnesol treatments were the same as described above. The medium was aspirated after a 72-hour-incubation at 37°C, and biofilms were then washed three times with PBS (pH 7) and placed in 2.5% [vol/vol] glutaraldehyde overnight at 4°C. All experiments were performed in triplicate on three separate occasions.

For field emission scanning electron microscope (FE-SEM) observation, the samples were washed with distilled water twice, dehydrated in gradient ethanol with increasing ethanol concentrations (30%, 50%, 70%, 80%, 90%, and 100%) twice, dried in a desiccator, and sprayed with gold powder. The morphological changes were analyzed using a LEO 1530VP FE-SEM (LEO, Oberkochen, Germany)[26].

For confocal laser scanning microscopy (CLSM) observation, the samples were washed with distilled water twice, and stained with L-13152 LIVE/DEAD BacLight™ bacterial cell stain containing 6 μmol/L SYTO-9 dye and 30 μmol/L propidium iodide (Molecular Probes Inc., Eugene, OR). Bacterial cell stain (100 μL) was added into 1 mL medium for 15 minutes at 37°C in the dark. After staining, biofilms were examined using a Zeiss LSM710 confocal laser scanning microscope (Carl Zeiss, Inc., Oberkochen, Germany) with 495 nm argon ion lasers. Biofilms were observed using a 40 × water-immersion objective lens (40 × /1.2 w). The thickness of the biofilms was measured from the top to the bottom of the biofilm, and the maximum thickness of each region was calculated using the Z-stack of Zeiss ZEN software (Carl Zeiss, Inc., Oberkochen, Germany), which is a scanning image parameter. Each sample was scanned at intervals of 1 μm, and images were acquired randomly from at least five different regions of each surface analyzed. The regions were from the upper left, upper right, lower left, lower right, and middle fields.

Measurement of *S. mutans* biofilm formation

The wells of 96-well, flat-bottom microtiter plates (Cellstar Greiner Bio-One, Frickenhausen, Germany) were filled with 100 μL of standardized *S. mutans* suspension. Biofilm preparation and farnesol treatments were the same as described above. For the biofilm formation assay, the biofilms were treated with different concentrations of farnesol (0, 250, 500, or 1,000 μmol/L) followed by the replenishment of their medium twice daily until the end of the experiment. Farnesol-untreated control biofilms were treated with deionized water instead of farnesol. After 72 hours, the medium was removed and the wells were carefully washed three times with PBS (pH 7).

The biofilm survival rate of the farnesol-treated and untreated control groups were compared using an MTT (KeyGen Bio Co., Nanjing, China) assay[27]. Briefly, 100 μL of MTT solution (2 mg/mL of MTT in sterile PBS) was placed in each well and the plates were incubated at 37°C for 3 hours in a dark place. The MTT solution was then gently removed, and intracellular formazan crystals were dissolved by adding 150 μL of dimethyl sulfoxide (Ameresco, OH, USA). Colorimetric analysis using a microtiter plate reader (Bio Tek Instruments Inc., VT, USA) at wavelength of 490 nm indicated the changes in metabolic activity of the biofilms.

Measurement of *S. mutans* biofilm oxygen sensitivity

The wells of 96-well, flat-bottom microtiter plates were filled with 100 μL of standardized *S. mutans* suspension. Biofilm preparation and farnesol treatments were the same as described above. Before the oxygen sensitivity assay, the biofilms were treated with 1,000 μmol/L farnesol twice daily. After the biofilm had been growing for 72 hours, hydrogen peroxide was added to the biofilms in a final concentration of 58.8 mmol/L (0.2%, v/v) for 30, 60, or 120 minutes. The biofilm survival rate of the farnesol-treated and untreated control groups were compared using an MTT assay, as described above[27].

Measurement of the expression levels of genes potentially targeted by farnesol

Total RNA was isolated from the 72-hour-old biofilms from the farnesol-treated and untreated control groups using a modified one-step method[28]. After centrifugation, the cells were washed three times using PBS (pH 7) and re-suspended in 200 μL of TE buffer (10 mmol/L Tris HCL, 1 mmol/L EDTA; pH 8) with lysozyme (5 g/L) (Sigma, Germany) on ice for 30 minutes. Then, 20 μL 10% SDS was added to the samples and they were boiled for 1 minute followed by

the addition of 100 μL of TRIzol reagent (Invitrogen) for 5 minutes. After the samples were placed on ice for 2 minutes, 100 μL of chloroform was added and the tubes were vortexed for 20 seconds before being centrifuged again. RNA was finally precipitated from the aqueous phase with isopropanol, and the resulting pellets were dried and suspended in 20 μL of diethylpyrocarbonate-treated water.

