Phuong T.M. Nguyen*, Bac H. Vo, Nhung T. Tran and Quyen D. Van

Anti-biofilm activity of α-mangostin isolated from *Garcinia mangostana* L.

DOI 10.1515/znc-2015-0187
Received August 4, 2015; revised September 23, 2015; accepted November 12, 2015

Abstract: This study was carried out to further examine the anti-biofilm activity of α-mangostin (αMG) isolated from *Garcinia mangostana* L. grown in Vietnam, against a strongly biofilm producing *Streptococcus mutans*, a major causative agent of dental caries. The obtained data indicated that topical applications (twice-daily, 60 s exposure each) of 150 μM αMG during biofilm formation on the surfaces of hydroxyapatite disks (sHA) by *S. mutans* UA159 resulted in 30.7% reduction in biofilm accumulation after 68 h of growth. The treatment did not affect the viability of *S. mutans* cells in the biofilms. The surface activities of two key enzymes responsible for biofilm formation, i.e. the glycosyltransferases GtfB and GtfC, were reduced by 20 and 35%, respectively (*vs*. vehicle control, $P < 0.05$). Interestingly, αMG specifically targeted *S. mutans* in mixed biofilms, resulting in the decrease of the *S. mutans* population and total biofilm biomass. αMG was also found to accumulate within the biofilm of *S. mutans* up to 4.5 μg/biofilm, equal to a concentration of >10 μM/biofilm. In conclusion, this study confirmed anti-biofilm activity of αMG against *S. mutans*. A brief exposure to αMG may suppress biofilm formation by targeting key enzymes imvolved in biofilm formation.

Keywords: α-mangostin; biofilm; *Streptococcus mutans*.

1 Introduction

Biofilm-forming bacteria are responsible for about two-thirds of human bacterial infections [1]. It has been estimated that bacteria embedded in biofilms are 100–1000-fold more resistant to the effects of commonly used antimicrobials than planktonic bacteria [2–5]. Thus, effective anti-biofilm agents are urgently needed, especially such from natural sources (e.g. derived from plants) that may pose less risk of toxicity and resistance development, as many of these are naturally applied or consumed by humans. α-Mangostin (αMG), a xanthone purified from *Garcinia mangostana* L. (mangosteen tree, Guttiferae family) has been reported to be an effective antimicrobial agent against planktonic cells of *Streptococcus mutans*, a strongly biofilm-forming and acid-producing cariogenic organism [6]. Recently we found that the compound was effective in reducing the formation of *S. mutans* biofilms and facilitates its mechanical removal [7]. In this study, we continue the work on the anti-biofilm activity of αMG against *S. mutans* using *in vitro* models of both single and mixed biofilms.

***Corresponding author: Phuong T.M. Nguyen,** Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Caugiay, Hanoi, Vietnam, Fax: +84-4-38363144, E-mail:phuong_nguyen_99@yahoo.com
Bac H. Vo, Nhung T. Tran and Quyen D. Van: Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Caugiay, Hanoi, Vietnam

2 Experimental

2.1 Extraction and isolation of αMG

αMG (Figure 1) was isolated from the peels of *G. mangostana* L. grown in Vietnam as described previously [6]. In brief, a 12 g residue of the *n*-hexane fraction was separated by silica gel column chromatography (Merck Kieselgel 60, 70–230 mesh) using using elution by *n*-hexane – ethyl acetate – methanol (6:3:0.1, v/v) followed by *n*-hexane – chloroform – ethyl acetate – methanol (4:1:0.5:0.3) to yield a single compound, αMG, as yellow crystals (105 mg). The purified αMG was identified by ^{13}C and ^{1}H NMR and by HPLC. The purity of the sample exceeded 98%.

The compound was dissolved in 25% aqueous ethanol, which was also used as a vehicle control. Treatments with 25% ethanol did not affect the viability of *S. mutans* in a biofilm as compared to untreated controls.

