An In Vitro Study of the Effects of Sports and Energy Drinks on *Streptococcus mutans* Biofilm Formation and Metabolic Activity

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ABSTRACT

Purpose: Sports and energy drinks are being increasingly consumed and contain large amounts of sugars, which are known to increase *Streptococcus mutans* biofilm formation and metabolic activity. The purpose of this in vitro study was to investigate the effects of sports and energy drinks on *Streptococcus mutans* biofilm formation and metabolic activity.

Methods: *S mutans* UA159 was cultured with and without a dilution (one to three ratio) of a variety of sports and energy drinks in bacterial media for 24 hours. The biofilm was washed, fixed, and stained. Biofilm growth was evaluated by reading absorbance of the crystal violet. Biofilm metabolic activity was measured by the biofilm reducing XTT to a water-soluble orange compound.

Results: Gatorade Protein Recovery Shake and Starbucks Doubleshot Espresso Energy were found to significantly increase biofilm ($P \leq 0.05$) and metabolic activity by between 22 to 30 fold

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and two to three fold, respectively. However, most of the remaining drinks significantly inhibited biofilm growth and metabolic activity.

Conclusions: Several sports and energy drinks, with sugars or sugar substitutes as their main ingredients, were demonstrated to inhibit *Streptococcus mutans* biofilm formation. Among the drinks evaluated, Gatorade Protein Recovery Chocolate Shake and Starbucks Doubleshot Energy appear to have cariogenic potential, since they increased the biofilm formation and metabolic activity of *S mutans.*

KEYWORDS: SPORTS DRINKS, ENERGY DRINKS, *STREPTOCOCCUS MUTANS*, BIOFILM

Sports drinks have traditionally been marketed, since the 1960s, to improve athletic performance, due to their high content of carbohydrates and electrolytes. Energy drinks, containing large amounts of carbohydrates in the form of high fructose corn syrup, sucrose, or sucralose, were introduced in the United States around 2007 and appealed to consumers who needed a boost in energy, concentration, athletic performance, and/or metabolism.[1-3] Since that time, the utilization of sports and energy drinks has skyrocketed. Thirty to fifty percent of children and young adults in the United States consume energy drinks on a regular basis, and 51 to 62 percent of this population drink at least one sports drink each day,[1,4] with half of this population consisting of children younger than 12 years of age.[4] O'Dea [5] reported that 56.4 percent of adolescents used sports drinks and 42.3 percent used energy drinks during a specific two-week period prior to the study.

These trends show no sign of shifting. Energy drinks were the fastest-growing beverage market in the United States in the 2000's..[2] The American Academy of Pediatrics (**AAP**) has stated that sports and energy drinks are being inappropriately marketed to children and young adults.[6] Marketing for sports and energy drinks has been geared toward athletic children to optimize athletic performance, increase energy, and improve concentration.

Carbohydrates serve as the primary energy source for bacteria in dental plaque, which increases the cariogenic potential of the oral flora.[7] A previous study has shown a direct correlation between the consumption of sucrose and the frequency of dental caries.[8] Huumonen et al. [9] found that rats fed a 43 percent sucrose diet had considerably larger carious lesions in the first molars than those fed 15 or 30 percent sucrose diets. A systematic review of caries risk and

sucrose consumption found that 16 of the 36 papers studied indicated a moderate relationship between sucrose consumption and caries development, even with fluoride exposure.[10]

Carbohydrates have also been described as the main contributor of biochemical and physiological changes in dental biofilms. After ingesting fructose, sucrose, or glucose, oral biofilm pH falls rapidly. Most carbohydrate-electrolyte sports drinks have pH levels below the critical pH of 5.5 for enamel demineralization, with most ranging from 3.16 to 3.70.[11,12] This alteration in the environment triggers a change in the plaque microflora, promoting the appearance of cariogenic bacteria and dental demineralization, leading to dental caries.[13] Consequently, the acidic nature of these beverages and their potential to erode enamel should be of great concern to both dental practitioners and their consumers.[14]

