Impact of Sugar Content in Ready-to-Drink Green Tea and Exposure Duration on Cariogenic Biofilm pH

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Abstract

Objective: To explore the impact of sugar supplements in ready-to-drink green tea (GT) beverages on the pH levels of cariogenic biofilms over different exposure durations.

Material and Methods: *Streptococcus mutans* biofilms were exposed to GT categorized by sugar content: no-sugar (GT/NS), < 5% sugar (GT/S < 5%), >5% sugar (GT/S > 5%), and 5% sucrose (positive control). Biofilm pH was measured at baseline, 5, 20, 40, and 60 min. The impact of sugar content and exposure times on biofilm pH was assessed using the Generalized Estimating Equation and the least significant difference tests ($p < 0.05$).

Results: The mean biofilm pH at baseline was 4.28 ± 0.01. Sugar content and exposure duration significantly influenced biofilm pH, with a strong interaction effect observed ($p < 0.001$). Post-hoc analysis revealed that biofilm pH in all GT groups was significantly higher than 5% sucrose at all exposure times, surpassing the critical enamel demineralization pH threshold of 5.5. The biofilm pH in GT/NS and GT/S > 5% significantly increased until 40 min post-exposure, while in GT/S < 5%, the rise stabilized at 20 min. At 60 min, biofilm pH for GT/NS, GT/S < 5%, GT/S > 5%, and 5% sucrose was 6.25 ± 0.11, 5.91 ± 0.18, 5,70 ± 0.21, and 4.83 ± 0.05, respectively. Notably, pH in GT/S > 5% significantly decreased from 40 min ($p = 0.006$) and was significantly lower than in GT/NS ($p = 0.023$).

Conclusion: The pH of *S. mutans* biofilm exposed to GT with high sugar concentrations, even over extended periods, is unlikely to drop below the threshold for enamel demineralization. Nevertheless, adding sugar to GT may promote *S. mutans* growth, as suggested by a significant pH difference between high- and no-sugar GT.

Keywords: Beverages, Biofilms, Sugars, *Streptococcus mutans,* Tea

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Introduction

Dental caries remains a significant oral health problems globally (1). The disease is caused by an imbalance between the enamel demineralization and remineralization processes. A key contributor to the demineralization of tooth enamel is *Streptococcus mutans (S. mutans),* recognized as one of the major cariogenic pathogens by virtue of its capacity to produce short chain carboxylic acids such as lactic and acetic acid as metabolites of sucrose metabolism (2). The accumulation of these acids within the biofilm leads to a decline in pH and once it falls below the critical level of 5.5, caries formation is initiated (3). Given this evidence, sucrose is widely recognized as a significant factor in the development of dental caries (4).

Currently, green tea stands as one of the most popular beverages globally, driven by health perceptions, and supported by evidence of its potential to lower the risk of various diseases (5-8) as well as its ability to hinder the development of dental caries (9-10). Indeed, the exploration of green tea's benefits on oral health dates back to the mid-1970s in Japan, where it was observed to decrease the caries increment among school children (11).

In many regions of the world, sweetened beverages, such as ready-to-drink green tea (GT), are extensively consumed and are readily accessible at various retail outlets. According to the Thailand Food Market Report 2018, it was observed that sweetened GT was the most favored choice among consumers, with a substantial market share of 56.6%. This was followed by sweetened ready-to-drink black tea, unsweetened GT, sweetened ready-to-drink white tea, and other flavors, with market shares of 18.5%, 10.7%, 13.3%, and 0.9%, respectively (12).

Research suggest that the anti-cariogenic effects of green tea primarily stem from its high concentration of polyphenols, particularly catechins, such as epigallocatechin gallate (EGCG) (13, 14). Additionally, it is known that green tea extracts reduce the number of *S. mutans* in plaque and saliva (15-16). This is likely to be due to the maintenance of high pH conditions within the biofilm milieu as it has been shown that green tea extracts increase the alkalinity of plaque biofilm and maintains it above pH 5.5 even after consumption of acidic food (15,17). Another *in vitro* study highlighted EGCG at 1 mg/mL inhibited the rate of glucose-induced acid production significantly impeding the growth of *S. mutans* (18).

