FULL PAPER *Bacteriology*

Antimicrobial activity of tea catechin against canine oral bacteria and the functional mechanisms

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ABSTRACT. Epigallocatechin gallate (EGCG) is the major polyphenolic compound of green tea. Polyphenolic compounds were extracted from the leaf of *Camellia sinensis* (Japanese green tea), and the minimum inhibitory concentration against canine oral bacteria was measured. Subsequently, we investigated the inhibitory effects of polyphenolic compounds and EGCG on the growth of canine oral bacteria. EGCG showed antimicrobial activity against a model bacterium, *Streptococcus mutans*. Our results indicate that EGCG can inhibit the growth and biofilm formation of *S. mutans* and that EGCG does not interact with streptococcal lipoteichoic acid (LTA). Furthermore, our findings suggest that EGCG interacts with other component(s) of the bacterial membrane aside from streptococcal LTA to inhibit biofilm formation and damage biofilms.

KEY WORDS: biofilm, EGCG, polyphenolic compounds, streptococcal lipoteichoic acid

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Tea is the most consumed beverage in the world, except for water [7]. Green tea, made from *Camellia sinensis* leaves, is a non-fermented tea and has more beneficial health effects than black tea or oolong tea [17]. Green tea contains several polyphenolic compounds, including flavins and polyphenols. Catechins are the most frequent and abundant polyphenolic compounds [12, 22]. The major green tea catechins include epigallocatechin gallate (EGCG) and epicatechin gallate (ECG), which are produced from the esterification of other catechins (C) and epicatechins (EC) with gallic acid. EGCG is the most abundant [9], accounting for 50 to 65% of total catechins, as reviewed by Zaveri [36] and Nagle *et al.* [24]. EGCG possesses a range of biological and medicinal properties, including antioxidant, anti-carcinogen, anti-obesity, antibacterial, antiviral and anti-enzymatic effects [28].

It has been reported that oral disorders, such as periodontitis, occur frequently in dogs [15, 16, 33]. Canine periodontitis occurs with plaque accumulation and subsequent gingivitis, and with bone resorption with aging. In the early stages, oxygen-resistant and other streptococci can adhere to the oral cavity. Plaque flora changes with increasing numbers of obligate anaerobic Gram-negative bacteria. The genus *Streptococcus* has been recognized as an initial colonizer [26].

Streptococcus mutans has various unique characteristics for survival in oral cavities of humans [1] and dogs [5, 6, 21]. The numbers of salivary *S. mutans* were different among various dog populations [21]. It has been reported that the quantity of caries-causing bacteria (*S. mutans*) is related to the environment in which dogs are kept [6, 21]. *S. mutans* is an important bacterium for biofilm formation [1, 5, 6], indicating that it would also be a model bacterium for testing antimicrobial substances in dogs [6]. One of the most documented characteristics of the virulence of *S. mutans* is its ability to produce glucosyltransferases, which synthesize intracellular polysaccharides and extracellular polysaccharides (EPS). The EPS, specifically the water-insoluble glucans, mediates the adherence of *S. mutans* and other oral bacterial species to tooth surfaces. This contributes to the formation of dental plaque biofilms [27] and allows the adhering bacteria to evade host defenses. The two major classes of these cell surface glycopolymers are teichoic acids (TA) and lipoteichoic acids (LTA), which are phosphate-rich molecules found in a wide range of Gram-positive bacteria [19, 29, 31]. They have been implicated in many persistent and chronic diseases, such as cystic fibrosis, endocarditis and infections, caused by biofilms growing on incorporated foreign materials, e.g. stents, indwelling catheters, bone implants and artificial valves [20]. Infections associated with implant surfaces or necrotic tissues like bone grafts can be fatal for the patient. Bacteria in biofilms are encased in a polysaccharide glycocalyx, which provides them with

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Table 1. Minimum inhibitory concentration (MIC) of extracted polyphenolic compounds from Japanese green tea and EGCG against various isolates from oral cavity of dogs