The RNA samples were treated with DNase (Takara), and reverse transcription was performed on 5 μg of total RNA using avian myeloblastosis virus reverse transcriptase XL (Takara) following the manufacturer's instructions. Primers (***Table 1***) were all designed by Shanghai Generay Bio-Tech Co., Ltd., using 16S RNA as a reference. The synthetic cDNA described above was used for *q*-PCR analysis, which was performed on an ABI 7300 Fast real-time PCR machine (Applied Biosystems, Rotkreuz, Switzerland), using FastStart Universal SYBR green master mix (Roche, Applied Science, Penzberg, Germany). Amplification was achieved using the following cycle settings: 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds, and 95°C for 15 seconds. A melting curve was analyzed *via* amplification to ensure the absence of primer dimers. Expression of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method[29], with 16S rRNA used as a reference.

Statistical analysis

All quantitative experiments were done in triplicate for statistical analyses. The data were analyzed using analyses of variance (ANOVAs), and paired *t*-test was performed for intra-group comparisons. When significant differences were detected, pairwise comparisons among all the groups were calculated. Data were analyzed using the SPSS17.0 software (SPSS Inc., Chicago, IL, USA). Comparisons resulting in *P* values of less than 0.05 were considered to be statistically significant. In the *q*-PCR analysis, genes were considered to be differentially expressed if the values for $2^{-\Delta\Delta Ct}$ were upregulated at least 2.0-fold compared with the control group in three independent experiments.

Results

Farnesol inhibits caries development *in vitro*

The hardness analysis results showed that farnesol inhibited enamel demineralization caused by *S. mutans*. Before the inoculation of *S. mutans*, there were no significant differences in micro-hardness among these enamel blocks in the five study groups ($P > 0.01$). After the inoculation, however, the micro-hardness of the enamel surface was decreased in the blocks covered with farnesol-untreated *S. mutans* biofilms ($P < 0.01$). Additionally, blocks from the three farnesol-treated *S. mutans* biofilm groups had significantly higher enamel surface micro-hardness than those from the-untreated control biofilms, respectively ($P < 0.05$). Furthermore, a significant difference was also observed in the enamel surface micro-hardness between the group treated with 1,000 μmol/L farnesol and the other two groups treated with the lower concentrations of farnesol, respectively ($P < 0.05$) (***Table 2***).

As another measure of the effects of farnesol treatment

Table 1 **Primers for RT-PCR and *q*-PCR analysis**

Primer	Sequence (5′-3′)	Products (bp)
luxS-F	GAGTTTGGACCTAAAGGCGATCTT	313
luxS-R	CTTTGTAATTCCCACAGGACTCAAT	
brpA-F	GAAGGGCTGGTTCAGTTGGT	415
brpA-R	CACCGTCAATAGTCGTTCCTTCT	
ffh-F	TGGGAATGGGAGACTTGCTTA	305
ffh-R	GCTCGGAGTTAGGAGGTCAG	
recA-F	CCAGATTCAGGAGAACAGGGTC	478
recA-R	ATTCACCTGTACGAGAAATGCCT	
nth-F	CGCTTTATCCAGATGCTGTTCC	390
nth-R	AAATACGGCTGACATGGGTGT	
smx-F	TATCTGCCAAAGGTCCCACG	417
smx-R	AACGGCGATTGGAAGAAGG	
16S rRNA-F	GCAGTAGGGAATCTTCGGCA	442
16S rRNA-R	GTATCTAATCCTGTTCGCTACCCAC	

on caries development, the calcium levels in the supernatant from the culture media of the enamel blocks described above were examined. The calcium analysis results showed that the calcium concentrations in all the supernatants were elevated at 24, 48, and 72 hours. After 24 hours of culture in medium, the calcium concentrations of both the farnesol-treated and untreated control groups reached a steady-state (***Table 3***). The calcium concentrations in the supernatants were lower in the group treated with 1,000 μmol/L farnesol than those in the groups treated with 250 or 500 μmol/L farnesol or in the untreated control group, respectively ($P < 0.05$).

