The Tea Catechin Epigallocatechin Gallate Suppresses Cariogenic Virulence Factors of Streptococcus mutans

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Streptococcus mutans, the primary etiologic agent of dental caries, possesses a series of virulence factors associated with its cariogenicity. Alternatives to traditional antimicrobial treatment, agents selectively inhibiting the virulence factors without necessarily suppressing the resident oral species, are promising. The anticariogenic properties of tea have been suggested in experimental animals and humans. Tea polyphenols, especially epigallocatechin gallate (EGCg), have been shown to inhibit the growth and glucosyltransferases activity of S. mutans. However, their effects on biofilm and cariogenic virulence factors of oral streptococci other than glucosyltransferases have not been well documented. In this study, we investigated the biological effect of EGCg on the virulence factors of S. mutans associated with its acidogenicity and acidurity. The antimicrobial effects of EGCg on S. mutans biofilm grown in chemically defined medium were also examined. EGCg inhibited growth of S. mutans planktonic cells at an MIC of 31.25 μg/ml and a minimal bactericidal concentration (MBC) of 62.5 μg/ml. EGCg also inhibited S. mutans biofilm formation at 15.6 μg/ml (minimum concentration that showed at least 90% inhibition of biofilm formation) and reduced viability of the preformed biofilm at 625 μg/ml (sessile MIC80). EGCg at sub-MIC levels inhibited acidogenicity and acidurity of S. mutans cells. Analysis of the data obtained from real-time PCR showed that EGCg significantly suppressed the ldh, eno, atpD, and aguD genes of S. mutans UA159. Inhibition of the enzymatic activity of F1F0-ATPase and lactate dehydrogenase was also noted (50% inhibitory concentration between 15.6 and 31.25 μg/ml). These findings suggest that EGCg is a natural anticariogenic agent in that it exhibits antimicrobial activity against S. mutans and suppresses the specific virulence factors associated with its cariogenicity.

Dental caries is a multifactorial infectious disease dependent on diet, nutrition, resident oral flora, and host response. Unlike most infectious diseases exhibiting classic virulence factors such as endotoxin (lipopolysaccharide), the etiology of dental caries is unique, being associated with the bacterial metabolism of carbohydrates, leading to prolonged periods of plaque acidiﬁcation and demineralization of the tooth enamel. Bacterial virulence factors contributing to dental caries result in stable biofilm formation, stress tolerance (e.g., acid tolerance), and efficient acid production from carbohydrates (7, 36, 37, 53). Despite the complexity of oral flora, oral streptococci, including Streptococcus mutans and Streptococcus sobrinus, have generally been considered the primary etiologic agents of dental caries (1, 38). One of the most often documented characteristics of the virulence of S. mutans is its ability to produce glucosyltransferases (GTFs), which synthesize intracellular polysaccharides (IPS) and extracellular polysaccharides (EPS). The EPS, especially water-insoluble glucans, mediate the adherence of S. mutans and other oral bacterial species to tooth surfaces, contributing to the formation of dental plaque biofilms (21, 60). Other crucial virulence factors of S. mutans include the membrane-bound F1F0-ATPase system, the agmatine deiminase system, enolase, and lactate dehydrogenase. The membrane-bound F1F0-ATPase system pumps protons from cells, while maintaining the internal pH value. This is considered the primary determinant of acid tolerance in S. mutans (5, 14, 33, 34). Another inducible pathway for alkali production is through the agmatine deiminase system (AgDS), which increases the competitive fitness of S. mutans, contributing to its persistence and pathogenesis (19, 20). Enolase is responsible for the production of phosphophoolpyruvate (PEP), which functions as the key component of the PEP:carbohydrate phosphotransferase system (PTS). Lactate dehydrogenase (LDH), responsible for lactate production, further contributes to the virulence of S. mutans (36). Mutants of S. mutans, deficient in specific virulence factors, were more sensitive to environmental stress and less cariogenic than their parent strains (11, 27, 36, 39, 71). These findings suggest that suppression of virulence-associated genes and enzymes could be appealing for the prevention of dental caries.