Streptococcus mutans is one of the most cariogenic bacterium in the oral cavity.[15] *S mutans* lives, multiplies, and competes for nutrients in dental biofilm and metabolizes sucrose and glucose into insoluble glucan and large amounts of lactic acid. This lowers the pH of the environment, creating the risk for carious lesions to develop.[16] Direct relationships between *S mutans* and foods or beverages containing sucrose have been observed.[17] Consumption of such carbohydrates over a long period of time can increase the number of *S. mutans* in the oral cavity.[13]

Sucrose is viewed as the most cariogenic dietary carbohydrate due to its ability to serve as a substrate for production of bacterial polysaccharides and organic acids in dental plaque. Extracellular polysaccharides, such as *S mutans* glucan, support bacterial adherence to tooth surfaces, contribute to integrity of dental biofilms, and promote lower fasting pH levels. The addition of sucrose to biofilms causes lower pH levels, increased *S mutans* numbers, and increased cariogenicity over biofilms formed in the absence of sucrose.[13,18] Therefore, the presence of large amounts of sucrose in sports and energy drinks is concerning. Many sports and energy drinks are sucrose-based, while others contain high fructose corn syrup (**HFCS**) or sucralose. Due to the large amounts of sucrose and fructose in these drinks, research on this subject is imperative to counsel patients on the caries risk associated with these beverages. Determining the effect of these beverages on *S. mutans* biofilm formation and metabolic activity will help health care providers educate parents about how the consumption of sports and energy beverages relates to caries potential.

The purpose of this study was to evaluate if *S. mutans* in vitro biofilm formation and metabolic activity are affected by the presence of sports and energy drinks.

METHODS

BACTERIAL STRAIN, MEDIA, DRINKS, AND CHEMICALS

S. mutans strain UA159 (ATCC 700610) was used in the present study. The strain was stored at negative 80 degrees Celsius in tryptic soy broth (**TSB**, Acumedia, Baltimore, Md., USA) containing 20 percent glycerol before use. mitis salivarius sucrose bacitracin (**MSSB**, Anaerobe Systems, Morgan Hill, Calif., USA) agar plates were used to initially grow the bacterium.[19] Unless otherwise stated, TSB was used and the bacteria were grown in five percent CO_2 at 37 degrees Celsius. The sports and energy drinks were purchased from a local grocery store. The ingredients and sweetener in each drink are indicated in Table 1.. Pure HFCS (Food Club, Topco Associates, Skokie, Ill., USA) was obtained from a local grocery store, sucrose was obtained from Fisher Scientific (Fair Lawn, N.J., USA), and sucralose was obtained from Sigma Chemical Company (St. Louis, Mo., USA). The initial pH values of each undiluted drink and drink dilutions (at a ratio of one to three) in TSB were measured (Table 2).

BIOFILM FORMATION

To determine biofilm formation, an overnight *S mutans* culture in TSB was treated with various concentrations of the drinks and HFCS, sucrose, and sucralose. A one-to-three ratio of drink to TSB medium was used based on the dilution effect of saliva on drinks consumed.[20] The drinks (diluted at a ratio of one to three; 190 µL) and 10 µL of the overnight culture of *S mutans* (approximately 10^6 colony forming units per ml) were added and incubated for 16 hours at 37 degrees Celsius in sterile 96-well microtiter plates. The biofilm that formed in the wells of the microtiter plate was washed twice with saline, fixed with 10 percent formaldehyde (Sigma) for 30 minutes, washed twice again with saline, and stained with 0.5 percent crystal violet for 30 minutes. After washing the biofilm three times with saline, crystal violet was extracted from the biofilm cells by 200 µL of 2-propanol (Fisher Scientific) for ONE hour. The extract was diluted (at a one-to-five ratio) with 2-propanol and read at 490 nm using a microplate spectrophotometer with 2-propanol as a blank control.[19] Controls included various concentrations (0.67, 1.25, 2.5, five, and 10 percent) of HFCS, sucrose, and sucralose added to TSB and bacteria, TSB without

bacteria, and TSB and 0.12 percent chlorhexidine and bacteria, since all but one of the test beverages contained HFCS, sucrose, or sucralose.