Canine isolates	MIC (mg/m*l*)	
	Polyphenolic compounds[a] from Japanese green tea	EGCG[b]
Porphyromonas endodontalis INU-1	0.8	0.1
Porphyromonas salivosa Festa-S	0.4	0.1
Porphyromonas gulae Festa-G	0.4	0.05
Prevotella intermedia INU-B4	0.1	0.025
Prevotella melaninogenica INU-BL1	0.2	0.05
Fusobacterium nucleatum INU-F2	0.2	0.05
Lactobacillus acidophilus INU-L3	0.2	0.05
Streptococcus spp. INU-7A3	0.1	0.0125
Streptococcus spp. INU-8SO1	0.1	0.0125
Streptococcus spp. INU-9SOA3	0.1	0.0125
Peptostreptococcus spp. INU-PS	0.4	0.025

a) Polyphenolic compounds were using 95% ethanol at 80°C for 4 hr to extract. It is a mixer that includes various components, such as tannins (EGCG and other catechins), minerals, nitrogenous components, caffeine and lipids, etc. b) EGCG is one of major polyphenolic compounds, which had been purified.

protection against the host defenses, antimicrobial drugs and biocides [3].

In this study, we investigated inhibition of growth of canine oral bacteria. Streptococci were highly sensitive to EGCG. Growth inhibition, anti-biofilm formation and anti-biofilm activity of catechins against *S. mutans* as a model bacterium were examined. Electron microscopic observations of *S. mutans* exposed to EGCG were also performed. Finally, the interaction between streptococcal LTA and EGCG was measured by a quartz crystal microbalance (QCM) binding assay.

MATERIALS AND METHODS

Bacterial strains and culture conditions: Various oral bacteria were isolated from gingival plaque taken from maxillary premolars of the dogs with periodontal disease (Table 1) as previously described [14]. The bacteria were grown in GAM broth (Nissui Co., Tokyo, Japan) for 24 to 48 hr at 37°C anaerobically. *S. mutans* was isolated by Hirose *et al.* as previously described [10] and was grown in Brain Heart Infusion (BHI) broth (Merck KGaA, Darmstadt, Germany) for 24 hr at 37°C.

Catechins: Polyphenolic compounds were isolated from the leaf of *C. sinensis* by extraction using 95% ethanol (80°C for 4 hr) as previously described [12]. Five major catechins, epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC), epicatechin (EC) and catechin (C), were detected at amounts of 17.8, 11.8, 4.2, 2.8 and 0.4%, respectively [12]. Purified forms of these five major catechins were also purchased (Nagara Science Co., Ltd., Gifu, Japan). The purity of the five major catechins was 98%.

Growth inhibition test: Canine oral bacteria were used for the growth inhibition test. The pre-cultured bacteria were diluted to 10^4 CFU (colony forming units) per m*l*, and then, a mixture of polyphenolic compounds [12] containing either the five major catechins and other components or only purified EGCG was added to 1 m*l* of bacterial suspension. *S. mutans* (model bacterium) was added to each of the catechin (EGCG, ECG, EGC, EC or C) solutions, which had final concentrations of 0.2, 0.1, 0.05, 0.025 or 0.0125 mg/m*l*, and were mixed and incubated at 37°C. One-hundred microliters of cell suspension, which had been treated with catechins for 3 or 5 hr, was used for a short-time killing assay and seeded on plates to incubate for 48 hr at 37°C. After incubation, the colonies were counted. Furthermore, cells were incubated with EGCG for 24 hr to measure the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Controls were prepared by mixing 1 m*l* of bacterial suspension, 0.9 m*l* of BHI broth and 0.1 m*l* of Hanks' Balanced Salt Solution (HBSS, pH 7.4; Gibco, Grand Island, NY, U.S.A.). The MICs of the polyphenolic compound mix and EGCG are defined as the lowest concentrations that inhibited visible growth after overnight incubation. The MBC is defined as the lowest concentration of EGCG that killed 99.9% of the initial inoculum in a given time using a plate count assay of viable cells.