Fluoride has long been proven to be an effective cariostatic agent (15, 17, 44) via the inhibition of demineralization and the enhancement of remineralization at the crystal surface, and the inhibition of bacterial activity (16). However, in the case of high bacterial challenge and/or xerostomia, fluoride alone is insufficient to prevent the progression of caries. In addition, adverse effects such as fluorosis have led to the limited use of fluoride for public health in many countries (16, 66). Therefore, the development of an alternative cariostatic agent with minimal side effects has been promising.

Tea, an aqueous infusion of dried leaves of the plant Camellia sinensis (family: Theaceae), is the most popular beverage consumed by humans worldwide (18). Numerous in vitro and animal studies have demonstrated that polyphenols from...
Bacterial susceptibility assays. The MIC and minimum bactericidal concentration (MBC) of EGCg against S. mutans UA159 was determined by a microdilution method in both CDM and BHI, as described previously (45).

The kinetics of the bacterial killing effect of EGCg against S. mutans UA159 was determined with the method modified from that of Koo et al. (35). Tubes containing S. mutans UA159 (1 \times 10^6 CFU/ml) and EGCg (31.25 to 125 \mu g/ml) in CDM were incubated at 37°C. Cell samples were removed at 0, 0.5, 1, 2, 4, and 8 h, serially diluted in phosphate-buffered saline (PBS, 50 mM, pH 6.8), and plated onto BHI agar with a spiral plater (Autoplate model 4000; Spiral Biotech, Inc., Norwood, MA). All plates were incubated anaerobically at 37°C for 48 h, and the number of colonies was then determined with a colony counter (Synbiosis Acolyte 7510; Microbiology International, Frederick, MD). Control contained S. mutans UA159 grown in the absence of EGCg. We constructed the killing curves by plotting the log_{10} CFU/ml versus time over 8 h. A bacterial effect was defined as a ≥ 3-fold CFU/ml decrease from the original inoculum.

Biofilm susceptibility assay. The effect of EGCg on S. mutans biofilm formation was examined by the microdilution method described previously (68). S. mutans UA159 (1 \times 10^6 CFU/ml) was grown in CDM supplemented with 1% (wt/vol) sucrose and EGCg (0 to 1,000 \mu g/ml) at 37°C for 24 h. The culture supernatant from each well was then decanted, and the adherent biofilm was washed three times with PBS, fixed with methanol for 15 min, and stained with 0.1% (wt/vol) crystal violet (Sigma) for 5 min. Subsequently, the wells were shaken at room temperature for 30 min, and the absorbance at 595 nm was recorded. The minimum biofilm inhibition concentration (MBIC) was defined as the lowest EGCg concentration that resulted in at least 50% (MBIC50) or 90% (MBIC90) inhibition of the formation of biofilm as compared with the untreated control. A parallel study was also performed with BHI medium supplemented with 1% (wt/vol) sucrose.

The effect of EGCg on the viability of in vitro S. mutans biofilm was determined by the microdilution method modified from that of Pierce et al. (51). A 200-\mu l quantity of an S. mutans UA159 cell suspension (1 \times 10^6 CFU/ml) in CDM containing 1% (wt/vol) sucrose was added to the wells of a 96-well microtiter plate for biofilm formation. After anaerobic incubation at 37°C for 24 h, the growth medium was removed without disrupting the integrity of the biofilms. The formed biofilms were then washed three times with PBS to remove nonadherent cells. CDM supplemented with EGCg (9.77 to 1,250 \mu g/ml) was added to wells containing biofilm and incubated at 37°C for 24 h. The control wells contained CDM without EGCg. After incubation, the culture media were removed, and the treated biofilms were washed three times with PBS. The metabolic activity of the biofilms was determined by the addition of 120 \mu l of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT)–menadione solution (0.2 mg/ml XTT and 16 \mu M menadione). All plates were further incubated in the dark at 37°C for 2 h. A 100-\mu l quantity of the colored cell-free supernatant in each well was then transferred to another blank microtiter plate, and absorbance at 490 nm was determined. The sessile MIC (SMIC50 and SMIC90) was defined as the lowest EGCg concentration resulting in at least 50% or 80% reduction compared with that of the untreated control. A parallel study was also performed with BHI medium supplemented with 1% (wt/vol) sucrose.

