Effects of Cola-Flavored Beverages and Caffeine on *Streptococcus mutans* Biofilm Formation and Metabolic Activity

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Objective: *To examine the effects of cola-flavored beverages and caffeine on growth and metabolism of Streptococcus mutans biofilm. This study was designed to determine if carbonated beverages or caffeine can increase S. mutans growth and biofilm formation and metabolic activity in vitro, potentially leading to increased S. mutans-associated cariogenicity in children that consume them.* ***Study Design:*** *Six different cola-flavored products, plus pure caffeine, and pure high fructose corn syrup (HFCS), at different concentrations similar to those in the beverages were tested. A 16-hour culture of S. mutans was treated with different dilutions in bacteriological media. To test for the effect on biofilm formation, the biofilm was stained with crystal violet. The absorbance was determined to evaluate biofilm growth. Biofilm metabolic activity was measured based on biofilm having the ability to reduce XTT to a water-soluble orange compound.* ***Results:*** *The inclusion of HFCS in the beverages, as well as pure HFCS, significantly enhanced bacterial biofilm formation and metabolic activity. Pure caffeine and the presence of caffeine in beverages did not significantly increase biofilm formation, but pure caffeine significantly increased metabolism, and Diet Coke had significantly greater metabolic activity than Caffeine-Free Diet Coke.* ***Conclusions:*** *HFCS increases both the biofilm formation and metabolism of S. mutans, and caffeine in some cases increases metabolism of S. mutans.*

Key words: Soda, Cola, Streptococcus mutans, Biofilm, Metabolic Activity

INTRODUCTION

Streptococcus mutans is the major etiological agent of human tooth decay[1]. It is part of a group of similar species of bacteria that are collectively known as mutans streptococci. These species are present in not only the mouth, but also in the pharynx and intestines[2]. *S. mutans* is one of the major contributors in the development of dental caries. Studies have shown that *S. mutans*, along with *S. sobrinus*, are significantly associated with early childhood caries (ECC) [3]. ECC is a very costly and pervasive disease that is defined by the AAPD as "the presence of one or more decayed, missing (due to caries) or filled tooth surfaces in any primary tooth in a child under the age of six[4]." ECC is also often an indicator for the development of dental caries on the permanent dentition[5]. NHANES III found that by age 5, around 40% of all American children are already affected by either mild or severe early childhood caries[6].

The caries forming activity of *S. mutans* on teeth are carried out through the formation of dental biofilm containing *S. mutans* and subsequent production of lactic acid. Biofilm is a layer of slime that contains polymers, bacterial cells, and debris from a person's diet[2]. This biofilm grows in thickness, especially without good patient oral hygiene. It can end up being several hundreds of cells thick if not removed[2]. Biofilm is formed via a multistep process. It starts out as an acquired salivary pellicle that contains no bacteria. Secondly, planktonic bacteria colonize the pellicle, via reversible binding to the salivary proteins. Finally, in what is known as the maturation stage, via polysaccharide or protein receptors, the first group of bacteria allows for binding of a second group of colonizers including *S. mutans.* This aggregation is crucial for the formation of a mature biofilm[7]. Biofilms offer specific advantages for bacteria, including resistance to antibiotics and protection from salivary immune factors due to the high density of cells. These advantages may be a contributing factor for the estimated 65% of human infections that come from a biofilm entity[8,9].

The sucrose-dependent virulence of an *S. mutans* biofilm is primarily due to the activity of glucosyltransferases (GTF), glucans, and glucan-binding proteins (GBP). Tooth enamel has cell-free and cell-associated GTFs attached to the salivary pellicle, which, when

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exposed to sucrose, synthesize glucans. These glucans, through the use of GBPs, aid in the attachment of *S. mutans* to tooth enamel. They also allow for the ability of bacteria to attach to one another, thus creating a stronger and more virulent biofilm[10]. Though most studies have been done with sucrose, at least one study has shown up-regulation of GTF by high fructose corn syrup (HFCS)[11], which, like sucrose, is a combination of glucose and fructose.