Biofilm formation test: The effect of EGCG on biofilm formation of *S. mutans* was measured by using the Minimum Biofilm Eradication Concentration-High Throughput Plate (MBEC^{TM}-HTP, Innovotech, Inc., Edmonton, AB, Canada). The pre-cultured *S. mutans* was diluted to a final concentration of 10^7 CFU/m*l*. One-hundred microliters of the bacteria dilution was mixed with 100 μl of 0.4 mg/m*l* EGCG and was added into the 96-well microtiter plate. The plate was covered with a lid equipped with 96 pegs, the surface of which was covered with hydroxyapatite, with each peg dipping into the bacterial suspension. Thus, biofilms could be formed on the surface of the pegs. The control was the bacterial suspension incubated under EGCG-free conditions. The plates

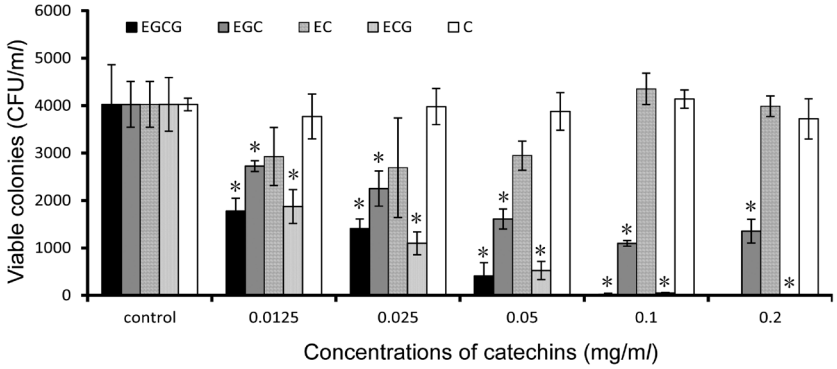

Fig. 1. Antimicrobial activity of catechins against *S. mutans*. The bacteria cultures added different concentrations of catechins to incubate for 5 hr at 37°C. The viable colonies were calculated in colony forming units and analyzed by *t*-test. Standard deviations indicated by error bars were calculated from 3 independent experiments. * Significantly different from the untreated control (*P<0.05).

were incubated at 37°C with shaking for 12, 24 and 36 hr. After different periods of incubation, the pegs were broken off, and the biofilms were disrupted from the surface of the pegs in 200 μl of physiological saline solution (PSS) with a sonicator. Twenty-microliter aliquots of the cell suspensions were then inoculated on BHI agar plates. The plates were incubated for 48 hr at 37°C, and colonies were counted. All measurements were done in triplicate.

Biofilm susceptibility assay: Biofilms of peg surfaces were formed from 24 hr cell suspensions of *S. mutans* containing 10^7 CFUs/m*l*. The biofilms formed on the pegs were inserted into 0.2 mg/m*l* of EGCG solution for 6 hr and 8 hr at 37°C. The survival of the bacteria was assessed as described above using the colony count method.

Observation of EGCG-treated bacteria surface using field emission-scanning electron microscope (FE-SEM): A 10 μl cell suspension of *S. mutans* was treated with EGCG at 37°C for 24 hr, mixed with 10 μl distilled water on a micro-glass and then dried. The dried cells were treated with saturated 70% ethanol for 5 min and saturated 100% ethanol for another 5 min. After air-drying, the sample was examined using a FE-SEM (SU8000; Hitachi High-Technologies Corporation, Tokyo, Japan).

The pegs treated with EGCG were removed from the plate, on which biofilms had formed, and were rinsed in 0.9% physiological saline for 1 min to remove planktonic culture. The samples were fixed with 2.5% glutaraldehyde (Kanto Chemical Co., Inc., Osaka, Tokyo, Japan) in 0.1 M cacodylic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 4°C for 16 hr. The pegs were washed with 0.1 M cacodylic acid and distilled water for approximately 10 min each. Saturated 70% ethanol was applied for 15 to 20 min and then air-dried at least for 24 hr. The pegs were mounted on a stage and examined using an FE-SEM.