Glycolytic pH drop and acid tolerance assay. The effect of EGCg on S. mutans glycolysis was measured by the method modified from that of Song et al. (63). S. mutans UA159 was harvested at mid-logarithmic phase, washed with 0.5 mM potassium phosphate buffer containing 37.5 mM KCl and 1.25 mM MgCl2 (pH 6.5), and resuspended (optical density at 600 nm (OD_{600}) = 0.5) in the same solution containing sub-MIC levels of EGCg (3.9 to 15.6 \mu g/ml). The control mixture contained no EGCg. Glucose was added in the mixture to give a final concentration of 1% (wt/vol) sucrose. The decrease in pH, as a result of glycolytic activity of S. mutans UA159 cells, was monitored at 15-min intervals over a period of 75 min (Corning pH meter 240; Corning Inc., New York, NY).

The effect of EGCg on the acid tolerance of S. mutans UA159 was determined by measurement of the viability of bacteria after 2 h of exposure at pH 5.0 (63, 64). S. mutans UA159 was grown in TYEG medium until the cells reached the mid-logarithmic phase (OD_{600} = 0.5). The cells were collected by centrifugation and resuspended (OD_{600} = 0.2) in TYEG medium buffered with 40 mM phosphate-citrate buffer (pH 5.0) containing sub-MIC levels of EGCg, and incubated at 37°C for 2 h. The control mixture contained no EGCg. Samples were removed before and after incubation at pH 5.0 for viable counts as described above.

Cell permeabilization. S. mutans UA159 cells were permeabilized according to the method modified from Hooper and Hudak (29). S. mutans UA159 grown in BHI broth (for F_{1}F_{0}-ATPase and enolase assays) or TYEG medium (for AgD assay) until late exponential phase; 30-ml aliquots were harvested by centrifugation (4,000 \times g, 5 min, 4°C). Cells were resuspended in 2.5 ml of 75 mM Tris-HCl–10 mM MgSO_{4} (pH 7.0). Toluene (Fluka, Steinheim, Germany) was
TABLE 1. Specific primers used for real-time PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (L and R)</th>
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<tbody>
<tr>
<td>16S rRNA</td>
<td>AGCGTGTGCGCATATGTTCTAC</td>
</tr>
<tr>
<td>atpD</td>
<td>TTTGATGGTCGGGTAAG</td>
</tr>
<tr>
<td>aguD</td>
<td>TGGTGCTGCTCTTGCTAATG</td>
</tr>
<tr>
<td>ldh</td>
<td>GCGACGGCTCTTGTCTTAG</td>
</tr>
<tr>
<td>eno</td>
<td>CAGCGCTCTTGCATCCAC</td>
</tr>
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added to a final concentration of 10% (v/vol), and the cell suspensions were vigorously vortexed and incubated for 5 min at 37°C. The cell suspensions were further frozen in liquid nitrogen and thawed twice at 37°C. The permeabilized cells were harvested by centrifugation (4,000 × g, 5 min, 4°C), resuspended in 75 mM Tris-HCl–10 mM MgSO₄ at pH 7, and stored at −80°C until use. For F₁Fₒ-ATPase assay, we determined F₁Fₒ-ATPase activity by using permeabilized cells of S. mutans UA159 according to the method described by Belli et al. (2). Permeabilized cells were pretreated by EGCg (7.8 to 62.5 µg/ml) at room temperature for 15 min. The F₁Fₒ-ATPase activity was measured in terms of the release of inorganic phosphate in the following reaction mixture: 100 mM Tris-maleate buffer (pH 7.0) containing 5 mM adenosine-5′-triphosphate (ATP), 10 mM MgCl₂, and permeabilized cells. The released phosphate was then precipitated (4), and results were expressed as enzymatic activity relative to that of the untreated control.