It is well known that soft drinks have a negative impact on systemic and oral health[12,13]. One of the major concerns about soft drinks is their role in the formation of early childhood caries. There is a positive correlation between soda consumption and risk of caries[14], and children who have higher rates of soda consumption have been shown to have higher rates of dental caries[15]. Bowen, et. al., found that the cariogenicity of cola is higher than milk and even sucrose alone[5]. One of the major reasons for this cariogenicity may be due to the presence of a caries-promoting sugar, HFCS, which is present in most non-diet soft drinks[16]. One of the factors that is thought to favor the cariogenicity of the soft drinks is the fact that fermentable carboydrates cause a decrease in pH through fermentation and thus production of lactic acid by the action of acidogenic bacteria[17], such as *S. mutans*. The acidity, primarily through phosphoric acid, present in both diet and "full flavor" drinks can also cause tooth erosion that weakens the enamel through chemical demineralization[18] thereby providing a more favorable environment for *S. mutans*[19], which thus makes teeth more susceptible to tooth decay. Both regular and diet beverages can cause enamel erosion, though in one study, Coca-Cola Classic demonstrated a higher erosive potential than did Diet Coke[20]. Thus, the cariogenicity of full-flavor soft drinks is influenced by sugar, which ultimately lowers pH, weakening enamel and providing a favorable environment for the bacteria to grow[5].

Another health concern associated with the consumption of soft drinks is the presence of caffeine. Caffeine is a psychoactive substance, and worldwide it has a higher consumption than any other psychoactive substance[21]. Though caffeine may lead to certain systemic health concerns[21], it does not seem to increase the cariogenic potential of the diet[22], and in fact it may actually interfere with the adsorption of *S. mutans* to hydroxyapatite in enamel, thus increasing resistance[23]. In a recent antibacterial coffee study, caffeinated coffee has been shown to have higher inhibitory effects against *S. mutans* than decaffeinated coffee[24]. Thus, it will be beneficial to study the effects of caffeinated versus caffeine-free beverages on *S. mutans* biofilm.

Soft drink consumption is very prevalent (56% of children consume soft drinks on a given day[25]) throughout the United States, and has increased over the past 30 years by 123%, from an average of 5 fluid oz/day to 12 fluid oz/day[25]. In children, this consumption is widely thought to be a highly contributory factor to the presence of ECC. It is essential that we learn of the effects of cola-flavored soft drinks on the biofilm formation of *S. mutans* for further preventative efforts. Thus, the aim of the study was to determine the effects of the soft drinks themselves, as well as the effects of some of their individual ingredients on the growth and metabolism of the bacteria. We hypothesize that the addition of the sugar-containing cola-flavored products and HFCS to *S. mutans* cultures will: 1) increase growth and biofilm activity; and 2) increase metabolic activity, due to the fact that fermentable carbohydrates have been shown to increase caries activity in teeth[5,14,15]. We also hypothesize that the addition of caffeine to *S. mutans* cultures will inhibit biofilm formation and growth, as a few studies have shown caffeine having a negative effect on caries activity[23,24].

MATERIALS AND METHOD

S. mutans strain UA159 (ATCC 700610) was used in the study. The strain was stored at -80°C in tryptic soy broth (TSB, Acumedia, Baltimore, MA) with 20% glycerol before use. Mitis Salivarius Sucrose Bacitracin (MSSB, Anaerobe Systems, Morgan Hill, CA) agar plates were used to initially grow the strains. Unless otherwise stated, TSB was used and the growth conditions were 5% CO_2 at 37°C[26]. The cola soft drinks (Table 1) were purchased from a local grocery store and opened for 24 hours to remove carbonation. The drinks were chosen based on caffeine content (3 contain caffeine and 3 do not) and carbohydrate content (2 contain sugars and 2 do not). The sugar content in both beverages is purely composed of HFCS. Pure caffeine was obtained from Sigma Chemical Co., St. Louis, MO.