EGCG interaction with streptococcal LTA: The quartz crystal microbalance (QCM) is a very sensitive mass measuring device, which measures changes in resonant frequency upon a weight increase on the surface of sensor crystal oscillator. The use of QCM transducers offers sensitive, *in situ* detection of hybridization events, without the need for optical or redox indicators [34]. The QCM (Single-Q, AS ONE Co., Ltd., Osaka, Japan) has an automatic injection mechanism, mixer, sensor crystal oscillator and low capacity reaction vessel. The vibration frequency changes has 200 Hz was defined as substances have attached to sensor crystal oscillator. Firstly, 500 μl of HBSS was added to the low-capacity reaction vessel and mixed at 6,000 revolutions per minute (rpm) to stabilize at 37°C. Secondly, 5 μl of EGCG (16 mg/m*l*) was injected into the low capacity reaction vessel to stabilize. Subsequently, 5 μl of block agent that did not react with the substances was injected to fix the rest of sensor where it was not bound with EGCG. Finally, 5 μl of streptococcal LTA (1 mg/m*l*) from *S. mutans* (Sigma-Aldrich Japan Corporation, Tokyo, Japan) was injected to measure the frequency change.

Statistical analysis: The function program in Microsoft Excel (Microsoft Corporation) was used to conduct *F*-tests and *t*-tests for our results (estimation of bacterial cell numbers). The type of function used to analyze results of Figs. 1, 2 and 3. After statistical analysis, *P*-values of less than 0.05 were considered statistically significant.

RESULTS

Growth inhibition of canine oral bacteria: Various bacteria isolated from oral cavity were sensitive to the polyphenolic compounds mix and EGCG (Table 1). MIC ranges were 0.1–0.8 mg/m*l* for polyphenolic compounds and 0.0125–0.1 mg/m*l* for EGCG. Oral streptococci showed significant growth inhibition compared to controls which were in the absence of the polyphenolic compounds mix and EGCG.

Bactericidal effect of catechins: As shown in Fig. 1, the growth of *S. mutans* was inhibited by three kinds of catechins (EGCG, ECG and EGC), and the order of their inhibitory effect is EGCG >ECG >EGC. EC and C did not show inhibitory function against *S. mutans*. The MIC and MBC

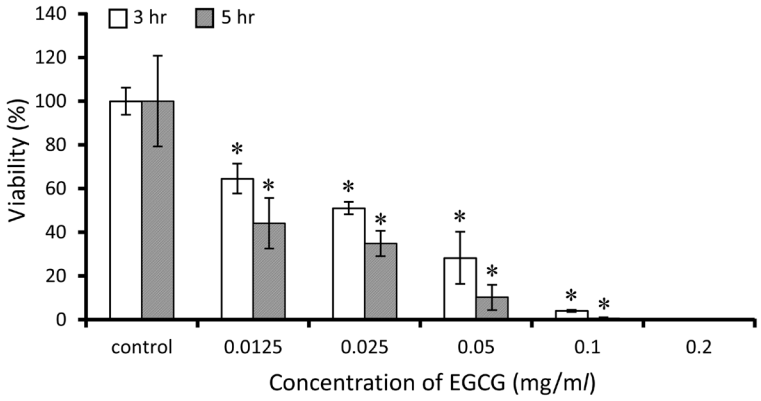

Fig. 2. Antimicrobial activity of EGCG against *S. mutans*. The bacteria culture that added different concentrations of EGCG was incubated for 3 hr and 5 hr at 37°C. The viability (%) is represented as a percentage of the colonies that EGCG cell culture is compared with control. The result of viability analyzed by *t*-test. Standard deviations indicated by error bars were calculated from 3 independent experiments. * Significantly different from the untreated control (*P<0.05).