Enolase assay. Enolase activity was determined using the cell lysates of S. mutans UA159 according to the method described by van Lovern et al. (67). Permeabilized cells were pretreated with EGCg (7.8 to 62.5 µg/ml) at room temperature for 15 min, and we measured enolase activity directly by monitoring the formation of PEP at 240 nm. The reaction mixture (200 µl) contained 180 µl of 20 mM KPO₄ buffer (pH 6.5) with 2 mM MgSO₄, 10 µl of n- (−)-2-phosphoglycerate (final concentration of 17.6 mM), and 10 µl of pre-treated permeabilized cells. Results were expressed as enzymatic activity relative to that of the untreated control.

Agmatine deiminase (AgD) assay. Permeabilized S. mutans UA159 cells were pretreated with EGCg (7.8 to 62.5 µg/ml) at room temperature for 15 min, and AgD activity was measured by colorimetric determination of N-carbamoylputrescine production from agmatine as previously described by Grissow et al. (19). Results were expressed as enzymatic activity relative to that of the untreated control.

Lactate dehydrogenase (LDH) assay. S. mutans UA159 cells were collected at late exponential phase and incubated in Tris-HCl buffer (pH 7.0) containing 0.5 mg/ml of lysozyme at 37°C for 1 h. The lysate was further sonicated by means of a cup horn (Thermo Fisher Scientific Inc.) on ice for 2 cycles of ultrasonication for 10 s each. The lysate was then centrifuged at 12,000 × g for 15 min, and we measured LDH activity directly by monitoring the formation of LDH at 340 nm (13). The reaction mixture (200 µl) contained 180 µl of 50 mM phosphate-buffered saline (pH 6.9) with 0.167 mM NADH and 10 mM sodium pyruvate; 10 µl of fructose 1,6-diphosphate (final concentration of 1 mM); and 10 µl of pre-treated LDH. Results were expressed as enzymatic activity relative to that of the untreated control.

RNA isolation, purification, and reverse transcription. For determination of the effect of EGCg on selected virulence factors at the transcriptional level, S. mutans UA159 was grown in either CDM or BHI medium supplemented with sub-MIC levels of EGCg (15.6 µg/ml and 62.5 µg/ml in CDM and in BHI, respectively). Cells were collected during the late exponential phase by centrifugation, and RNA was immediately stabilized with an RNAprotect bacterial reagent (Qiagen, Valencia, CA). Cells were then pelleted, resuspended in 100 µl of lysis buffer (20 mM Tris-HCl, 3 mM EDTA, 20 mg/ml lysosome, 60 mM NaCl, proteinase K, 1,000 U/ml mutanslysin [pH 8.0]), and incubated at 37°C with gentle agitation for 45 min. The lysate was further sonicated by means of a cup horn (Thermo Fisher Scientific Inc.) on ice for 2 cycles of ultrasonication for 60 s and then purified in an RNeasy minikit (Qiagen). Reverse transcription was performed by use of a 1st Strand cDNA synthesis kit with random hexamer primers (Invitrogen, Madison, WI).