Considering that green tea has the potential to mitigate the development of dental caries (15, 19), the addition of sugar to the beverage, a well-known caries risk factor, raises concerns about its impact on the beneficial anti-caries effect of green tea. The *in vitro* study had proved that the addition of sucrose to green tea infusion did not significantly alter the ability of green tea neither in reducing the biofilm biomass nor modifying biofilm architecture (20).

Additionally, the length of time the sweetened beverage is in contact with biofilms may influence pH levels and subsequently affect the risk of caries development. A previous study showed that one hour after tea consumption, salivary pH remained significantly lower than baseline only in the group that consumed tea containing jaggery—a traditional unrefined sugar made from sugarcane or palm sap. In contrast, salivary pH in the unsweetened tea group had returned to baseline (21).

However, to the best of our knowledge, no previous study has examined the combined effects of sugar content in GT and exposure time on the cariogenic attributes of *S. mutans* biofilm. Hence, the objective of this study was to investigate the impact of sugar content in ready-to-drink green tea (GT) and exposure duration on the pH levels of cariogenic biofilms.

Materials and Methods

Ready-to-drink green tea

Ready-to-drink green tea (GT) products from four different brands were categorized based on sugar content: no sugar (GT/NS), less than 5% sugar (GT/S < 5%), and more than 5% sugar (GT/S > 5%). These categories were used in this study (Table 1), with a 5% aqueous sucrose solution, the mean pH was 7.10 ± 0.20, serving as the positive control.

Table 1. The sugar content of ready-to-drink green tea used in this study.

Ready-to-drink green tea		Mean pH ± SD	Sugar	
			Amount	Type
No sugar	Brand 1	6.51 ± 0.06		
GT/NS	Brand 2	6.36 ± 0.09		
	Brand 3	6.33 ± 0.10		
	Brand 4	5.83 ± 0.35		
With sugar	Brand 1	6.34 ± 0.12	14g/400mL (3.5%)	fructose, sucrose
GT/S<5%	Brand 2	5.66 ± 0.42	19g/440mL (4.3%)	sucrose
GT/S>5%	Brand 3	6.34 ± 0.06	28g/500mL (5.6%)	sucrose
	Brand 4	5.78 ± 0.09	30g/500mL (6.0%)	sucrose

GT = ready-to-drink green tea, GT/NS = GT with no sugar, GT/S < 5% = GT containing less than 5% sugar, and GT/S > 5% = GT containing more than 5% sugar

Sucrose-containing artificial saliva

Sucrose-containing, sterilized, artificial saliva was prepared as described by Cavazana TP, et al (22), with minor modifications. In brief, one liter of artificial saliva was prepared with 2 g of yeast extract, 5 g of bacteriological peptone, 1 g of mucin-type III, 10 g of sucrose, 0.35 g of NaCl, 0.2 g of $CaCl_2$, and 0.2 g of KCl and was aliquoted as needed. The pH of the artificial saliva was adjusted to 6.8, using NaOH.

Biofilm formation

A single reference strain of *S. mutans* UA159 obtained from the archival collection of the Department of Microbiology, Faculty of Dentistry, Chulalongkorn University was used in this study as representative cariogenic organism. *S. mutans* were cultured in Brain Heart Infusion (BHI) broth at 37°C, 5% CO_2 until reaching the log-phase ($OD_{600nm} \approx 0.5$). Cells were then collected (approximately $5x10^8$ CFU/mL) and

re-suspended in sterilized sucrose-containing artificial saliva. One mL of the latter bacterial suspension was added to each well of a 24-well plate in duplicate. The plates were incubated at 37°C, 5% CO_2 for 96 h to allow biofilm development, with sterilized sucrose-containing artificial saliva refreshed every 24 h (Fig. 1) (22).