2.2 Preparation and treatment of single biofilms

Streptococcus mutans UA159 (ATCC 700610), a proven virulent cariogenic strain selected for genomic sequencing, was used in this study. Biofilms of *S. mutans* were formed on hydroxyapatite disk (sHA) surfaces (12.7 mm in diameter, 1 mm in thickness, Clarkson Chromatography Products, South Williamsport, PA, USA), as previously described [8]. The biofilms were grown in ultra-filtered (10 kDa cut-off membrane; Prep/Scale, Millipore, Billerica, MA, USA) buffered tryptone-yeast extract broth (UFTYE; 2.5% tryptone and 1.5%

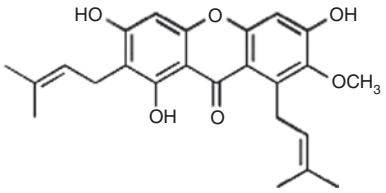

Figure 1: Chemical structure for αMG. Molecular formula: $C_{24}H_{26}O_6$. Molecular mass: 410.466.

yeast extract with the addition of 4.35 g/L of potassium phosphate and 1 g/L of $MgSO_4 \cdot 7H_2O$, pH 7.0) with 1% sucrose at 37°C and 5% CO_2. *Streptococcus mutans* cells in exponential growth phase were inoculated into UFTYE and applied to wells containing sHA placed vertically in a custom-made holder. Biofilms were allowed to form on sHA and were treated for the first time with the test agent or vehicle control after 6 h of incubation. Subsequently, the biofilms were exposed at 8 a.m. (20 h-incubation) and 6 p.m. (30 h-incubation), to two additional treatments in the following morning (8 a.m.; 44 h and 6 p.m.; 54 h incubation, respectively). The biofilms were exposed to the treatments for 60 s, then dip-washed in 0.89% (w/v) NaCl solution to remove excess agent, and thereafter transferred to fresh culture medium [8, 9]. For biomass analysis, an aliquot of the homogenized suspension was centrifuged at 9500 × *g* for 10 min at 4°C, and the cell pellet was washed twice with water, then dried in an oven at 105°C for 24 h and weighed.

2.3 Multispecies biofilm preparation

The mixed biofilm model was prepared as described by Xiao et al. [10]. *Streptococcus mutans* UA159 (ATCC 700610), *Actinomyces naeslundii* ATCC 12104, and *Streptococcus oralis* ATCC 35037 were used for mixed biofilm formation. These representative organisms found in the oral cavity have been previously used to mimic ecological biofilms [10–12]. *Streptococcus oralis* and *A. naeslundii* are well established early colonizers in the mouth, while *S. mutans* is a proven virulent (cariogenic) oral pathogen [13]. Furthermore, *S. oralis* ATCC 35037 produces soluble glucans and can be highly acid tolerant [14]. *Actinomyces naeslundii* may be associated with development of root caries; the strain 12104 is acidogenic and produces exopolysaccharide (EPS) [15, 16]. The mixed-species biofilm model is based on a batch culture approach using sHA. At 29 h of biofilm growth, mixed-species biofilms were transferred to UFTYE containing 1% sucrose to induce environmental changes to simulate a cariogenic challenge. Treatments were started at 8 a.m. (43 h, 67 h) and 6 p.m. (53 h). The culture medium was then changed twice daily (at 10 a.m. and 8 p.m.) until the end of the experimental period. At 29 h, *S. oralis* (a non-cariogenic species) was the predominant species, but an ecological shift occurs after the introduction of 1% sucrose and *S. mutans* becomes the dominant species in this mixed-species biofilm [12]. Mixed biofilms at 76 h were then harvested for biochemical analyses. Biofilms were removed and homogenized by sonication in a sterile 0.89% (w/v) NaCl solution (30-s pulse at an output of 7 W; Branson Sonifier 150; Branson Ultrasonics, Danbury, CT, USA). A homogenized suspension was used to determine the dry weight and the number of viable cells (by plating on blood agar using serial dilution and determining the total number of CFU per biofilm). The three species were distinguished from each other by observation of colonial morphology in conjunction with microscopic examination of cells from selected colonies [10]. Figure 2 depicts the treatment scheme for the mixed biofilm model.