Quantitative real-time PCR. Real-time PCR was used for quantification of atpD (part of the F₁Fₒ-ATPase operon), aguD (part of the agmatine deiminase system operon), ldh (LDH), and eno (enolase) mRNA expression, with 16S rRNA as an internal control. All primers for real-time PCR were designed with Primer3 (25) and obtained commercially from Sigma-Aldrich Corp. (Table 1). Real-time PCR amplification was performed on the iCycler iQ detection system (Applied Biosystems, Foster City, CA). The reaction mixture (25 µl) contained 1× SYBR green PCR Master Mix (Applied Biosystems), 1 µl of template cDNA, and forward and reverse primers (10 µM each). Thermal cycling conditions were designated as follows: initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. An additional step, from 95°C for 15 s to 60°C for 1 min (0.05°C s⁻¹), was performed to establish a melting curve. Threshold cycle values (Cₜ) were determined, and the data were analyzed by StepOne software v2.0 (Applied Biosystems) according to the ΔΔCₜ method.

Statistical analysis. All experiments were performed in triplicate and reproduced at least three separate times. Differences between the experimental group and the untreated control group were analyzed by SPSS (version 15.0 for Windows). One-way analysis of variance (ANOVA) was performed, and a post hoc Tukey test was used for the comparison of multiple means. Significance was set at a P value of <0.05.

RESULTS

Antimicrobial activity of EGCg against S. mutans UA159 planktonic culture and biofilm in vitro. EGCg inhibited the in vitro growth of S. mutans UA159 (MIC = 31.25 µg/ml) in the chemically defined medium and had an MBC of 62.5 µg/ml (Table 2). The kinetics of the antimicrobial effect of EGCg against S. mutans UA159 is shown in Fig. 1. At 62.5 µg/ml, EGCg was bactericidal against S. mutans UA159, with a more

FIG. 1. Kinetic killing effect of EGCg on S. mutans UA159 plank- tonic cell cultures. The concentrations of EGCg ranged from 31.25 µg/ml to 125 µg/ml. The untreated control was S. mutans UA159 cells grown in chemically defined medium in the absence of EGCg. Data are means ± standard deviations (SD) (n = 3).
inhibited by EGCg at sub-MIC levels, 7.8 \( \times 10^{-5} \) for all EGCg groups were significantly different from those of untreated controls at 30, 45, 60, and 75 min (Tukey test, \( P < 0.01 \)). (B) Acid tolerance was determined by measuring the survival rate of \( S. mutans \) UA159 cells at pH 5.0. *, EGCg at test concentrations (1.95 to 15.6 \( \mu \)g/ml) significantly inhibited the survival rate (means \( \pm \) SD, \( n = 4 \)) of \( S. mutans \) UA159 cells compared with the untreated control (\( P < 0.05 \)).

The acidity of \( S. mutans \) UA159 cells was also suppressed by EGCg at sub-MIC levels. As shown in Fig. 2B, the survival rate of \( S. mutans \) UA159 cells at pH 5.0 was significantly reduced in the presence of EGCg (1.95 to 15.6 \( \mu \)g/ml) compared with the untreated control. Dosage-dependent inhibition was observed, and the survival rate was significantly reduced by EGCg at concentrations as low as 1.95 \( \mu \)g/ml (\( P < 0.05 \)).

**EGCg inhibits the virulence factors of \( S. mutans \) UA159 in vitro.** The virulence factors of \( S. mutans \) UA159 examined in this study included those encoded by \( atpD, eno, ldh, \) and \( aguD \). In CDM, EGCg at 15.6 \( \mu \)g/ml significantly suppressed expression of \( atpD, eno, ldh, \) and \( aguD \) by \( \geq 50\% \) compared with expression in the untreated control (\( P < 0.05 \)). A similar result was observed in BHI medium supplemented with 62.5 \( \mu \)g/ml of EGCg (Fig. 3). In addition to the suppression of virulence factors at the transcriptional level, EGCg inhibited F1Fo-ATPase activity, with half-maximal inhibitory concentrations (IC\(_{50}\)) between 15.6 \( \mu \)g/ml and 31.25 \( \mu \)g/ml (Fig. 4A) (\( P < 0.05 \)). A similar IC\(_{50}\) was noted for LDH (Fig. 4B). However, less than 10\% reduction of enolase or AgD activity was observed. A similar IC\(_{50}\) was noted for LDH (Fig. 4B). However, less than 10\% reduction of enolase or AgD activity was observed.