Different morphological changes in *S.mutans* biofilm

The morphological changes of the farnesol-treated and-untreated control biofilms were characterized by microscopy (***Fig. 1***). At lower magnification, FE-SEM showed that the biofilms formed in the farnesol-untreated control group were bulky and appeared to be structurally stable (***Fig. 1A***). On the other hand, the biofilms formed in the farnesol-treated group were less voluminous and their cells aggregated into clusters (***Fig. 1B***). At higher magnification, FE-SEM showed that there was less extracellular matrix in the farnesol-treated biofilms than in the-untreated control biofilms. Additionally, although the *S. mutans* still kept their primary shapes in the farnesol-treated biofilms, the proportion of long-chained shaped bacteria was decreased inside those biofilms as compared to that in the-untreated control biofilms (***Fig. 1C-D***).

The morphological changes in *S. mutans* biofilms between the farnesol-treated group and the untreated control groups were also analyzed using CLSM (***Fig. 2***). The biofilms in the farnesol-untreated control group were continuous and uniform. Inside the farnesol-untreated control biofilms, *S. mutans* appeared to be distributed evenly with small aggregates (***Fig. 2A***). After treatment with farnesol, biofilms appeared to be discontinuous and dispersed. The farnesol-treated *S. mutans* aggregated into large clumps (***Fig. 2B***). The thickness of biofilm in the farnesol-treated groups was thinner than that in the untreated control. These results suggest that farnesol contributes to the accumulation of *S. mutans* biofilms, while reduces the thickness of these biofilms.

Farnesol inhibits the formation of *S. mutans* biofilms

The effects of farnesol on biofilm formation were analyzed in our study using three different farnesol concentrations (***Fig. 3***). The *S. mutans* biofilm formation was significantly inhibited in the groups treated with 500 or 1,000 μmol/L farnesol as compared with that in the groups treated with only 250 μmol/L farnesol or in those that were untreated controls ($P < 0.01$). Additionally, compared with the values in the untreated control group, the OD_{490} values of *S. mutans* biofilms were decreased by 26.4% and 37.1% in the groups treated with 500 and 1,000 μmol/L farnesol, respectively. The results suggest that the inhibitory effect of farnesol on *S. mutans* biofilm formation is related to its concentration.

Farnesol treatment increases oxygen sensitivity of *S. mutans* biofilms

After 30, 60, and 120 minutes of incubation in 0.2% hydrogen peroxide, the oxygen sensitivity of farnesol-treated and untreated control *S. mutans* biofilms (***Fig. 4***) was examined to determine whether this quality is affected by farnesol treatment. During 0 to 30 minutes of incubation in 0.2% hydrogen peroxide, the survival rate of *S. mutans* in the farnesol-treated group was lower than that in the-untreated control group (***Fig. 4***). After the biofilms were treated with 0.2% hydrogen peroxide, the survival rate of *S. mutans* in the farnesol-treated group remained stable for 30, 60, and 120 minutes. Additionally, in contrast to the biofilms exposed to farnesol, the survival rate of *S. mutans* in the untreated control group decreased significantly after 30 minutes of incubation in 0.2% hydrogen peroxide, and

Table 2 **Enamel surface microhardness (SMH) before and after farnesol treatment ($x \pm s$, N/mm^2)**

Groups	Before	After	Before- After
SMH1	327.40±7.28	200.60±11.45	126.80±4.63**
SMH2	329.12±9.87	220.21±9.11	108.91±5.42**
SMH3	340.22±11.04	246.12±7.05	94.10±7.31**
SMH4	333.04±9.61	174.80±10.47	158.24±9.66**
SMH5	332.28±13.10	328.58±7.20	3.70±8.15

The micro-hardness of enamel surfaces covered BHI broth alone or with farnesol-treated or vehicle control *S. mutans* biofilms was evaluated. SMH, Enamel surface microhardness; SMH1, *S. mutans* + 250 μmol/L farnesol; SMH2, *S. mutans* + 500 μmol/L farnesol; SMH3, *S. mutans* + 1,000 μmol/L farnesol; SMH4, *S. mutans* + 0 μmol/L farnesol; SMH5, no *S. mutans* + 0 μmol/L farnesol. The variation between the micro-hardness before and after treatments was analyzed using ANOVAs and paired *t*-test was performed for intra-group comparisons: *$P < 0.05$, **$P < 0.01$.