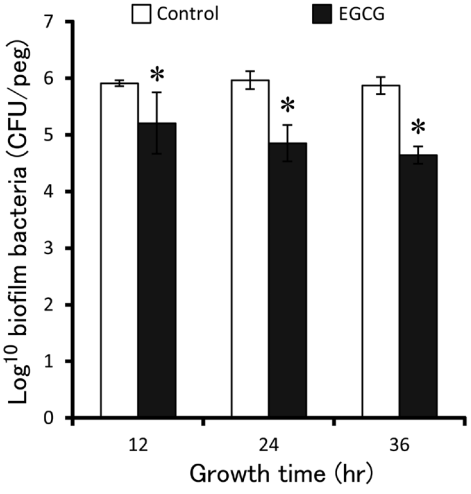

Fig. 3. Inhibitory effect of EGCG on biofilm formation. The pegs inserted into 10^7 CFU/m*l* planktonic cultures that had added final concentration of 0.2 mg/m*l* of EGCG to incubate for 12, 24 and 36 hr to form biofilm, respectively. The bacteria were moved from biofilm of pegs and seeded on agar plate to count and analyzed by *t*-test. Standard deviations indicated by error bars were calculated from 3 independent experiments. * Significantly different from the untreated controls (*P<0.05).

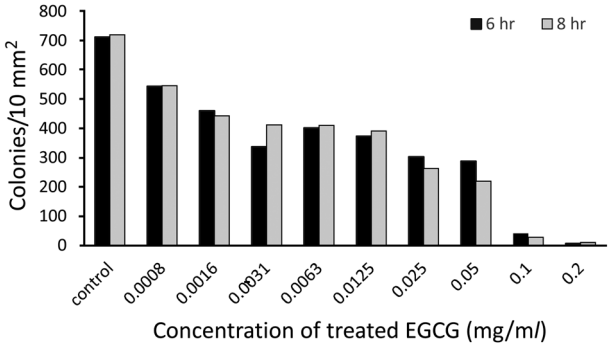

Fig. 4. Effect of EGCG on living microbes after biofilms formation. Biofilm pegs treated different concentrations of EGCG for 6 hr or 8 hr at 37°C, and then, bacteria were moved from biofilm of pegs to count living cell within per 10 square millimeter where seeded on ager plate.

values of EGCG against *S. mutans* were 0.125 and 0.1 mg/m*l*, respectively.

The viabilities of *S. mutans* bacterial cells treated with 0.0125 to 0.1 mg/m*l* of EGCG clearly decreased in the short-time killing assay (Fig. 2). The percentage of viable bacterial cells, which were incubated at concentrations of 0.0125 to 0.1 mg/m*l* of EGCG for 3 hr and 5 hr comparing with controls, had decreased to 64.7–4.0% and 45.3–0.0%, respectively.

Inhibitory effect of EGCG on biofilm formation: The inhibitory effect of EGCG on biofilm formation of *S. mutans* was measured by counting colonies that were recovered from biofilms formed on the surface of pegs, as described in Materials and Methods. The number of CFUs obtained from biofilm, which had formed in the presence of EGCG, was less than that formed in the control culture (*t*-test, P<0.05) (Fig. 3), indicating that EGCG had an inhibitory effect on *S. mutans* biofilm formation. Thus, this result suggests that EGCG can interfere with some components of bacterial cells to inhibit biofilm formation.

Biofilm susceptibility to EGCG: The effect of EGCG on the *S. mutans* biofilm was measured using a biofilm susceptibility assay as described above. For this purpose, biofilms were first established on pegs, and the biofilm bacteria were statistically significantly eradicated in 6 hr of incubation at a concentration of 0.2 mg/m*l* of EGCG, and bacteria from the treated biofilm were seeded on a plate to count colonies within 10 mm^2 of agarose (Fig. 4). This anti-biofilm effect