Table 1: Ingredients, sugars, and caffeine content present in the various cola-flavored beverages studied.

Beverage (12 oz)	Ingredients	Sugars (amt/12oz)	Caffeine (amt/12oz)
Coca-Cola Classic	Carbonated water, high fructose corn syrup, caramel color, phosphoric acid, natural flavors, caffeine	39 g 0.11 g/mL	32 mg 0.09 mg/mL
Diet Coke	Carbonated water, caramel color, aspartame, phosphoric acid, potassium benzoate, natural flavors, citric acid, caffeine	0	42 mg 0.12 mg/mL
Coke Zero	Carbonated water, caramel color, phosphoric acid, aspartame, potassium benzoate, natural flavors, potassium citrate, acesulfame potassium, caffeine	0	32 mg 0.09 mg/mL
Caffeine Free Coca-Cola	Carbonated water, high fructose corn syrup, caramel color, phosphoric acid, natural flavors	39 g 0.11 g/mL	0
Caffeine Free Diet Coke	Carbonated water, caramel color, aspartame, phosphoric acid, potassium benzoate, natural flavors, citric acid	0	0
Caffeine Free Coke Zero	Carbonated water, caramel color, phosphoric acid, aspartame, potassium benzoate, natural flavors, potassium citrate, acesulfame potassium	0	0

Growth and Biofilm Formation

To determine growth and biofilm formation (biofilm mass), an overnight *S. mutans* culture (approximately 10^8 CFU/ml) in TSB was treated with various concentrations of the colas, caffeine, and HFCS diluted in TSB. The drinks, caffeine, and HFCS were diluted with TSB (190 ul total volume), along with 10 ul of the overnight culture

of *S. mutans* and incubated for 16 h at 37°C in 96-well microtiter plates. The drinks were diluted 1:2 to 1:128. The concentrations of HFCS and caffeine were determined based on the concentrations present in the drinks (see above table), based on four doubling dilutions above and below the concentrations within the drinks selected. Total growth was assessed by reading the absorbance at 595 nm. 120 ul of the planktonic cells were transferred to another plate and read at 595 nm. The remaining biofilm was washed twice with saline, fixed with 10% formaldehyde (Sigma) for 30 min, washed twice again with saline, and stained with 0.5% crystal violet (Sigma) for 30 min[26]. After washing the biofilm three times with saline, the crystal violet was extracted from the biofilm cells with 200 ul of 2-propanol (Fisher Scientific Co., Fair Lawn, NJ) for 1 h. The extract was diluted 1:5 with 2-propanol and read at 490 nm with 2-propanol used as a blank control. Controls included a sterility control consisting of TSB without bacteria, TSB plus bacteria alone as a negative control, and TSB containing 0.12% chlorhexidine plus bacteria as a positive control.

Biofilm metabolic activity

S. mutans biofilm metabolic activity was measured by a method described by Pierce et al. [8] originally developed for *Candida albicans*, but adapted by this lab for *S. mutans*[26]. The assay is based on biofilm cells reducing 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) to a water-soluble organic compound in the presence of menadione[26]. *S. mutans* biofilm prepared as above was grown in TSB without the drinks, in 96-well-plates for 16 h to establish the biofilm, followed by another 16 h growth in TSB supplemented with various concentrations of the drinks, caffeine, and HFCS (same as above for biofilm formation). Fresh XTT menadione media was prepared according to Pierce et al. [8], biofilm was washed twice with saline, 200 ul of XTT reagent added and the plate incubated in the dark with 5% CO_2 at 37°C for 2 h. After incubation, the XTT reagent from each well was transferred to another 96-well-plate to detect the color change by reading at 490 nm. In the meantime, the relative biofilm mass of identically prepared wells was detected by the crystal violet assay described above.