decreased even further after 60 minutes of incubation in 0.2% hydrogen peroxide. The OD_{490} value of the biofilms in the untreated control group was approximately 1/3 lower after 60 min of incubation in 0.2% hydrogen peroxide than that in the initial biofilm (0 minute). In the last, the OD_{490} value in the farnesol-untreated control group was close to that in the farnesol-treated group after 120 minutes of incubation in 0.2% hydrogen peroxide.

Gene expression in *S. mutans* biofilms

To determine if farnesol treatment affected the expression of *S. mutans* virulence-associated genes, the levels of *luxS*, *brpA*, *ffh*, *recA*, *nth*, and *smx* expression in farnesol-treated and -untreated control biofilms were investigated. The *q*-PCR results demonstrated that the transcription levels of *luxS*, *brpA*, *ffh*, *recA*, *nth*, and *smx* were all downregulated in the farnesol-treated biofilms, as compared with that in the untreated control biofilms (***Fig. 5A***). The *q*-PCR assay showed that the transcription levels of *luxS*, *brpA*, *ffh*, *recA*, *nth*, and *smx* were reduced approximately 1-fold in the farnesol-treated biofilms as compared to that in the untreated control biofilms (***Fig. 5B***, $P < 0.05$).

Discussion

Dental caries, which continues to affect the majority of the world's population, is attributed to a disturbance in the equilibrium between tooth substance and microbial deposit, and results in a loss of tooth mineral by the action of bacterial acidic metabolites. Formation of microbial biofilms on the tooth surfaces is the initial dynamic process of dental caries, thus making the control of dental biofilms essential in the prevention of carious lesions[30–31].

Investigating natural products may provide innovative therapeutic agents to treat dental caries. Previous studies have reported that certain concentrations of farnesol affect the growth, metabolism, and polysaccharide contents of *S. mutans* biofilms[32]. However, to our knowledge, no data are available about the exact mechanism by which farnesol affects *S. mutans* biofilms[11], nor have there been any study about the inhibitory effects of farnesol to *S. mutans* biofilms on enamel surface, and the morphological changes or oxygen sensitivity of *S. mutans* biofilms exposed to farnesol.

A few studies have shown that surface micro-hardness is one of the key physical properties of enamel that can access the effect of chemical agents on hard teeth[26,33]. The demineralization process caused by *S. mutans* biofilms decreases enamel surface micro-hardness, and eventually leads to caries lesion. Previous studies have confirmed that micro-hardness evaluation (micro indentation hardness testing) is sensitively for accessing the demineralization levels of an enamel surface[33]. In this study, we took the lower premolars from the similarly aged patients and chose enamel blocks with similar micro-hardness for comparison. Our results showed that the reduction of surface enamel micro-hardness by *S. mutans* was reduced by farnesol treatment, suggesting that farnesol prevent *S. mutans*-related caries development and can be used as a potential agents against *S. mutans*[34].

In this study, we also used FE-SEM and CLSM to investigate whether any morphological differences could be observed between the farnesol-treated and-untreated control biofilms. It is well known that the extracellular matrix, including the polysaccharide matrix, can play a critical role in affecting the virulence of plaque, and influence the physical and biochemical properties of biofilms[35]. We found that the farnesol-treated biofilms appeared to be looser, thinner, and have less extracellular matrix than the-untreated control biofilms. The FE-SEM and CLSM results from our study further confirmed that farnesol reduce the

Table 3 **Calcium concentration in the *S.mutans* biofilm supernatants ($x \pm s$, N /mm^2)**

Groups	24 hours	48 hours	72 hours
C1	80.17±0.92	92.25±1.30*	87.75±1.19*#
C2	72.51±1.40	84.27±1.76*	80.57±1.09*#
C3	56.71±1.37	67.26±0.83*	64.74±1.83*
C4	112.13±2.42	109.25±2.12*	113.03±2.02
C5	6.38±0.73	6.54±0.73	3.10±0.33*#