2.4 Determination of surface GtfC activity

Activities of glucosyltransferase (Gtfs) can be determined using either a solution or surface adsorbed enzyme assay. An assay using adsorbed Gtfs provides an estimate of the real activity of these enzymes because the secreted Gtfs will bind avidly to the pellicle formed on the tooth and bacterial surfaces. Therefore, assays of surface Gtf activities were carried out in this study. GtfC was obtained from a recombinant strain carrying the appropriate gene [7], i.e. strain *S. mutans* WHB 410 harboring the g*tf*C gene. The GtfC enzymes (E.C. 2.4.1.5) were obtained and purified to near homogeneity by hydroxyapatite column chromatography. The GtfC was adsorbed onto hydroxyapatite beads, and following adsorption, the beads were washed three times with buffer to remove loosely bound material and then exposed to the respective compounds for 30 min. The beads were then exposed to 300 µl of [U-^{14}C-glucose]-sucrose at 0.03 mCi/ml (final concentration, 100 µM sucrose), and a specific activity of 400–700 mCi (14.8–25.9 GBq)/mmol (New England Nuclear, Boston, MA, USA) for 4 h up to which time the incorporation of ^{14}C-label into EPS was linear. Radiolabeled EPS formed was precipitated by 95% ethanol for 18 h at –20°C and quantified by scintillation counting.

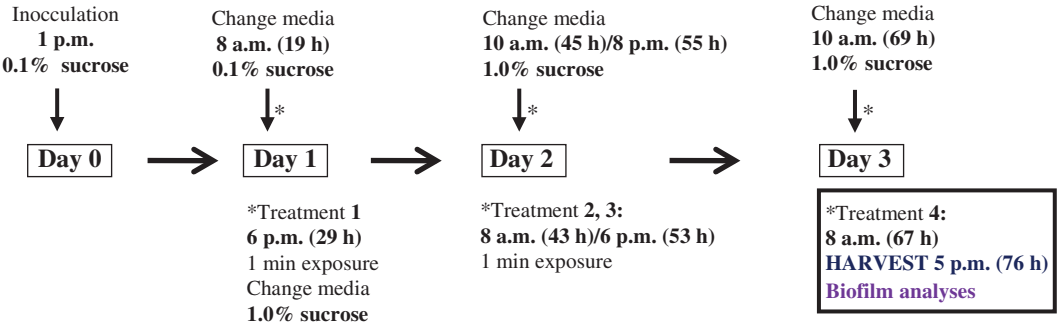

Figure 2: Experimental design of the treatment and analysis of mixed biofilms of *S. mutans*, *A. naeslundii* and *S. oralis*. The mixed population of the three bacterial species was cultured in UFTYE with 0.1% sucrose. At 29 h of culture, the formed biofilms were transferred to UFTYE containing 1% sucrose to induce an environmental change resulting in a cariogenic challenge. Four αMG treatments at 29 h, 43 h, 53 h, 67 h were applied. Mixed biofilms were harvested at 76 h for biochemical analyses.

2.5 Determination of GtfB surface activity absorbed on *S. mutans* cells

GtfB was purified to near homogeneity by hydroxyapatite column chromatography from the recombinant *S. milleri* strain KSB8 harboring the *gtf*B gene from *S. mutans* UA159. The assay of GtfB activity on the bacterial surface was modified from methods previously described [17]. *Streptococcus mutans* cells were grown in UFTYE medium containing 1% glucose to OD_{600} = 0.5 – 0.6. The cells were then harvested by centrifugation at 9500 × *g* for 10 min at 4°C and washed twice with absorption buffer containing 50 mM KCl, 1 mM K_3PO_4, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$ and 0.1 mM PMSF. The cell suspension in this buffer was then probe-sonicated in 2 cycles of 15 s and stopped on ice for 30 s. Cells at a density of OD_{540} = 1.3, equal to 11.14 × 10^9 cells/ml, were used for binding GtfB (15 units GtfB /1 ml). After 40 min of incubation with the enzyme at 37°C on a shaker, cells were washed with the absorption buffer described above, containing additionally 0.02% NaN_3, and subsequently treated with the test compound for another 40 min at 37°C with gentle shaking. The activity of GtfB bound to the surface of the cells was determined by incubation with [U-^{14}C-glucose]-sucrose substrate and further processing as described above.