BIOFILM METABOLIC ACTIVITY

S mutans biofilm metabolic activity was measured by a method described by Pierce et al.,[21] which was originally based on yeast 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 and adapted for use with *S mutans.*[19,22] *S mutans* biofilm was grown in TSB without the drinks in 96-well microtiter plates for 16 hours to establish the biofilm, followed by another 16 hours of growth in TSB supplemented with each of the drinks diluted at a ratio of one to three in TSB. Fresh XTT solution containing menadione was prepared, biofilm was washed twice with saline, XTT/menadione reagent was added, and the plate was incubated in the dark in five percent CO_2 at 37 degrees Celsius for two hours. After incubation, the XTT reagent was transferred to another 96-well-plate to detect the color change by measurement at 490 nm.

STATISTICAL METHODS

Each experiment was repeated three times. Biofilm and XTT data were represented as the ratio to the TSB control. Comparisons against the TSB control were made by determining if the ratio to TSB was significantly different from one (equal means for the groups and the TSB control are represented by a ratio equal to one). No significant effect is represented by a ratio of one. Statistical comparisons (using SPSS 24.0 software for Windows, IBM Corp., Armonk, N.Y., USA) among drinks for differences in biofilm and XTT data were made using one-way analysis of variance. Tukey's multiple comparisons procedure was used to control the overall significance level at five percent. Analyses were performed after a natural logarithm transformation of the data due to the non-normal distribution of the data.

RESULTS

In general, the sports drinks had higher initial pH values than the energy drinks, even when they were diluted at a one-to-three ratio in TSB (Table 2). Most of the beverages significantly inhibited *S mutans* biofilm formation (Figures 1 and 2). The positive control (0.12 percent

chlorhexidine) totally inhibited *S mutans* biofilm formation and metabolic activity. However, Gatorade Protein Recovery Chocolate Shake (sports drink category) and Starbucks Doubleshot Energy (energy drink category) were the only drinks in each category to demonstrate statistically significant increases ($P \leq 0.05$) in *S mutans* biofilm formation when compared to the TSB control. Most of the other beverages had ratios of approximately or less than one. Gatorade Protein Recovery Shake had an approximate 30-fold increase, and Starbucks Doubleshot Energy had an approximate 22-fold increase compared to the TSB control. Other drinks produced significantly less ($P \leq 0.05$) biofilm than the control.

The different sweeteners in the drinks were assayed for their ability to affect *S mutans* biofilm formation. As sucrose concentrations increased (from 0.67 percent to 10 percent), there was an overall increasing trend in biofilm formation (Figure 3). Sucralose exhibited an overall significant decrease in biofilm formation from 0.67 percent to 10 percent ($P \leq 0.05$). HFCS enhanced biofilm formation, but this was not statistically significant.

Gatorade Protein Recovery Chocolate Shake and Starbucks Doubleshot Energy also significantly enhanced ($P \leq 0.05$) metabolic activity (approximately two- and three-fold increases, respectively; Figures 4 and 5), while all other beverages significantly inhibited *S. mutans* metabolism. Sucrose, HFCS, and sucralose at all concentrations demonstrated increased metabolic activity and exhibited an overall trend for enhancing metabolic activity as their concentrations increased (Figure 6).

DISCUSSION

Sports and energy drinks contain large amounts of carbohydrates and sugars (up to 58 g per 12 ounces). These sugars are in the form of fermentable sucrose, sucralose, or HFCS. Dental plaque microorganisms metabolize carbohydrates to use as an energy source, increasing *S. mutans* metabolic activity in the oral cavity and the cariogenic potential of these organisms. In this study, we concluded the sports and energy drinks tested in general inhibit biofilm formation and metabolic activity of *S. mutans*, except for Gatorade Protein Recovery Chocolate Shake and Starbucks Doubleshot Energy drink.

