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Beverages Containing Plant-Derived Polyphenols Inhibit Growth and Biofilm Formation of *Streptococcus mutans* and Children's Supragingival Plaque Bacteria

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Abstract: Objectives: Polyphenols in edible berries and tea plant (*Camellia sinensis*) suppressed virulence factors of oral pathogens. We investigated if the commercially marketed plant polyphenols-containing beverages inhibited growth and biofilm formation of *Streptococcus mutans* and children's dental plaque. Methods: Supragingival plaque collected from 16 children (7–11 years) were suspended in TSB for testing. Test beverages included 26 marketed packaged teas, ready-to-drink bottled raspberry flavored teas and cranberry juice cocktails with and without added sugars. Their effects on in vitro growth and biofilm formation of *S. mutans* and children's plaque bacteria were determined after 24–48 h at 37 °C anaerobically in CDM with or without sucrose. Results: Brewed infusions from black, green and cinnamon or raspberry flavored teas bags inhibited growth and biofilm formation of children's plaque bacteria. Compared to controls, bottled raspberry flavored teas and cranberry juice cocktails significantly inhibited growth and biofilm formation of test bacteria. Added sugar did not significantly impact the inhibition ($p > 0.05$). Biofilms formed in these beverages were loosely attached and easily dislodged from surfaces. Conclusions: Beverages rich in antimicrobial plant polyphenols reduce plaque adherence, may benefit oral health and are preferred over other sugary beverages. The concept of oral diseases prevention using natural foods/diet is innovative, practical and acceptable.

Keywords: natural anticariogenic agents; children's dental plaque biofilm; beverages with plant polyphenols; caries prevention in children; diet and caries management; *Streptococcus mutans*; healthy beverages; beverage and oral health

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1. Introduction

Despite the introduction of fluoride, dental caries remains one of the most prevalent chronic diseases in children and adults in the United States [1]. Next to the common cold, dental caries is the major cause of days lost from work and school, resulting in a major impact on economic productivity and the education of American children. Dental caries is a multifactorial disease that involves host defense, dietary carbohydrates, dental plaque bacteria and the duration of bacterial acid challenge of the enamel [2,3]. Dental plaque has been implicated as the prime etiologic factor for dental caries. It is a complex microbial biofilm community consisting of more than 900 microorganisms adherent to tooth surfaces. Plaque bacteria, especially *Streptococcus mutans*, ferment dietary carbohydrates to organic acid, which can demineralize the tooth enamel, leading to dental caries [4,5]. In addition, the acid-tolerant *S. mutans* also possesses glucosyltransferases (GTFs), which hydrolyze sucrose and synthesize water-insoluble sticky extracellular polysaccharides, contributing to the architecture and adhesive nature of dental plaque biofilm. Over the years, many attempts have been made to seek effective means to prevent or reduce acid production and the adherence of plaque biofilm for the prevention and control of dental caries [6]. *S. mutans* has often been used as the indicator oral bacterial species for the evaluation of biofilm inhibitors.

Diet, food and nutrition play important roles in a person's overall well-being. In recent decades, advances in research and education have facilitated the identification and development of food products with disease-preventing and health-promoting benefits beyond their traditional nutritional value, i.e., the "functional foods". Although research has shown that poor oral health may increase a person's risk for systemic diseases [7], less attention has been focused on foods and oral health. Diet, especially sugars, play an important role in influencing the caries experience. While most diet or food components especially sugars, have traditionally been viewed from a caries causing standpoint, various research groups have reported their caries-protective properties targeting cariogenic virulence factors such as biofilm adhesion, growth, metabolic activity, gene expression and mechanical integrity. The potential of using dietary natural products over the use of broad-spectrum antimicrobial chemicals as virulence-targeted anticaries therapy has been emphasized [8–12].

In recent years, due to the rapid increase in the prevalence of childhood obesity, much attention has been focused on children's food and beverage consumption and its effect on health. As lifestyles have changed, there has been a 50% increase in the consumption of soft drinks over the past few decades, especially among children and adolescents. In addition, parents embrace fruit juices, juice drinks and smoothies for their children because these products are perceived as healthier alternatives to sugar-sweetened carbonated beverages. According to the US Food and Drug Administration, to be labeled as a fruit juice, a product must be 100% fruit juice. This includes juices reconstituted from concentrate. Any beverage that is less than 100% fruit juice must list the percentage of the product that is fruit juice, and the beverage must include a descriptive term, such as "drink", "beverage" or "cocktail". The currently marketed beverages including juices or juice drinks are frequently sweetened with added sugar, exceeding the daily recommended maximum amount of 19 g. Increasingly, there are concerns regarding high sugar and caloric contents, which may lead to obesity, thus affecting a person's general health [13–15]. The excessive consumption and prolonged exposure of sugar-sweetened beverages in the oral cavity promote acid production by plaque bacteria and pose a significant risk for dental caries, especially in pediatric populations [16–18]. Dietary changes and inadequate oral hygiene have also led to increasing dental erosion in young people. Unfortunately, the implications of the consumption of sugar-sweetened beverages (SSB) as a cause of dental caries are not usually emphasized to consumers.