Statistical analyses

Each experiment for the individual beverages, caffeine, and HFCS was repeated three times. Summary statistics (mean, standard deviation, standard error of the mean, range) were calculated for each beverage at each dilution for biofilm mass, biofilm metabolic activity, and the ratio of specific biofilm metabolic activity/mass. 1-way ANOVA and Fisher's Protected Least Significant Difference multiple comparison tests were performed to compare the groups. A 5% significance level was used for all tests. The distribution of the data was examined, and a rank transformation was used.

Based on the results of previous studies, the expected within-group standard deviations for biofilm mass, biofilm metabolic activity, and the activity/mass ratio were 0.03, 0.01, and 0.03, respectively. Therefore, this study was designed to have 80% power to detect biofilm mass, biofilm metabolic activity, and activity/mass ratio difference of 0.09, 0.03 and 0.09, respectively, between any two beverages or a beverage and the negative control, assuming a non-significant drink-by-dilution interaction.

RESULTS

Measurement of Total Absorbance

Prior to removing the planktonic bacterial cells to measure the biofilm formation, the total absorbance of each well was measured. Pure caffeine and pure HFCS both demonstrated significantly greater total absorbance compared to the *S. mutans* control (Figure 1). On the other hand, Diet Coke, as well as Caffeine Free Diet Coke and Caffeine Free Coke Zero demonstrated significantly less biofilm formation compared to the control (Figure 1).

Measurement of Biofilm Formation

The results of the experiment demonstrated that all products that contain HFCS enhance total growth of the biofilm (Figure 2). Both of the HFCS-containing products demonstrated significantly higher ($p \leq 0.05$) biofilm mass than the bacterial control. Pure HFCS also demonstrated significantly higher biofilm mass compared to the control. When comparing specific beverages, Coca-Cola demonstrated significantly higher biofilm formation than Diet Coke, Caffeine-Free Diet Coke, and Caffeine-Free Coke Zero. The only sugar-free beverage that did not exhibit significantly higher biofilm mass than Coca-Cola was Coke Zero.

In terms of caffeine and biofilm formation, the results were not as conclusive, but caffeine does appear to increase biofilm formation. Coke Zero had a significantly greater biofilm formation than Caffeine-Free Coke Zero. However, no other pair of caffeinated and non-caffeinated beverages displayed this effect. Caffeine-Free Diet Coke did have significantly less biofilm formation than the control, but other than Coca-Cola, none of the caffeinated beverages displayed significantly greater biofilm formation than the control. Also, pure caffeine did not significantly increase biofilm formation.

Measurement of Planktonic Bacteria

After measuring the total absorbance, prior to measuring biofilm formation, the planktonic bacterial cells were removed from above the biofilm and measured. Only pure caffeine and pure HFCS demonstrated a higher concentration of planktonic bacteria as compared to the bacterial control (results not shown). This correlates well with our findings of caffeine having significantly greater total absorbance, though not having significantly greater biofilm formation.

Measurement of Metabolic Activity

HFCS displayed a significant impact on the metabolism of *S. mutans* biofilm metabolic activity. Both Coca-Cola and Caffeine-Free Coke, the only two sugar-containing beverages studied, demonstrated a significantly greater amount of metabolic activity compared to the bacteria control (Figure 3). In addition, pure HFCS exhibited significantly greater activity than the control. In comparing the beverages, both Coca-Cola and Caffeine Free Coke indicated a significantly greater amount of metabolic activity as compared to all of the other beverages, none of which contain HFCS (Figure 3).