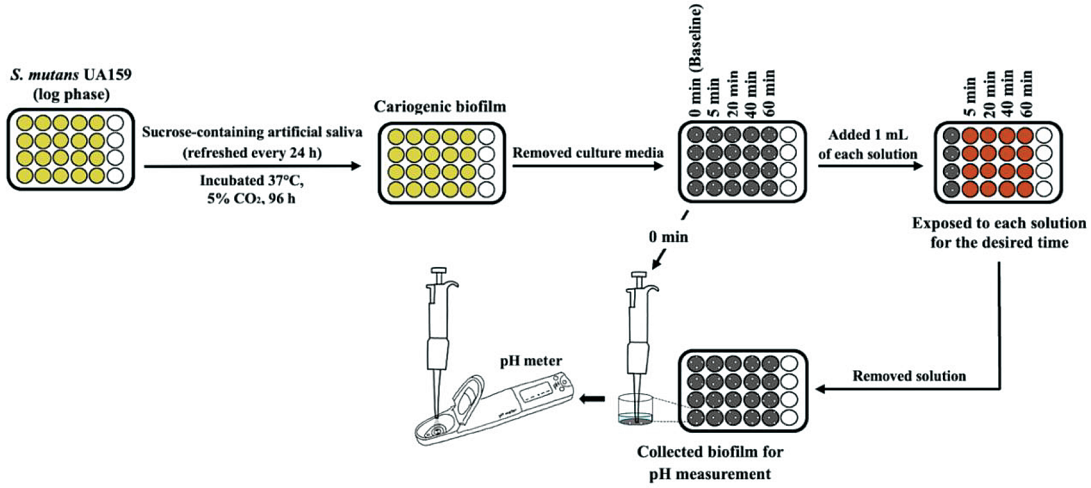

Fig. 1 Schematic flow chart of the study.

Ready-to-drink green tea treatment and pH measurement

The plaque pH measurement was performed according to the method of Garg D, et al. (23) with minor modifications. The pH of a 96-hour *S. mutans* biofilm was measured using a pH meter (LAQUAtwin pH-22, HORIBA Advanced Techno, Japan) at baseline (0 min), and then after treated with each GT beverage and a 5% sucrose solution for 5, 20, 40, and 60 min (Fig. 1). The experiments were performed independently on six occasions with duplicate determinations on each occasion to determine the mean value.

Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics Version 28.0 software (IBM Corporation, Armonk, New York, USA). The difference in mean pH values between the groups and at different time intervals (repeated measures) was statistically analyzed using the Generalized Estimation Equation (GEE), followed by the least significant difference (LSD) test to assess the interaction between two factors and the difference in each pair. The significant difference was determined when the p-value was less than 0.05.

Results

The mean biofilm pH of *S. mutans* at baseline (0 min) was 4.28 ± 0.01. In GT/NS group, the pH value progressively increased from 6.01 ± 0.11 at the 5th min to 6.27 ± 0.11 at the 40th min, followed by a slight decrease to 6.25 ± 0.11 at the 60th min. Both sugar-containing green tea groups, GT/S < 5% and GT/S > 5%, exhibited a similar pH profile, gradually increasing until the 40th min. At the 60th min, the biofilm pH in all

GT groups decreased, with GT/S > 5% group was 5.70 ± 0.21 nearing the critical pH of enamel (pH = 5.5, Fig. 2). However, throughout the 5- to 60-min period, the biofilm pH in all GT groups remained above 5.5, unlike 5% sucrose (a positive control), which stayed below this criterion (Fig. 2).

The GEE analysis revealed that both sugar content and exposure time were statistically significant (χ^2 = 163.93, p-value < 0.001 and χ^2 = 92.19, p-value < 0.001, respectively), with a significant interaction effect observed between them (χ^2 = 68.89, p-value < 0.001).