2.6 Accumulation of αMG in biofilms

Biofilms were harvested after 68 h of growth. The biofilm biomass was detached by sonication and collected by centrifugation at 9,500 × g for 10 min at 4°C. The cell pellet was washed twice with deionized water, and αMG was desorbed from the biofilm pellets with absolute ethanol (Sigma-Aldrich, St. Louis, MO, USA). αMG was determined by HPLC (Alliance series 2695, detector PDA 2996;Waters, Milford, MA, USA). Commercially available αMG (Sigma-Aldrich) was used as a standard. The parameters used in the HPLC were: column: Sunfire-C18 RP (4.6 × 150 mm), 5 µm, mobile phase: H_2O plus 0,1% formic acid (channel A), and acetonitrile (channel B), flow rate: gradient 1 ml/min, detector PDA: 315 nm.

2.7 Statistical analyses

Data are presented as the mean ± standard deviation (SD). Student's t-test was used to calculate the significance of the difference between the mean expression of experimental and control samples. The level of significance was set at 5%.

3 Results and discussion

3.1 αMG inhibits biofilm formation by *S. mutans* in a single bioflim model

In the single bioflim model, *S. mutans* was treated with αMG at a concentrationof 150 µM, which had been selected based on the previously reported data [7]. The aim was to determine whether there is a difference in the inhibition level between single and mixed biofilms upon treatment with αMG. A reduction in single biofilm biomass, up to 30.7% (P < 0.05), was observed (Table 1), while cell viability was not significantly changed. The obtained data are in agreement with our previous finding of the reduction of EPS in αMG treated *S. mutans* biofilms [7].

3.2 αMG inhibits Gtf B and surface GtfC activity of *S. mutans*

Streptococcus mutans produces at least three Gtfs, including *GtfB, GtfC*, and *GtfD*. GtfB synthesizes mostly insoluble glucans containing elevated amounts of α-1,3-linked glucose, while GtfC synthesizes a mixture of insoluble and soluble glucans (rich in α-1,6-linked glucose), and GtfD synthesizes predominantly soluble glucans [9]. EPS formed on microbial surfaces enhances adhesive interactions between *S. mutans* and other organisms, while increasing overall cell cohesion [18, 19]. In presence of sucrose, the enzymes catalyze the attachment of glucosyl residues to a growing glucan chain. Insoluble glucans create the biofilm "skeleton" and provide binding sites for bacteria. Therefore, GtfB and GtfC are especially crucial in biofilm formation and maintenance [12, 20, 21]. Thus, the inhibition of these enzymes has many implications for the interference with biofilm development. Gtfs (particularly GtfB) also bind many oral microbes (e.g. *Actinomyces viscosus, Lactobacillus casei*, and *Candida albicans*), even those that do not synthesize Gtfs, thereby converting these organisms into glucan producers [17, 19]. In this experiment, the adsorbed GtfB and GtfC activities were determined to observe a realistic effect of αMG on these enzymes. As seen in Figure 3, αMG inhibited the activities of both adsorbed Gtfs (about 22% for GtfC adsorbed to sHA, and 35% for GtfB adsorbed on bacterial cells). Although the inhibition level of αMG on adsorbed Gtfs found was less than that reported for soluble Gtfs [7], the these data again confirm that Gtfs are targets of αMG.

Table 1: Biomass and cell viability of αMG-treated single biofilms.

Biofilm samples	Biomass (mg)	Total CFU × 10^8/ml (mean)
Vehicle	5.31 ± 0.44	2.623 ± 0.61
150 µM αMG	3.68 ± 0.30	2.515 ± 0.69

Values (SD, *n* = 9; P < 0.05), for each parameter. Single biofilms at early initial formation (6 h) were treated with αMG at concentrations of 150 µM twice daily for brief exposures (60 s). Sixty-eight-hour-old single biofilms were harvested for analysis. Data are derived from triplicate samples in three separate experiments.