These two drinks, both containing sucrose and cow's milk, enhanced biofilm formation and metabolic activity under the conditions used in these experiments. Although much controversy exists on whether or not milk is cariogenic, the results of the present study are

comparable to recent reports that indicate the addition of an external source of carbohydrates, such as sucrose to milk, increases its cariogenic potential and the extent of caries progression into dentin.[23] In addition to that, we recently reported that human breast milk alone can increase *S. mutans* biofilm formation, which is most likely due to the effect of lactose on biofilm. Others have indicated increased biofilm formation of *S. mutans* by increasing sucrose concentrations.[24]

Sports and energy drinks have numerous ingredients, and it is highly likely that a common ingredient found in all these drinks may help inhibit the biofilm formation and metabolic activity. It is speculated the vitamin content in the sports drinks and the caffeine content in energy drinks may inhibit biofilm formation and metabolic activity. Coffee has been reported to inhibit bacterial growth, and caffeine increases the antibacterial activity .[25,26] Specifically, caffeine significantly inhibits *S. mutans* biofilm formation and metabolic activity.[27] More research should also be done on the various ingredients in these drinks to isolate which one specifically may inhibit biofilm formation and metabolic activity. Nonetheless, adequate alternatives to sports and energy drink consumption should be encouraged by oral health care providers when providing nutritional counseling to their patients.

CONCLUSIONS

Based on the results of this study, the following conclusions can be made:

1. Various sports and energy drinks, with different types of sugars or sugar substitutes as their main ingredients, were demonstrated to inhibit biofilm formation and metabolic activity of *S. mutans*.
2. Gatorade Protein Recovery Chocolate Shake and Starbucks Doubleshot Energy, both containing cow's milk and sucrose, appear to have cariogenic potential since they both increased the biofilm formation and metabolic activity of *S mutans*.

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Table 1. Composition of Sports and Energy Drinks

Sports drinks	**Ingredients**	**Sugars (g)**	**Carbs (g)**
Gatorade Series O1 Prime	Water, isomaltulose, sucrose, dextrose, maltodextrin, citric acid, natural and artificial flavor, xanthan gum, salt, sodium citrate, monopotassium phosphate, niacinamide (vitamin B3), calcium pantothenate (vitamin B5), blue1, pyridoxine hydrochloride (vitamin B6), red 40	23	25
Gatorade Thirst Quencher– Original	Water, sucrose, dextrose, citric acid, natural flavor, salt, sodium citrate, monopotassium phosphate, gum Arabic, yellow 6, glycerol, ester of rosin, brominated vegetable oil	14	14
Gatorade G2 Low Calorie	Water, sucrose, citric acid, salt, sodium citrate, natural and artificial flavor, monopotassium phosphate, sucralose, acesulfame potassium, red 40, blue 1	5	5
Gatorade Protein Recovery Shake (Chocolate)	Water, maltodextrin, milk protein concentrate, sucrose, whey protein concentrate, dextrose, cocoa (processed with Alkali), natural and artificial flavor, cellulose gum and gel, dipotassium phosphate, salt, mono and diglycerides, carrageenan, sucralose	20	45
Powerade	Water, high fructose corn syrup, less than 0.5% of: citric acid, salt and potassium citrate and magnesium chloride and calcium chloride and potassium phosphate, gum acacia, natural flavors, glycerol, ester of rosin, yellow no. 5, yellow no. 6, vitamin B3 (niacinamide), vitamin B6 (pyridoxine hydrochloride), vitamin B12	14	14
Powerade Zero	Water, less than 1% of citric acid, salt and potassium citrate and magnesium chloride and calcium chloride and potassium phosphate (electrolyte sources), natural flavors, sucralose, acesulfame potassium, vitamin B3, vitamin B6, vitamin B12, red 41, blue 1	0	0