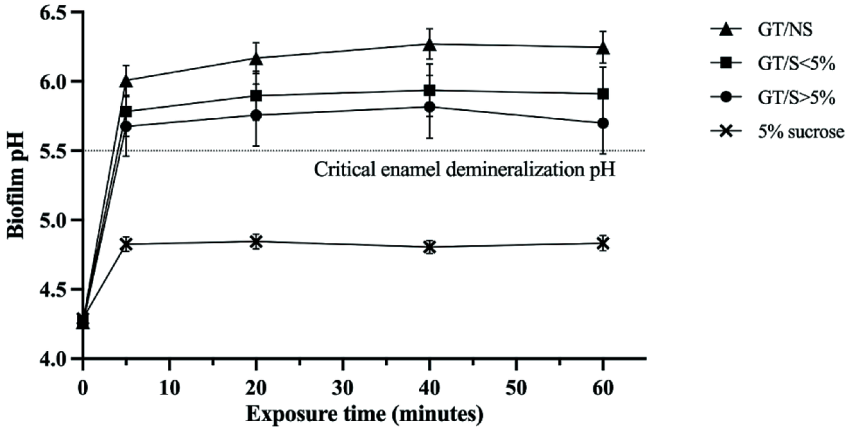

Fig. 2 Mean biofilm pH determined after exposure to each ready-to-drink green tea (GT) groups and 5% sucrose solution for 5, 20, 40, and 60 min. The experiment was repeated independently six times, each with duplicates. GT/NS = GT with no sugar, GT/S < 5% = GT containing less than 5% sugar, and GT/S > 5% = GT containing more than 5% sugar.

Pairwise comparisons using the least significant difference (LSD) revealed that the biofilm pH in all GT groups was significantly different from 5% sucrose (p-value < 0.001, Table 2). At the 60th min, the biofilm pH in the GT/NS was also significantly different from the GT/S > 5% group (p-value = 0.023, Table 2).

Table 2. Mean difference of biofilm pH in all beverage groups at each exposure group by time.

Group x Time		Mean Difference (SE)	p-value	95% CI	
				Lower	Upper
5 min	GT/NS vs GT/S < 5%	0.23 (0.20)	0.264	-0.169	0.619
	GT/NS vs GT/S > 5%	0.33 (0.23)	0.151	-0.122	0.787
	GT/NS vs 5% sucrose	**1.18 (0.12)**	**<0.001***	**0.953**	**1.412**
	GT/S < 5% vs GT/S>5%	0.11 (0.27)	0.689	-0.418	0.633
	GT/S<5% vs 5% sucrose	**0.96 (0.18)**	**<0.001***	**0.607**	**1.308**
	GT/S>5% vs 5% sucrose	**0.85 (0.21)**	**<0.001***	**0.434**	**1.266**
20 min	GT/NS vs GT/S<5%	0.27 (0.20)	0.179	-0.124	0.668
	GT/NS vs GT/S>5%	0.41 (0.24)	0.086	-0.059	0.882
	GT/NS vs 5% sucrose	**1.33 (0.12)**	**<0.001***	**1.088**	**1.562**
	GT/S<5% vs GT/S>5%	0.14 (0.27)	0.608	-0.395	0.675
	GT/S<5% vs 5% sucrose	**1.05 (0.18)**	**<0.001***	**0.705**	**1.401**
	GT/S>5% vs 5% sucrose	**0.91 (0.22)**	**<0.001***	**0.483**	**1.344**
40 min	GT/NS vs GT/S<5%	0.34 (0.21)	0.112	-0.078	0.748
	GT/NS vs GT/S>5%	0.45 (0.24)	0.061	-0.021	0.929
	GT/NS vs 5% sucrose	**1.47 (0.12)**	**<0.001***	**1.237**	**1.693**
	GT/S<5% vs GT/S>5%	0.12 (0.28)	0.674	-0.436	0.674
	GT/S<5% vs 5% sucrose	**1.13 (0.19)**	**<0.001***	**0.764**	**1.496**
	GT/S>5% vs 5% sucrose	**1.01 (0.22)**	**<0.001***	**0.576**	**1.446**
60 min	GT/NS vs GT/S<5%	0.34 (0.22)	0.120	-0.087	0.762
	GT/NS vs GT/S>5%	**0.55 (0.24)**	**0.023***	**0.075**	**1.019**
	GT/NS vs 5% sucrose	**1.42 (0.13)**	**<0.001***	**1.169**	**1.660**
	GT/S<5% vs GT/S>5%	0.21 (0.28)	0.456	-0.342	0.762
	GT/S<5% vs 5% sucrose	**1.08 (0.19)**	**<0.001***	**0.701**	**1.454**
	GT/S>5% vs 5% sucrose	**0.87 (0.22)**	**<0.001***	**0.438**	**1.297**