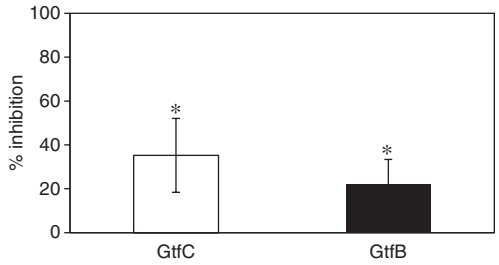

Figure 3: Effect of 150 μM αMG on activities of GtfB (attached to cells) and GtfC (attached to sHA) of *S. mutans* UA159. The percentage of inhibition was calculated setting the vehicle control to 100% Gtf activity. Data are expressed as mean ± standard deviation for experiments run in triplicate in three separate experiments.

Phenolic compounds are known to have anti-Gtf activity [22]. This likely accounts for the inhibitory activity of αMG, a polyphenolic xanthone.

3.3 αMG affects *S. mutans* growth and its dominance in mixed biofilms

Dental plaque is a typical example of a biofilm. The ecological dental plaque concept has been discussed at length in dental research [11, 12, 23–26]. The oral cavity is a diverse environment, being home to roughly 700 different bacterial species [27]. The complexity of the oral microbiome fosters the establishment of mixed oral biofilms that developdynamically under the influence of different conditions that can be present transiently or persistently in the oral cavity (e.g. consumption of dietary sugars). By including multiple bacterial species in a single community, biofilms obtain numerous advantages, such as passive resistance, metabolic cooperation, byproduct influence, quorum sensing, an enlarged gene pool with more efficient DNA sharing, and many other synergies, which give them a competitive advantage [26, 28]. Although no biofilm model can adequately mimic all the conditions present in a real biofilm, an ecological approach with consortia of mixed bacteria is another way to understand how the bacteria relate to biofilm development and react to a treatment. In an attempt to understand the effect of αMG on *S. mutans* in the oral biofilm community, a multispecies biofilm model was established. Data in Table 2 reveal the shifts in the microbial population after 76 h of growth with or without twice-daily αMG treatments for one minute. *S. mutans* was the dominant organism (>70% population) as expected, and *S. oralis* comprised 27% of the population, while almost no *A. neaslundii* cells were detected in the vehicle control samples. In the αMG-treated biofilms,

Table 2: Oral bacteria population in mixed biofilms after treatments with 150 μM αMG.

Organisms	Bacteria population (CFU/biofilm)			
	Vehicle	% population	Treatment	% population
S. mutans	2.36E + 0.7	72.17	0.69E + 0.7	57.50
SD	0.53E + 0.7		0.10E + 0.7	
S. oralis	0.91E + 0.7	27.82	0.51E + 0.7	42.50
SD	0.28E + 0.7		0.04E + 0.7	
A. neaslundii	0.25E + 0.1	0.01	0.00	0.00
SD	0.38E + 0.1		0.00	

At 29 h of growth, mixed-species biofilms were transferred to UFTYE medium containing 1% sucrose and treatments started with 1-min exposure per treatment. Further treatments followed at 43 h, 53 h, and 67 h. Mixed biofilms at 76 h were then harvested for biochemical analyses. Data are expressed as mean ± standard deviation from experiments run in triplicate in at least three separate experiments.

S. mutans was still dominant, but its abundance dropped significantly to 57% of the total population, while that of *S. oralis* was increased. This finding indicated that α-mangostin not only disrupts single-species biofilm formation, but also targets *S. mutans* specifically in a mixed species population. Here, Gtfs, especially GtfB on the surface of *S. mutans* cells, could be a specific target of αMG. As a result, binding sites to which bacteria may adhere and cohere among each other were likely also reduced.

The dry weight of mixed biofilms formed in the presence of αMG was determined as well. Data in Figure 4 indicate that treatment of αMG reduced biofilm formation up to almost 30% compared to the control (1.108 *vs*. 0.78 mg dry weight/biofilm). The reduction in the EPS biomass is

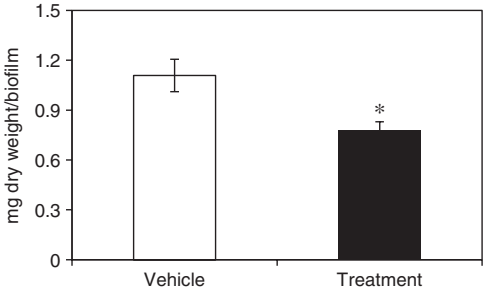

Figure 4: Mixed biofilm dry weight after treatment with 150 μM αMG. After 29 h of culture, mixed-species biofilms were transferred to UFTYE medium containing 1% sucrose and the first 60 s exposure to αMG was initiated. The next treatments were done at 43 h, 53 h, and 67 h. Mixed biofilms were harvested at 76 h for measurement of biofilm dry weight. Data are expressed as mean ± standard deviation for experiments run in triplicate in three separate experiments.