All Sport Body Quencher	Water, high fructose corn syrup, citric acid, monopotassium phosphate, sodium chloride, gum arabic, ascorbic acid, phosphoric acid, calcium chloride, yellow 6, natural and artificial flavors, ester gum	16	16
All Sport Body Zero	Water, citric acid, phosphoric acid, monopotassium phosphate, sodium chloride, ascorbic acid, gum arabic, natural flavors, sucralose, calcium chloride, rebiana, yellow 6	0	0
Energy drinks			
Red Bull Total Zero	Carbonated water, taurine, citric acid, sodium citrate, caffeine, glucuronolactone, aspartame, sucralose, acesulfame k, inositol niacinamide, calcium pantothenate, pyridoxine HCL, vitamin B12, xantham gum, natural and artificial flavors, colors; contains phenylalanine	0	0
Red Bull	Carbonated water, sucrose, glucose, citric acid, taurine, sodium citrate, magnesium carbonate, caffeine, glucuronolactone, inositol, niacinamide, calcium pantothenate, pyridoxine HCL, vitamin B12, natural and artificial flavors and colors	27	28
Monster	Carbonated water, sucrose, glucose, citric acid, natural flavors, taurine, sodium citrate, color added, panex ginseng root extract, l carnitine l-tartrate, caffeine, sorbic acid, benzoic acid, niacinamide, sodium chloride, D-glucuronolactone, inositol, guanine seed extract, pyridoxine HCL, sucralose, riboflavin, maltodextrin, cyanocobalamin	27	27
Rockstar	Triple-filtered carbonated water, sucrose, glucose, citric acid, taurine, natural and artificial flavors, sodium citrate, caffeine, caramel color, benzoic acid, sorbic acid, l-carnitine, inositol, niacinamide, calcium pantothenate, milk thistle extract, guarana seed extract, panex ginseng root extract, riboflavin, pyridoxine hydrochloride, cyanocobalamin	31	31

Rockstar Sugar Free	Triple-filtered carbonated water, citric acid, taurine, natural and artificial flavors, sodium citrate, caffeine, caramel color, benzoic acid, sorbic acid, acesulfame potassium, sucralose, l-carnitine, inositol, niacinamide, calcium pantothenate, milk thistle extract, guarana seed extract, panaz ginseng root extract, riboflavin, pyridoxine hydrochloride, cyanocobalamin	0	0
Full Throttle	Carbonated water, high fructose corn syrup, citric acid, sugar, natural and artificial flavors, sodium citrate, sodium benzoate, D-ribose, caffeine, acacia, niacinamide, calcium pantothenate, glycerol ester of rosin, yellow 5, pyridoxine hydrochloride, cyanocobalamin	58	58
AmP	Carbonated water, high fructose corn syrup, citric acid, orange juice concentrate, natural flavors, guarana seed extract, sodium benzoate, sodium hexametaphosphate, caffeine, gum arabic, niacinamide, ascorbic acid, taurine, calcium dissodium EDTA, panex ginseng root extract, riboflavin, calcium pantothenate, brominated vegetable oil, yellow no. 5, pyridoxine HCL, cyanocobalamin, blue no. 1	29	29
5 Hour Energy	Taurine, glucuronic acid, malic acid, N-acetyl l-tyrosine, l-phenylalanine, caffeine, citicoline, purified water, natural and artificial flavors, sucralose, potassium sorbate, sodium benzoate EDTA	0	0
Starbucks Doubleshot Energy (Vanilla)	Starbucks coffee (water, coffee), reduced-fat milk, skim milk, sugar, maltodextrin, dextrose, taurine, cellulose gel, natural flavor, panax ginseng root extract, inositol, sodium ascorbate, guarana seed extract, cellulose gum, niacinamide, sucralose, ascorbic acid, tricalcium phosphate, pyroxidine hydrochloride, riboflavin, vitamin a palmitate, vitamin D3	25	34
Venom	Carbonated water, corn syrup, glucose, citric acid, maltodextrin, taurine, sodium citrate, l-carnitine, inositol, caffeine, sodium benzoate, caramel color, potassium sorbate,	53	57

	natural and artificial flavors, niacinamide, sucralose, ginseng extract, pyridoxine hydrochloride, cyanocobalamin		

Table 2. pH Values of Sports and Energy Drinks

Sports drinks	**Undiluted**	**1:3 dilution in tryptic soy broth**
Gatorade		
Original Fruit Punch	2.85	6.51
Original Low Calorie	2.87	6.62
Pro Prime	2.53	5.39
Protein Recovery Shake	7.06	7.27
Original Cool Blue	2.71	6.3
Powerade		
Fruit Punch	3.38	6.36
Mountain Berry Blast	2.51	6.64
Zero Calorie - Fruit Punch	3.1	6.67
AllSport		
Fruit Punch	3.25	6.56
Razz Ice	2.61	6.71
Energy drinks		
Monster	3.27	5.32
AMP	2.39	5.9
5 Hour Energy	2.45	4.13
Full Throttle	2.73	5.85
Red Bull	3.19	5.3
Red Bull Zero	2.75	6.05
Venom	2.98	5.3
Rockstar	2.45	5.19
Rockstar Zero	2.83	5.34
Starbuck's Double Shot Espresso	7.28	7.31