GT = ready-to-drink green tea, GT/NS = GT with no sugar, GT/S < 5% = GT containing less than 5% sugar, and GT/S > 5% = GT containing more than 5% sugar, Time = Exposure time, SE = standard error, *p < 0.001, CI = confidence interval

Within the GT/NS group, pairwise comparisons showed that the biofilm pH significantly increased until the 40th min (p-value < 0.001, Table 3). In contrast, for GT containing sugar, the biofilm pH in GT/S < 5% significantly increased up to 20 min (p-value < 0.001,Table 3), while in GT/S > 5%, the increase was significant up to 40 min (p-value < 0.001,Table 3). However, in the GT/S > 5% group, the biofilm pH significantly decreased after 40 min (p-value = 0.006, Table 3). No significant differences in biofilm pH were observed within the 5% sucrose group (Table 3). All these results may reflect the effect of the sugar content in the beverage on the changes in pH over time.

Table 3. Mean difference of biofilm pH in all beverage groups between each exposure group by time

Group x Time		Mean Difference (SE)	p-value	95% CI	
				Lower	Lower
GT/NS	**5 min vs 20 min**	**-0.16 (0.02)**	**< 0.001***	**-0.202**	**-0.120**
	5 min vs 40 min	**-0.26 (0.03)**	**< 0.001***	**-0.320**	**-0.205**
	5 min vs 60 min	**-0.24 (0.04)**	**< 0.001***	**-0.310**	**-0.170**
	20 min vs 40 min	**-0.10 (0.02)**	**< 0.001***	**-0.148**	**-0.055**
	20 min vs 60 min	**-0.08 (0.03)**	**0.006***	**-0.135**	**-0.022**
	40 min vs 60 min	0.02 (0.02)	0.328	-0.023	-0.070
GT/S < 5%	**5 min vs 20 min**	**-0.11 (0.02)**	**< 0.001***	**-0.163**	**-0.066**
	5 min vs 40 min	**-0.15 (0.02)**	**< 0.001***	**-0.193**	**-0.112**
	5 min vs 60 min	**-0.13 (0.03)**	**< 0.001***	**-0.188**	**-0.066**
	20 min vs 40 min	-0.04 (0.02)	0.093	-0.083	0.006
	20 min vs 60 min	-0.01 (0.04)	0.756	-0.091	0.066
	40 min vs 60 min	0.03 (0.02)	0.280	-0.021	0.073
GT/S > 5%	**5 min vs 20 min**	**-0.08 (0.03)**	**0.011***	**-0.145**	**-0.019**
	5 min vs 40 min	**-0.14 (0.03)**	**< 0.001***	**-0.209**	**-0.073**
	5 min vs 60 min	-0.02 (0.05)	0.609	-0.117	-0.069
	20 min vs 40 min	**-0.06 (0.01)**	**< 0.001***	**-0.082**	**-0.036**
	20 min vs 60 min	0.06 (0.05)	0.237	-0.038	0.153
	40 min vs 60 min	**0.12 (0.04)**	**0.006***	**0.034**	**0.199**
5% sucrose	5 min vs 20 min	-0.02 (0.03)	0.489	-0.070	0.034
	5 min vs 40 min	0.02 (0.03)	0.485	-0.036	0.076
	5 min vs 60 min	-0.01 (0.03)	0.845	-0.074	0.060
	20 min vs 40 min	0.04 (0.04)	0.334	-0.039	0.116
	20 min vs 60 min	0.01 (0.04)	0.760	-0.063	0.087
	40 min vs 60 min	-0.03 (0.03)	0.321	-0.079	0.026

GT = ready-to-drink green tea, GT/NS = GT with no sugar, GT/S< 5% = GT containing less than 5% sugar, and GT/S > 5% = GT containing more than 5% sugar, Time = Exposure time, SE = standard error, *p < 0.001, CI = confidence interval

Discussion

Green tea is widely recognized for its potential to reduce the risk of dental caries (15,16,19). Despite its popularity, there is a gap in research on the effects of sweetened GT on biofilm pH, which is crucial for caries development. Hence the focus of our study was whether the sugar content in GT and its exposure time to biofilms could influence the positive attributes of green tea.