The supernatant of biofilms from the vehicle control and farnesol-treated groups were collected and their calcium concentrations were tested. The data were analyzed using a two-way ANOVA with interaction. Multiple comparisons within a certain group were performed using S-N-K method. *within a certain group, the 48 hours and 72 hours calcium concentrations were significantly different from the calcium concentration at 24 hours, $P < 0.05$; #within a certain group, the 24 hours and 72 hours calcium concentrations were significantly different from the calcium concentration at 48 hours, $P < 0.05$. The calcium concentrations in the supernatants were lower in the C3 than those in the C1, C2, or C4, respectively ($P < 0.05$). C, concentration of the supernatant; C1, *S. mutans* + 250 μmol/L farnesol; C2, *S. mutans* + 500 μmol/L farnesol; C3, *S. mutans* + 1,000 μmol/L farnesol; C4, *S. mutans* + 0 μmol/L farnesol; C5, no *S. mutans* + 0 μmol/L farnesol.

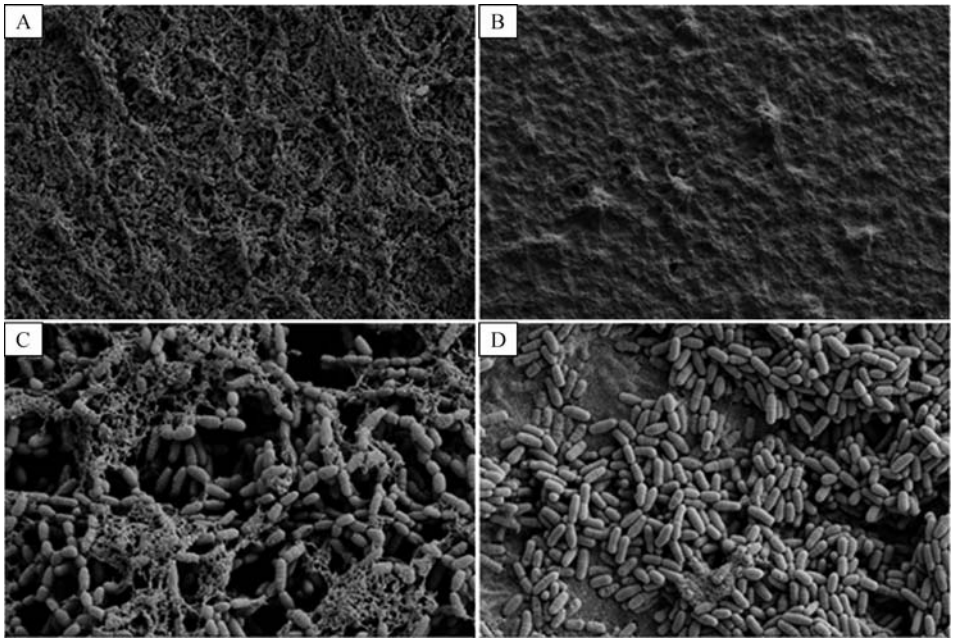

Fig. 1 **Morphology of *S. mutans* biofilms observed using FE-SEM.** Magnifications are 200 × for A and B, and magnifications are 6,000 × for C and D. The untreated control group (A and C): The morphology of untreated control *S. mutans* biofilms is shown. Farnesol-treated groups (B and D): The morphology of farnesol-treated *S. mutans* biofilms is shown. FE-SEM: field emission scanning electron microscopy.