As for caffeine, the relationship, as it did with biofilm growth, appears to have a positive impact on the magnitude of metabolic activity of *S. mutans* biofilm, though not as convincing as HFCS. In this case, pure caffeine displayed significantly higher metabolic activity as compared to the control. Diet Coke demonstrated a greater amount of metabolic activity than the two non-caffeinated sugar-free beverages (Caffeine-Free Diet Coke and Caffeine-Free Coke

Figure 1. Total absorbance of *S. mutans* grown in TSB containing various concentrations of multiple beverages, pure caffeine, and pure HFCS measured at 595 nm. Each bar represents the absorbance of a specific sample, compared to an *S. mutans* bacterial control. Asterisks represent values that are significantly greater or lesser than the bacterial control ($p < 0.05$). Caffeine and pure HFCS displayed significantly greater total absorbance at both concentrations as compared to the control, whereas Caffeine-Free Coke Zero, Caffeine-Free Diet Coke, and Diet Coke displayed significantly less as compared to the bacterial control.

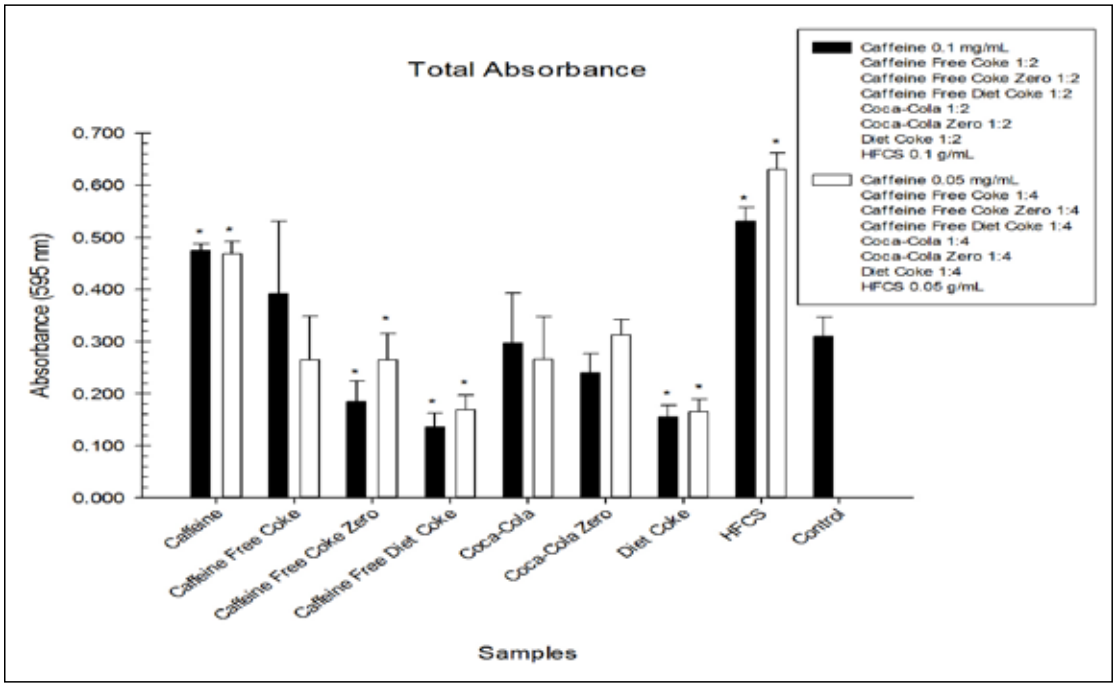


Figure 2. Effect of various concentrations of cola-flavored soft drinks, pure caffeine, and pure HFCS on overall *S. mutans* biofilm growth. Single asterisks indicate samples that were significantly greater than an *S. mutans* bacterial control. ** indicates samples that exhibited significantly less growth as compared to the control. @ indicates samples that displayed significantly less growth than Coca-Cola. $ indicates that Caffeine-Free Coke Zero displayed significantly less biofilm formation as compared to Coke Zero.