Figure 1. Biofilm formation of *Streptococcus mutans* UA159 after 24 hours of treatment with dilutions (at a one to three ratio) of various sports drinks in tryptic soy broth (TSB). The ratio of the absorbance (OD equals 490 nm) of crystal violet-stained *S mutans* biofilm treated with different sports drinks (at a dilution ratio of one to three in TSB), compared to the TSB control without any sports drinks, is shown with mean and standard error of the mean. The experiment was repeated three times, and asterisks indicate significant differences compared with the TSB control without any sports drinks (at a ratio of one). The horizontal line depicts a ratio of one.

Figure 2. Biofilm formation of *Streptococcus mutans* UA159 after 24 hours of treatment with dilutions (at a ratio of one to three) of various energy drinks in tryptic soy broth (TSB). The ratio of the absorbance (OD equals 490 nm) of crystal violet-stained *S mutans* biofilm treated with different energy drinks (at a dilution ratio of one to three in TSB), compared to the TSB control without any energy drinks, is shown with mean and standard error of the mean. The experiment was repeated three times, and asterisks indicate significant differences compared with the TSB control without any energy drinks (at a ratio of one). The horizontal line depicts a ratio of one.

Figure 3. Biofilm formation of *Streptococcus mutans* UA159 after 24 hours of treatment with different concentrations of high fructose corn syrup, sucrose, and sucralose (0.67, 1.25, 2.5, five, and 10 percent) in tryptic soy broth (TSB). The ratio of the absorbance (OD equals 490 nm) of crystal violet-stained *S mutans* biofilm treated with the different sweeteners, compared to the TSB control without any sweeteners, is shown with mean and standard error of the mean. The experiment was repeated three times, and asterisks indicate significant differences compared with the TSB control without any sweetener (at a ratio of one). The horizontal line depicts a ratio of one.

Figure 4. Metabolic activity of established *Streptococcus mutans* UA159 biofilm after 24 hours of treatment with dilutions (at a ratio of one to three) of various sports drinks in tryptic soy broth (TSB) using an XTT/menadione assay. The ratio of the absorbance (OD equals 490 nm) reflecting the metabolic activity of *S mutans* biofilm cells treated with different sports drinks (at a dilution ratio of one to three in TSB), compared to the TSB control without any sports drinks, is shown with mean and standard error of the mean. The experiment was repeated three times,

and asterisks indicate significant differences compared with the TSB control without any sports drinks (at a ratio of one). The horizontal line depicts a ratio of one.

Figure 5. Metabolic activity of established *Streptococcus mutans* UA159 biofilm after 24 hours of treatment with dilutions (at a ratio of one to three) of various energy drinks in tryptic soy broth (TSB) using an XTT/menadione assay. The ratio of the absorbance (OD equals 490 nm) reflecting the metabolic activity of *S mutans* biofilm cells treated with different energy drinks (at a dilution ratio of one to three in TSB), compared to the TSB control without any energy drinks, is shown with mean and standard error of the mean. The experiment was repeated three times, and asterisks indicate significant differences compared with the TSB control without any energy drinks (at a ratio of one). The horizontal line depicts a ratio of one.

Figure 6. Metabolic activity of established *Streptococcus mutans* UA159 biofilm after 24 hours of treatment with different concentrations of high fructose corn syrup, sucrose, and sucralose (0.67, 1.25, 2.5, five, and 10 percent) in tryptic soy broth (TSB) using an XTT/menadione assay. The ratio of the absorbance (OD equals 490 nm), reflecting the metabolic activity of *S mutans* biofilm cells treated with different sweeteners compared to the TSB control without any sweeteners, is shown with mean and standard error of the mean. The experiment was repeated three times, and asterisks indicate significant differences compared with the TSB control without any sweeteners (at a ratio of one). The horizontal line depicts a ratio of one.