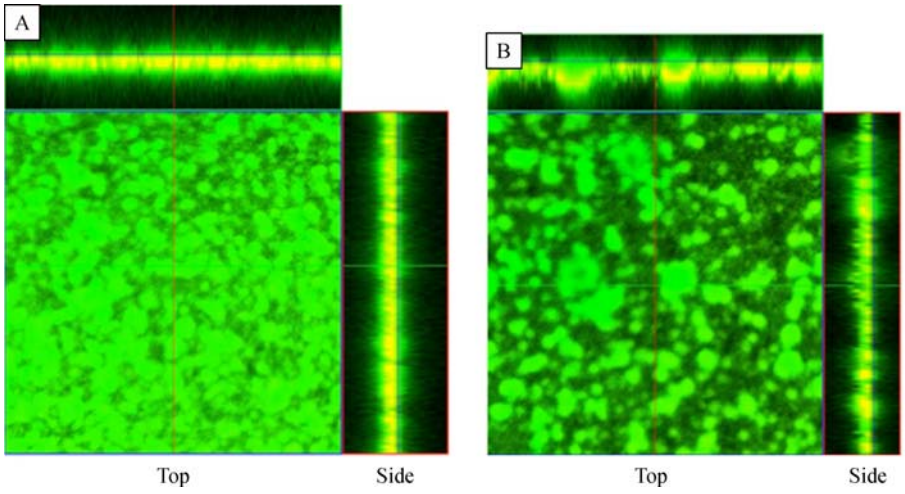

Fig. 2 **Morphology of *S.mutans* biofilms observed using CLSM.** Three-dimensional reconstructions of *S. mutans* biofilms by CLSM and its associated software for the compilation of x-y optical sections taken across the z axis. Magnification, 200 × . Images show views from the top and side. Yellow, viable cells combined with non-viable cells; green, viable cells. A: Images of *S. mutans* biofilms from the control group. B: Images of *S. mutans* biofilms from the farnesol-treated group. CLSM: confocal laser scanning microscope.

pathogenicity of *S. mutans* by inhibiting biofilm formation and the associated extracellular matrix production.

Previous studies have reported that the decreasing of long-chained cells is the initial morphological change in the cell apoptosis process, and this alternation is also one of the acid tolerance mechanisms of *S. mutans*[36]. Our microscopy results showed that there are fewer long-chained cells in the farnesol-treated biofilms than that in the-untreated control biofilms. The results suggest that the reduction of long-chained cells in the farnesol-treated biofilms could be a damage mechanism of farnesol against *S. mutans* biofilm.

In this study, we found that the transcription levels of *luxS* and *brpA* were downregulated in the farnesol-treated biofilms. The *luxS* and *brpA* genes are known for their roles in the regulation of biofilm formation and in the stress tolerance of *S. mutans*. Mutant strains lacking *brpA* have a limited ability to grow and accumulate on surfaces, while displaying increased sensitivity to acid[16,37]. Mutant strains lacking *luxS* are affected in nearly every aspect of their cellular physiology and

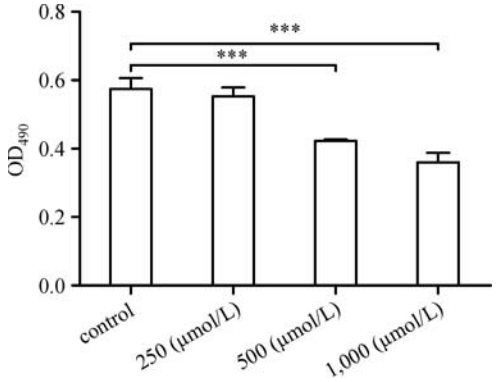

Fig. 3 **Effects of farnesol on *S. mutans* biofilm formation.** Cell densities of farnesol-treated and untreated control *S. mutans* biofilms were assayed by measuring the optical density at a wavelength of 490 nm (OD_{490}). The data were analyzed using a one-way ANOVA. The data represent mean±standard error. Multiple comparisons between experimental groups and the control group were performed using Dunnett method. $^{**}P<0.01$; $^{***}P<0.001$.

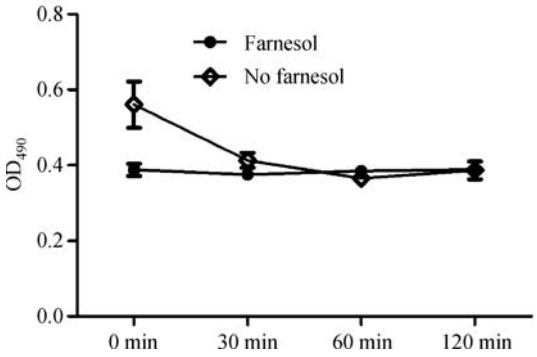

Fig. 4 **Effects of farnesol on oxygen sensitivity of *S. mutans* biofilms.** Mature biofilms from the farnesol-untreated control group and the farnesol-treated group were collected before (0 minute) and after (30, 60, 120 minutes) treatment with 0.2% (v/v) hydrogen peroxide. Viable cells from the farnesol-treated biofilms (●) and untreated control biofilms (l) were assayed using an MTT assay at different phases (0, 30, 60, and 120 minutes). The data represent mean±standard error.