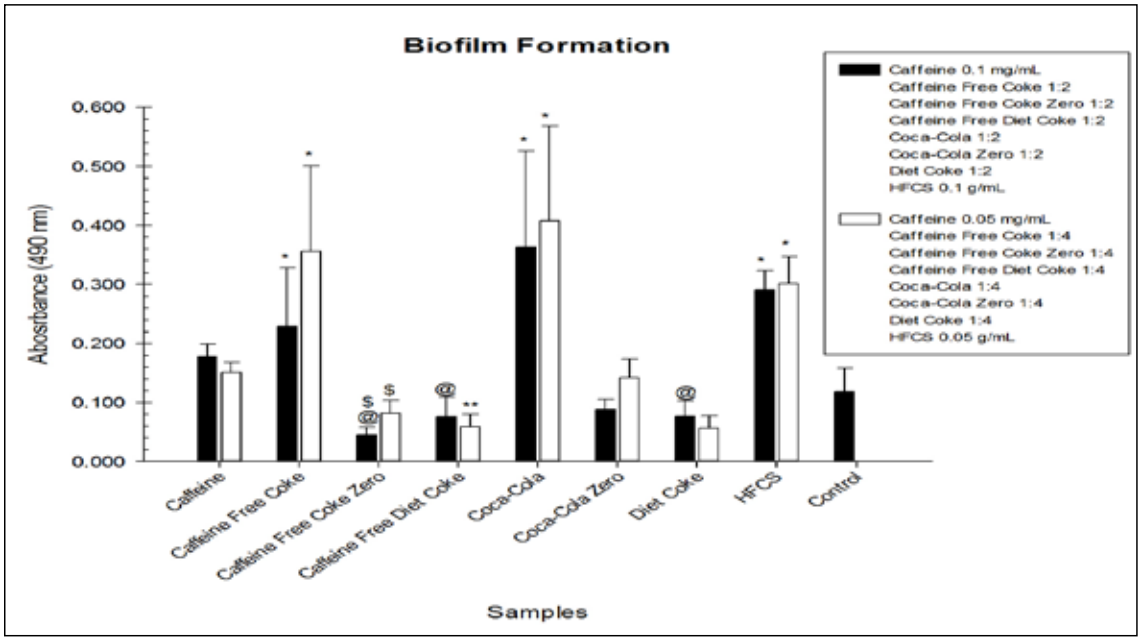
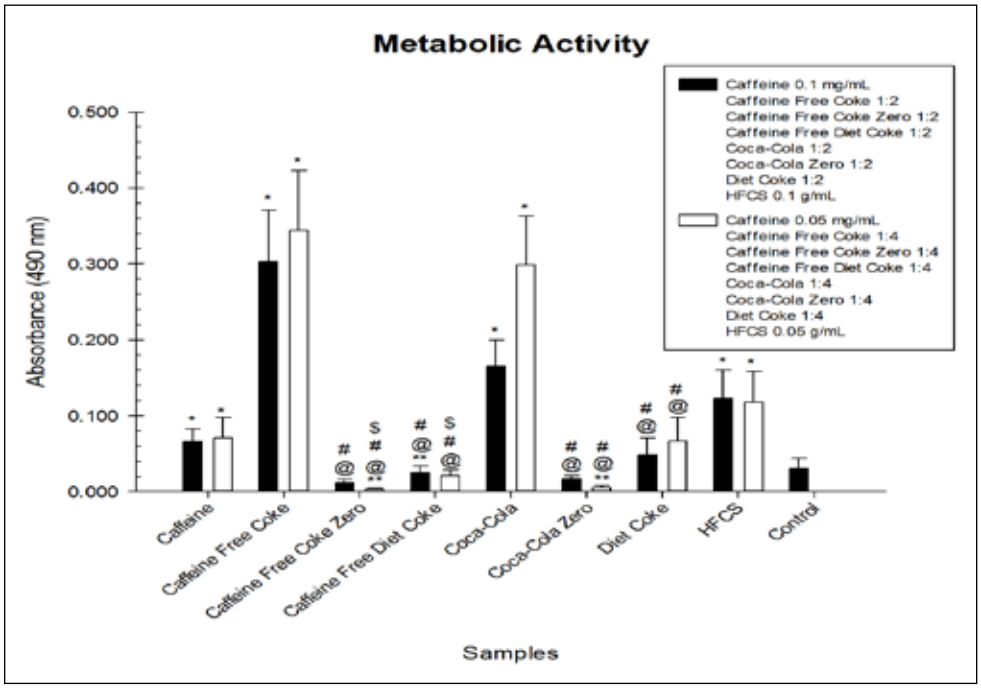