**DISCUSSION**

Tea catechins, especially the galloylated derivatives such as EGCg, have been documented to possess antimicrobial effects against oral streptococci (22, 23, 32, 40, 65, 70). The antimicrobial mechanism is mainly attributable to irreversible dam-
EGCg inhibits the cariogenicity of S. mutans. Previous studies have demonstrated that tea catechins, especially EGCg, could act as promising cariostatic agents, due to their antimicrobial activity and various biological effects on key virulence factors of oral streptococci (65, 70). In addition to its bacteriostatic and bactericidal effects, EGCg is able to inhibit GTF activity, thus disrupting the formation and integrity of oral biofilm (25, 49). EGCg and other catechins from green tea have also been reported to suppress salivary and bacterial amylase activities, leading to the inefficiency of carbohydrate metabolism (26). A previous study showed that EGCg was able to suppress the in vitro acid production of S. mutans, and rinsing with EGCg solution also inhibited the in situ acidity of dental plaque (28). Those authors suggested that the antimicrobial activity of EGCg against oral streptococci may contribute to its inhibitory effect on acid production. However, in the same study, the viability of S. mutans in sucrose-containing culture or adherent to hydroxyapatite was not affected by the test concentrations of EGCg, indicating that other mechanisms may contribute to the observed inhibition. In this study, we found that the in vitro acid production by S. mutans UA159 was inhibited by sub-MIC levels of EGCg, which confirmed that other biomolecular mechanisms may also be involved in inhibition of the acid production of S. mutans. The previously reported inhibitory effect of EGCg on LDH activity may explain its suppression of the acid production of S. mutans, despite the fact that LDH used in that study was commercially obtained from Leuconostoc mesenteroides (28). The in vitro suppression of the LDH activity of S. mutans UA159 observed in our study, in concert with the inhibition of ldh gene expression obtained from real-time PCR, suggests that the inhibitory effect of EGCg on acid production is attributable not only to its antimicrobial activity but also to the suppression of LDH at both the transcriptional and enzymatic levels.

Moreover, the inhibitory effect on acid production may result from the biological effect of EGCg on the bacterial glycolytic pathways. Not only may the suppression of enolase by EGCg at the transcriptional level exert direct inhibition on glycolysis, but also the reduction of downstream phosphoenolpyruvate (PEP) produced by enolase may suppress the PEP:carbohydrate phosphotransferase system (PTS), the major system for internalizing sugar into the cell during a depleted sugar situation (52). The net result would be a decrease in sugar internalization and suppressed glycolysis, leading to the reduced acid production by S. mutans cells, as observed in this study.

Since the pathology of dental caries is associated almost exclusively with bacterial metabolism of carbohydrates, the metabolites, especially acids produced after carbohydrate consumption, in turn impose physiological stress on the oral microflora. Due to its acidogenic and aciduric properties, S. mutans gains a competitive advantage over less-acid-tolerant species and emerges as the numerically predominant resident in the cariogenic plaque when environmental conditions are less favorable. Thus, stress tolerance, especially the acidity of S. mutans, is closely involved in its virulence. The acid tolerance of S. mutans is mainly attributed to the activity of F,F,−

FIG. 4. Effect of increasing concentrations of EGCg on the activity of enzymes associated with the acidogenicity and aciduricity of S. mutans UA159. Data are means ± SD (n = 3). * Significant difference compared with the untreated control (P < 0.05).
ATPase (3, 24). It has been demonstrated that increased F$_1$F$_0$-ATPase activity correlates with increased transcription of the F$_1$F$_0$-ATPase operon (53), and the operon of S. mutans can be regulated at the transcriptional level in response to growth pH (61). The inhibition of F$_1$F$_0$-ATPase enzymatic activity and atpD expression (a subunit of the proton translocator) observed in this study may lead to increased cytoplasmic acidity and decreased acid adaptation. Cytoplasmic acidification may impair the normal function of the serial enzymes involved in glycolysis and EPS production, compromising the competitive fitness of, or having a potential lethal effect on, S. mutans.