Table 3: αMG accumulation in treated single biofilms.

Biofilm samples	Peak area	αMG (μg/biofilm)
Vehicle	2909 ± 1238.654	0
150 μM αMG	29206 ± 3612.628	4.536 ± 0.01

Values (SD, $n = 9$), for each parameter, $P < 0.05$ (pair-wise comparison using Student's t-test). Twice daily with 60 s exposure for each treatment. Sixty-eight-hour-old single biofilms were harvested for analysis. αMG (μg) in each biofilm was determined using HPLC. Data are derived from triplicate samples in three separate experiments.

nearly the same as in the single biofilm model, suggesting that αMG targets *S. mutans* equally effectively in the mixed model.

3.4 αMG accumulation in biofilms of *S. mutans*

The data in Table 3 indicate that αMG is present to an amount of 4.5 μg/biofilm in 68 h old biofilms after 5 brief treatments with 150 μM αMG. This corresponds to a 10 μM concentration after only 5 brief treatments Long term use in oral health care products could possibly result in its accumulation to a concentration high enough to have significant inhibitory effects on biofilms. This bioaccumulation has also been demonstrated for fluoride in dental plaque (0.1 to 0.5 mM) [29], which helps to sustain its activity after the agent has been removed. Therefore, the retention of αMG in the plaque may help to perpetuate its effects after brief topical exposures, as would occur clinically during oral hygiene routine.

In conclusion, this study shows that αMG inhibits biofilm formation by *S. mutans* at concentrations not impairing bacterial growth or survival. The anti-biofilm activity of αMG appears to be related to the inhibition of glycosyltransferases associated with the formation of the polysaccharide matrix (EPS). Thus, αMG appears a promising anti-caries agent because it disrupts the matrix producing machinery, one of two major virulence factors of *S. mutans*, without changing the population of other species that may be beneficial for health. Further investigation of the anti-biofilm activity of αMG against other biofilm bacteria (e.g. *S. aureus*) are thus encouraged, the full picture of its mechanism of action should be established, and tests with *in vivo* model need to be carried out.

Acknowledgments: This work was supported by a Nafosted grant 106.05-2011.44, and IFS grant F4087/2. The authors are thankful to Prof. Hyun Koo, University of Pennsylvania, for his help in this research.

Conflict of interest statement: We declare that we have no conflict of interest.