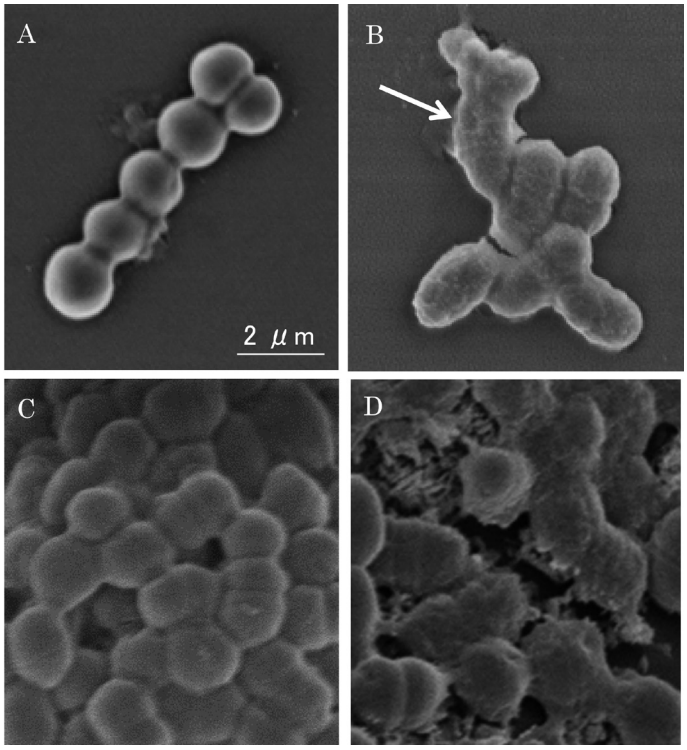

Fig. 5. The damage effect of EGCG on *S. mutans* cell morphology and biofilm. (A) Untreated control. (B) Treated with 0.2 mg/m*l* of EGCG for 24 hr. The arrow in Fig. 5B indicates "ring" phenomena around the damaged cells. (C) Untreated control of biofilm on MBEC^{TM}-HTP pegs. (D) Biofilm bacteria were treated with 0.2 mg/m*l* of EGCG for 24 hr.

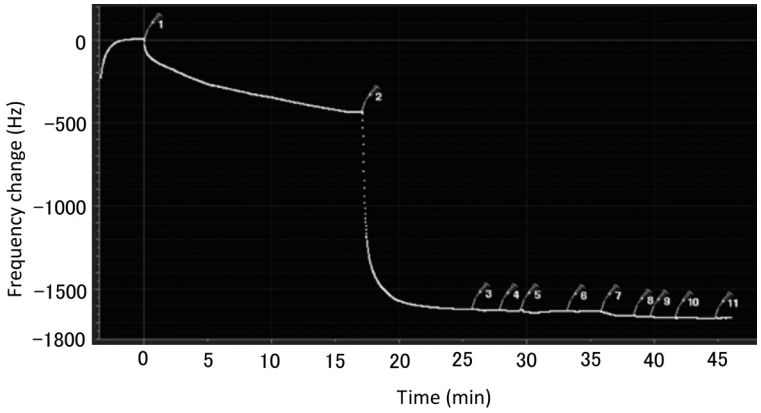

Fig. 6. Intermolecular interaction of EGCG and streptococcal LTA. This is one of the most typical data, in that the same experiment done three times. The numbers in the figure represent injecting times. 1, 5 μl of EGCG. 2, 5 μl of block agent. The numbers of 3 to 11 represent injected each 5 μl of streptococcal LTA.

of EGCG was dose-dependent. The anti-biofilm effect of EGCG was further enhanced after 8 hr incubation, when no colonies were recovered from biofilms treated with 0.2 mg/m*l* EGCG.

Bacterial cell surface damage after treatment with EGCG: The effect of EGCG on *S. mutans* cell morphology was observed using an FE-SEM. As shown in Fig. 5, untreated *S. mutans* cells had smooth surfaces. Cell surfaces treated with 0.2 mg/m*l* of EGCG became muddled, and some cell membranes broke, leading to leakage of the cytoplasm (Fig. 5B). These results demonstrate that the morphological changes to the cell surface were induced by EGCG.

A microscopy image of the *S. mutans* biofilm attached on the peg is shown in Fig. 5. In Fig. 5C, a regular form of the

bacteria can be observed on the peg, showing the biofilm as a pellicle. The effect of EGCG on *S. mutans* biofilm damage was similar to that in cell suspensions (Fig. 5D). Cells of the biofilm became muddled, and some cell membranes broke, leading to leakage of cytoplasm. The results indicate that the bacteria in the biofilm were damaged by EGCG.