In addition to the F$_1$F$_0$-ATPase system, the agmatine deiminase system (AgDS) is also able to increase the competitive fitness of S. mutans, contributing to the persistence and pathogenesis of this organism (19, 20). The AgDS system converts agmatine to putrescine, ammonia, and CO$_2$. The production of ammonia from agmatine increases the cytoplasmic pH, and the ATP generated in this process can also be used for growth or to extrude protons (20). The inhibition of aguD (antiporter of AgDS) expression observed in our study may further exacerbate the starvation stress of S. mutans cells due to reduced ATP production by AgDS.

Maintaining ΔpH across the cell membrane is the key factor allowing aciduric bacteria such as S. mutans to carry out glycolysis at low pH values within the biofilm, because glycolytic enzymes are not acid tolerant (2). Suppression of F$_1$F$_0$-ATPase and AgDS by EGCg may lead not only to energy starvation but also to disruption of ΔpH across the cell membrane (36), which in turn may trigger a series of physiological effects in the cell. As summarized in Fig. 5, we propose that cytoplasmic acidification and impaired acid tolerance as the result of suppression of AgDS and F$_1$F$_0$-ATPase may inhibit the normal function of various acid-intolerant enzymes. The optimum pH range of GTFs in S. mutans was reported to be 5.5 to 6.0 (59). The malfunction of GTFs at the lower pH value may lead to reduced production of EPS and IPS. The latter can be metabolized when exogenous fermentable substrate is depleted in the oral cavity (10). Therefore, the malfunction of GTFs may disrupt both bacterial adherence to the tooth surface and biofilm integrity and may augment the starvation stress of S. mutans cells due to the reduced preservation of IPS. Cytoplasmic acidity, in concert with the inhibition of enolase by EGCg, may also inhibit the normal process of glycolysis, as described above. This in turn will diminish the ATP pool and further suppress the activity of the proton translocator (F$_1$F$_0$-ATPase), aggravating cytoplasmic acidification. The inhibition of LDH at both transcriptional and enzymatic levels may also increase the levels of NADH and decrease the redox potential of the cell, leading to the NAD$^+$/NADH imbalance and/or accumulation of glycolytic intermediates in the cell, which is toxic for S. mutans (11, 27). The net result would be cytoplasmic acidification and disrupted glycolytic processes with diminished ATP pools, thereby triggering a series of cascaded biological effects at molecular levels, leading to compromised competence to environmental stress and impaired cellular functions, even cell death.

The interaction of EGCg with proteins, leading to the distortion of their tertiary structure, may account for the observed in vitro inhibition of EGCg in certain enzyme activity, e.g., F$_1$F$_0$-ATPase and LDH. However, the affinity for salivary proteins may limit its oral application (6). The reduced antimicrobial activity of EGCg in BHI medium, compared with that in CDM observed in our study, confirmed this point of view, which makes the inhibition of certain virulence factors at sub-MIC levels promising. The observed suppression of the virulence factors at the transcriptional level was comparable in both BHI and CDM, indicating that the interaction of EGCg with proteins may be unable to diminish the biological effect of
EGCG on S. mutans cells. However, further studies are needed to confirm this observation.

In summary, based on our data, EGCG represents a natural and alternative anticariogenic agent because (i) EGCG inhibits growth of both S. mutans planktonic and biofilm cultures, and (ii) EGCG inhibits various cariogenic virulence factors of S. mutans at the transcriptional and enzymatic levels, leading to reduced acidogenicity and compromised stress tolerance (especially acid tolerance). Considering the complexity of the acid stress and starvation stress elicited by EGCG, further studies with transcriptomic and proteomic approaches are warranted.

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