References

1. Koo H, Falsetta ML, Klein MI. The exopolysaccharide matrix: a virulence determinant of cariogenic biofilm. J Dent Res 2013;92:1065–73.
2. Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol 2010;8:623–33.
3. Koo H, Jeon JG. Naturally occurring molecules as alternative therapeutic agents against cariogenic biofilms. Adv Dent Res 2009;21:63–8.
4. Marquis RE, Burne RA. Biofilm acid/base physiology and gene expression in oral bacteria. Methods Enzymol 2001;337:403–15.
5. Nguyen PT, Abranches J, Phan TN, Marquis RE. Repressed respiration of oral Streptoccoci grown in biofilms. Curr Microbiol 2002;44:262–6.
6. Nguyen PT, Marquis RE. Antimicrobial actions of alpha-mangostin against oral Streptococci. Can J Microbiol 2011;57:217–25.
7. Nguyen PT, Falsetta ML, Hwang G, Gonzalez M, Koo H. α-Mangostin disrupts the development of *Streptococcus mutans* biofilms and facilitates its mechanical removal. PLoS One 2014;9:e111312.
8. Koo H, Schobel B, Scott-Anne K, Watson G, Bowen WH, Cury JA, et al. Apigenin and tt-farnesol with fluoride on *S. mutans* biofilms and dental caries. J Dent Res 2005;84:1016–20.
9. Koo H, de Guzman PN, Schobel BD, Smith AV, Bowen WH. Influence of cranberry juice on glucan-mediated processes involved in *Streptococcus mutans* biofilm development. Caries Res 2006;40:20–7.
10. Xiao J, Klein MI, Falsetta ML, Lu B, Delahunty CM, Yates JR 3rd, et al. The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. PloS Pathog 2012;8:e1002623.
11. Kleinberg I. A mixed-bacteria ecological approach to understanding the role of the oral bacteria in dental caries causation: an alternative to *Streptococcus mutans* and the specific-plaque hypothesis. Crit Rev Oral Biol Med 2002;13:108–25.
12. Koo H, Xiao J, Klein MI, Jeon JG. Exopolysaccharides produced by *Streptococcus mutans* glucosyltransferases modulate the establishment of microcolonies within multispecies biofilms. J Bacteriol 2010;192:3024–32.
13. Cabal B, Cafini F, Esteban-Tejeda L, Alou Bartolomé JF, Sevillano D, López-Piriz1 R, et al. Inhibitory effect on *in vitro Streptococcus oralis* biofilm of a soda-lime glass containing silver nanoparticles coating on titanium alloy. PLoS One 2012;7:e42393.
14. Klein MI, Xiao J, Lu B, Delahunty CM, Yates JR 3rd, Koo H. *Streptococcus mutans* protein synthesis during mixed-species biofilm development by high-throughput quantitative proteomics. PLoS One 2012;7:e45795.
15. Bergeron LJ, Morou-Bermudez E, Burne RA. Characterization of the fructosyltransferase gene of *Actinomyces naeslundii* WVU45. J Bacteriol 2002;182:3649–54.
16. Allen PZ, Bowen WH. Immunochemical studies on levans from several strains of *Actinomyces viscosus*. Arch Oral Biol 1990;35:55–62.

17. Vacca-Smith AM, Bowen WH. Binding properties of streptococcal glucosyltransferases for hydroxyapatite, saliva-coated hydroxyapatite, and bacterial surfaces. Arch Oral Biol 1998;43:103–10.
18. Cross SE, Kreth J, Zhu L, Sullivan R, Shi W, Qi F, et al. Nanomechanical properties of glucans and associated cell-surface adhesion of *Streptococcus mutans* probed by atomic force microscopy under in situ conditions, Microbiology 2007;153:3124–32.
19. Gregoire S, Xiao J, Silva BB, Gonzalez I, Agidi PS, Klein MI, et al. Role of glucosyltransferase B in interactions of *Candida albicans* with *Streptococcus mutans* and with an experimental pellicle on hydroxyapatite surfaces. Appl Environ Microbiol 2011;77:6357–67.
20. Schilling KM, Bowen WH. Glucans synthesized *in situ* in experimental salivary pellicle function as specific binding sites for *Streptococcus mutans.* Infect Immun 1992;60:284–95.
21. Bowen WH, Koo H. Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogeneic biofilms. Caries Res 2011;45:69–86.
22. Iio M, Uyeda M, Iwanani T, Nakagawa Y. Flavonoids as a possible preventive of dental caries, Agric Biol Chem 1984;48:2143–5.
23. Hardie JM. Oral microbiology: current concepts in the microbiology of dental caries and periodontal disease. Br Dent J 1992;172:271–8.
24. Marsh PD. Plaque as a biofilm: pharmacological principles of drug delivery and action in the suband supragingival environment. Oral Dis 2003;9:16–22.
25. Leme AF, Koo H, Bellato CM, Bedi G, Cury JA. The role of sucrose in cariogenic dental biofilm formation-new insight. J Dent Res 2006;85:878–87.
26. Jabra-Rizk AM. Pathogenesis of polymicrobial biofilms. Open Mycol J 2011;5:39–43.
27. Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH, et al. The human oral microbiome. J Bacteriol 2010;192:5002–17.
28. Wolcott R, Costerton JW, Raoult D, Cutler SJ. The polymicrobial nature of biofilm infection. Clin Microbiol Infect 2013;19:107–12.
29. Duckworth RM, Morgan SN, Murray AM. Fluoride in saliva and plaque following use of fluoride-containing mouthwashes. J Dent Res 1987;59:1187–91.