Our methodology mirrors a practical scenario wherein GT is consumed during the day after biofilm has accumulated on the tooth enamel surface. According to the ecological plaque hypothesis, the cariogenic biofilm escalates the likelihood of caries initiation, particularly in the presence of dietary sugars as it harbors pathogens such as *S. mutans,* which utilize sugar to generate acids that demineralizes the enamel (24). In the model described, we successfully developed a *S. mutans* biofilm with a baseline pH of 4.28 ± 0.01 below the critical pH of enamel (pH = 5.5). Although the acidic environment in this *in vitro* model may be more pronounced than in the oral cavity due to limitations in replicating the complex microbial ecosystem, the findings indicate that both sugared and unsweetened GT maintained biofilm pH above the critical threshold for enamel demineralization. This suggests that under less acidic, real-life conditions, the effect may be even more favorable. Nevertheless, clinical studies are needed to confirm these findings.

In an *in vitro* study using green tea infusion, the addition of sugar did not significantly alter its effect on biofilm architecture (20). Similarly, a clinical study in children showed that rinsing with brewed green tea containing 5% sucrose resulted in consistently higher biofilm pH compared to 10% sucrose at all measured time points (5, 10, 20, and 30 min), with a gradual increase over time. The study also compared milk containing 5% sucrose and found that the tea group had higher biofilm pH than the milk group, and both groups had higher values than the 10% sucrose group (25). These findings match our study, which found that after 5 min of exposure to GT with or without sugar, the biofilm pH increased above 5.5 in both sugared GT groups (GT/S < 5% and GT/S > 5%), whereas the pH reached 6 in the GT without sugar group (GT/NS). This effect could be attributed to catechins, particularly EGCG, which are abundant in GT (26).

Several studies have shown the antibacterial potency EGCG as well its ability to mitigate acid production (13,27-28). For instance, Han S., et al. revealed that pH regulatory mechanisms of EGCG are linked its buffer capacity and blocking sugar transportation through a phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS). This leads to inhibition of sugar metabolism, resulting in inadequate energy production and consequently reduced bacterial growth which in turn suppresses acid production within the biofilm milieu (28).

Our GEE analysis study revealed a significant interaction between sugar content and exposure time. Moreover, at the 60 min exposure period, GT/S > 5% significantly lowered biofilm pH compared to GT/NS, while GT/S < 5% did not. These results suggest that even in the presence of EGCG, higher sugar concentrations, and prolonged exposure period that may have contributed to a reduction in biofilm pH (Fig. 2; Table 2). This finding may be attributed to the elevated concentration of sucrose coupled with extended

exposure time, permitting sucrose to effectively outcompete the EGCG effects, and also surpass the physical barrier posed by extracellular polysaccharides of *S. mutans.* Moreover, the smaller molecular size of sucrose (342.30 g/mol, (29)) compared to EGCG (458.4 g/mol, (30)) likely enables it to penetrate more deeply into the biofilm. This enhanced penetration increases the likelihood of sucrose reaching and being metabolized by larger numbers of *S. mutans,* leading to acid production.

The heat generated during the production of GT is likely to be a key factor affecting the characteristics of catechins in these commercially available products (31-33). A previous study has reported that catechin contents in bottled and canned GT (250 mL) ranges from 3 to 60 mg, contrasting with 400 to 500 mg typically found in a cup of brewed hot green tea extracts (400 mL) (32). Despite the lower EGCG content in GT compared to other formulations, we noted that even with added sugar, GT retained its ability to effectively regulate pH. However, further research is required to validate our findings and determine their clinical relevance.

Conclusions

Our data suggest that the pH of *S mutans.* biofilm exposed to GT with high sugar concentrations, even for extended periods, is unlikely to drop below the cariogenic pH threshold required for enamel demineralization. Nevertheless, there is a significant temporal pH differential between high and no sugar GT implying that addition of sugar to GT may foster the growth of acidophilic, cariogens such as *S. mutans.*

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