Figure 3. Effect of different cola-flavored soft-drinks, pure caffeine, and pure HFCS on *S. mutans* biofilm metabolic activity. Single asterisks indicate samples that were significantly greater than an *S. mutans* bacterial control's metabolic activity. ** indicate samples that displayed significantly less metabolic activity as compared to the control. @ indicates samples that displayed significantly less metabolism than Coca-Cola. # indicates samples that displayed significantly less metabolism than Caffeine-Free Coke. $ indicates caffeine-free samples that displayed significantly less biofilm formation compared to Diet Coke.



Zero), which would suggest that the presence of caffeine in Diet Coke significantly increases biofilm metabolic activity. However, neither Diet Coke, nor Coke Zero provided a significantly greater amount of metabolism as compared to the bacterial control. Coke Zero, which contains caffeine, displayed significantly less metabolic activity compared to the control.

DISCUSSION

There have been numerous studies that have indicated the effects of sweetened beverages and specifically sodas on caries risk and development[5, 14, 15]. In this study, it was our aim to examine HFCS and caffeine, two of the most prevalent ingredients that are present in many soft drinks. Because caries formation occurs through the activity of *S. mutans* biofilm[1,2] and because *S. mutans* uses fermentable carbohydrates as its energy source[10,11], this study was designed to examine the role of both HFCS (a fermentable carbohydrate) and caffeine (because of its prevalence in soft drinks) on their role in *S. mutans* biofilm formation.

The study confirms our hypothesis that HFCS increases both biofilm formation and metabolic activity. This hypothesis was raised due to the fact that fermentable carbohydrates have been shown to increase caries activity in teeth[5,14,15]. Both of the samples in the current study that contained HFCS, as well as dilutions of HFCS, displayed significantly greater amounts of both biofilm formation and metabolic activity.

Caffeine's relationship with biofilm formation and metabolism, however, is not as straightforward. Neither pure caffeine nor any of the caffeinated beverages displayed significantly greater biofilm formation than the control. Though pure caffeine displayed greater metabolic activity than the control, none of the caffeinated beverages (other than Coca-Cola, which also contains HFCS) did. In one case, a caffeinated sample (Coke Zero) demonstrated a higher biofilm formation than its caffeine-free counterpart, and in another, a caffeinated sample (Diet Coke) exhibited significantly higher metabolism than both of the caffeine-free, sugar free variants. These examples suggest that caffeine plays a role in increasing biofilm formation and metabolic activity, though it is not as convincing as the role of HFCS. In either case, it does discount the hypothesis that caffeine has an inhibitory effect.

When it comes to beverage selection, parents and practitioners should be aware of the risks of soda consumption. The HFCS present in many soft drinks leads to systemic and dental problems, including promotion of cariogenic dental biofilm formation through the growth and metabolism of *S. mutans*. Caffeine is not only a psychoactive substance, but also appears to have a contributory effect on the growth and metabolism of *S. mutans* biofilm. Thus, due to the numerous potential problems associated with cola beverages, both systemically and orally, it should be recommended that children avoid such beverages entirely. However, we conclude that if carbonated cola beverages are to be consumed, it is best to choose sugar-free and caffeine-free choices. HFCS increases both the biofilm formation and metabolism of *S. mutans*, and caffeine is shown to, in some cases, increase metabolism of *S. mutans*.

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