Intermolecular interaction between streptococcal LTA and EGCG: In the QCM test, when 5 μl of EGCG was injected, the vibration frequency decreased by 440 Hz, and the amount of streptococcal LTA solution injected each time was 5 μl, the vibration frequency only reduced by 50 Hz (Fig. 6). This experiment indicates that EGCG and streptococcal LTA have no intermolecular interaction.

DISCUSSION

In this study, oral bacteria isolated from dogs were found to be sensitive to polyphenolic compounds and EGCG, with streptococci being highly sensitive. In our previous study, we demonstrated that polyphenolic compounds inhibited plaque deposition, gingivitis and other porphyromonas in dogs when the compounds were supplemented in dog food (0.8 mg/g) [13]. We suspected that EGCG was the most effective catechin, because MIC increased when using purified EGCG.

Bacteria are the most numerous microbes in the mouth. One of the model bacteria of oral cavities in humans, *S. mutans,* adheres to the surface of the tooth indicating an area of demineralization of enamel [2, 27]. Besides, it has been reported that carotid injection of *S. mutans* and other related bacteria in a dog model leads to multifocal choroiditis with retinal detachment [23]. In recent reports, green tea has been used as an ingredient in feeds for calves [11]. Catechins of green tea have a broad spectrum of antimicrobial activity against both of gram-positive and gram-negative bacteria. In particular, galloylated derivatives, such as EGCG, have been documented to possess antimicrobial effects against oral streptococci [8]. Our study showed that one component of catechins, EGCG, demonstrates the highest antibacterial activity against the growth of *S. mutans*. The MBC value was higher than MIC. In the comparison of biofilm colonies formed in EGCG-treated and untreated culture, the CFUs of biofilm bacteria formed in the EGCG-treated culture were obviously less than that formed in the untreated culture. It was suggested that EGCG not only inhibits the growth of *S. mutans*, but also damages surface adsorption ability on the tooth *in vitro*.

The antimicrobial mechanism of EGCG is mainly attributable to irreversible damage of the microbial cytoplasmic membrane [30]. Electron microscopic analysis showed that EGCG induced cell membrane lysis and cytoplasm leakage as shown in Fig. 5. Microbial biofilms commonly exhibit increasing levels of resistance to most antibiotics or therapeutic agents [4]. Biofilm cells have shown to be more tolerant of antibiotics comparing with planktonic bacteria, and this makes it hard to treat *S. mutans* with modern medicine [32, 37]. Our data certified further that bacteria in biofilms display lower susceptibility to EGCG than those in suspension. As shown in Fig. 5D, EGCG has a striking effect on *S. mutans* cells, which establish the biofilm.

Gram-positive bacteria develop a profound cell-envelope structure; they lack the normal outer membrane, and the cell wall is usually much thicker than that of gram-negative species, with multiple peptidoglycan layers [35]. The LTA is anchored to the plasma membrane and extends from the cell surface to the peptidoglycan layer. LTA and wall-teichoic acid create what has been aptly been described as a "continuum of negative charge," which extends from the bacterial cell surface beyond the outermost layers of peptidoglycan [16]. We performed a test on the intermolecular interaction with streptococcal LTA and EGCG, and the results indicated that EGCG did not bind to streptococcal LTA. Catechins are known to bind to various proteins (e.g., albumin, casein) to form macromolecular complexes *in vitro* [18, 25]. All results demonstrated that EGCG interacts with other component (s) of the bacterial membrane, which could be some proteins, to inhibit biofilm formation and damage bacterial cells and biofilms, not through streptococcal LTA. These findings highlight that the EGCG of green tea may be an attractive candidate for the prevention and treatment of oral caries. Further work to understand the relation between EGCG and components of the cell membrane is needed for the development of new means to fight the infections caused by canine oral bacteria in the future.

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