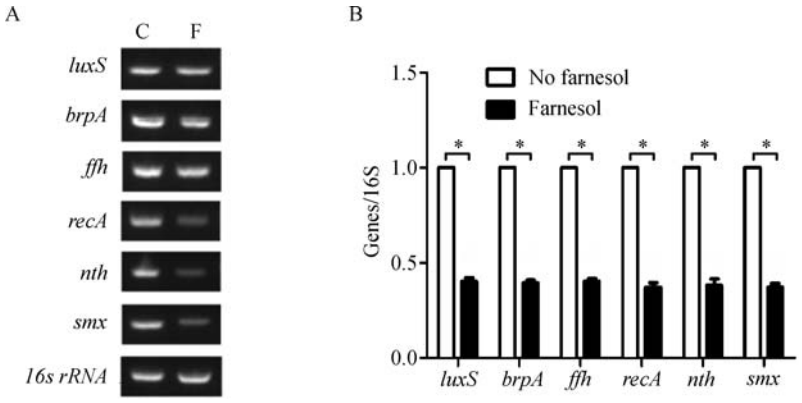

Fig. 5 **Gene expression in farnesol-treated and untreated control *S. mutans* biofilms.** The transcription levels of *luxS*, *brpA*, *ffh*, *recA*, *nth*, and *smx* were measured in the farnesol-treated and-untreated control groups. A: Expressions of *luxS*, *brpA*, *ffh*, *recA*, *nth*, and *smx* as determined by electrophoretic images of PCR products. C, farnesol-untreated control group; F, farnesol-treated group. B: Expressions of *luxS*, *brpA*, *ffh*, *recA*, *nth*, and *smx* as determined by *q*-PCR. *2-DDCt < 0.5 and $P<0.05$.

associated virulence, including carbohydrate and central metabolism, nucleic acid metabolism and DNA repair, amino acid and protein synthesis, stress responses, fatty acid synthesis, and other cellular processes[18]. Our findings suggest that farnesol inhibit the development of caries by downregulating the transcription levels of *luxS* and *brpA* of *S. mutans.*

It was reported that the biofilm formed by a *luxS* mutant strain adopted a notably more granular appearance, in contrast to the smooth and confluent layer formed by wild type *S. mutans*[38]. Accordingly, we found that when compared with the-untreated control biofilms, these farnesol-treated biofilms had down-regulated levels of *luxS* compared to the-untreated control biofilms and appeared to be less voluminous, with their cells aggregated into clusters. The result suggested that farnesol change the smooth layer to granular appearance by regulating *luxS* expression.

The low-pH survival strategy of *S. mutans* is to induce a DNA repair system to protect or repair DNA damage caused by the harmful effects of intracellular acidification[36–37]. Some proteins such as RecA, Nth, Smx, and Ffh are involved in this process. RecA is thought to be a recombinase protein, Nth an endonuclease, and Smx an apurinic-apyrimidinic (AP) endonuclease. The *S. mutans ffh* mutant strains exhibit a defect in the H^+/ATPase activity at acidic pH values, while H^+/ATPase protects wild-type *S. mutans* against environmental acid stress by regulating pH homeostasis[14]. In our study, we found that the transcription

levels of *ffh*, *recA*, *nth*, and *smx* were all downregulated in the farnesol-treated biofilms compared with the levels in control biofilms, contributing to the inhibitory effect of farnesol against *S. mutans* biofilms.

Our results also found that farnesol inhibits biofilm formation by decreasing the synthesis of extracellular matrix and by decreasing the proportion of long chain cells, which result in a lower tolerance to acidic stress. These consequences may stem from the farnesol-induced downregulation of *luxS*, *ffh*, *recA*, *nth*, and *smx*, which are involved in the regulation of acid tolerance in *S. mutans*[16,18,36–37].

In conclusion, this study shows that farnesol inhibits caries development and *S. mutans* biofilm formation by regulating the expression of virulence-related genes. The results suggest that using farnesol as a rinsing agent may prevent the biofilm infection known to cause dental caries. However, further studies will be needed to test its clinical effectiveness.

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