Due to these concerns and an increase in consumer awareness in health and overall well-being, beverage options with perceived health benefits have gained tremendous popularity. Among these beverages are the ready-to-drink (RTD) bottled teas with spices and herbs and fruit juices and juice drinks prepared from grapefruit, blueberries, pomegranates and apples, among others. It is well-known that dietary polyphenols can be found in both edible and nonedible plants, including fruits. Their health benefits, including antimicrobial, antioxidant, anticancer and anti-inflammatory properties, have been emphasized [10,19,20]. In vitro studies have shown that plant-based beverages, e.g., teas, coffees, wines and cranberry juice, contain antimicrobial compounds that inhibit oral pathogens [21–25]. There have also been reports suggesting the antigingivitis and anticaries properties of many widely consumed polyphenol-rich foods and beverages that may benefit oral health [8,26–28].

Tea, the polyphenol-rich beverage prepared from leaves of the plant *Camellia sinensis*, is the most widely consumed beverage in the world today. Populations with habits of drinking tea have decreased caries incidence, lower levels of cariogenic bacteria and healthier plaque scores [21,29]. In vitro studies have shown that tea polyphenols inhibit growth, acid production and biofilm formation of the cariogenic bacteria *Streptococcus mutans* [23,30]. The main antimicrobial tea polyphenol, epigallocatechin gallate (EGCG), has been reported to suppress gene expression of GTF and acid production enzymes of *S. mutans* [31]. Our previous study found that adults rinsing with Black Tea extract showed a significantly reduced plaque pH fall and a lower plaque index compared with those rinsing with water alone. Frequent short-term rinses with Black Tea also inhibited subsequent regrowth and glycolysis of human supragingival plaque bacteria [24].

Berry fruits, including cranberries, also represent a rich source of polyphenolic bioactives that may contribute to human health [32]. The American cranberry, *Vaccinium macrocarpon*, has been well-documented as a popular remedy for the self-treatment of urinary tract infections. The antiadhesion effects of proanthocyanidins (PACs) in cranberries prevent the attachment of uropathogenic *E. coli* to mucosal and epithelial surfaces. Additional studies have suggested that this effect may also show promise in the oral cavity [33]. Cranberry extract and its PACs are known to inhibit in vitro *S. mutans*, GTF enzyme and glucan synthesis, thus disrupting biofilms [25,34,35]. Inhibition of LPS-induced proinflammatory cytokines and chemokine responses has also been reported [36]. Analysis of our previous data has shown that US-marketed cranberry juice cocktails (CJC) and a cranberry extract caused rapid cellular aggregation of cariogenic bacteria such as *S. mutans* and their in vitro growth and biofilm formation. The cranberry extract was also bactericidal against periodontal pathogens including *P. gingivalis*, *Fusobacterium nucleatum* and a multispecies anaerobic biofilm [37]. Besides the marketed teas or fruit juices prepared from a single plant source, the US beverage market has seen a dramatic increase in flavored teas and bottled drinks with a variety of tea/fruit combinations. These products are popular among consumers, including children. Many of the flavoring ingredients in beverages are extracts from plants, such as cinnamon or berries, with documented antimicrobial properties against oral bacteria [12,38,39].

While processed foods and high-sugar diets have been associated with increased dental caries, certain foods and food constituents have been reported to have cariostatic properties or are associated with less-than-expected caries in experimental animals [9,40–42]. In daily life, one is often reminded of the deleterious effects of cariogenic foods/food components, and less emphasis is placed on foods that may reduce caries risks. Other in vitro studies have shown that certain food and beverage components possessed antiplaque potential by interfering with oral bacterial adhesion [8,27]. We have previously shown that raisins, dried plums, though sweet and sticky, did not lower plaque pH to less than pH 6 [43]. We have also reported that adults and children drinking milk following the consumption of dried ready-to-eat sugar-added cereal reduced in vivo dental plaque acidogenicity and returned plaque pH to neutral levels, which may minimize caries risk [44,45]. Woźniewicz et al. demonstrated that the 8-week daily consumption of a functional beverage containing ground cinnamon and cranberry and apple juices with no added sugar improved gingival and plaque indices without posing a risk of caries development in patients with gingivitis [27]. We hypothesize that teas and beverages rich in plant-derived antimicrobial polyphenols may benefit oral health by suppressing virulence properties of dental plaque bacteria. The influence of dietary habits on oral health is indisputable and understanding how marketed beverages may affect dental health may enhance consumer awareness in making healthy choices of beverages that may be cariogenic alternatives. In this study, more than two dozen selected, US-marketed bottled flavored teas and ready-to-drink (RTD) beverages containing dietary plant-derived polyphenols or berry extracts were evaluated for their effects on in vitro growth and biofilm formation of *S. mutans* and the supragingival plaque bacteria of 16 children between the ages of 7 and 11 years.

2. Materials and Methods

2.1. Children's Supragingival Plaque Samples

Supragingival plaque samples were collected from 16 children between the ages of 7 and 11 years at the Pediatric Dentistry Clinic, the University of Illinois at Chicago (UIC) College of Dentistry.

Plaque was collected from the buccal and lingual surfaces of all available teeth by means of a spoon-shaped excavator (EXCE 2, Hu-Friedy®, Hu-Friedy Mfg. Co., Chicago, IL, USA), smeared onto a sterile cotton swab (Huby®-340, Sanyo Co., Osaka, Japan) then placed into a 15-mL conical tube (Falcon®, Corning Inc., Corning, NY, USA) containing 1.75 mL of 0.03% Tryptic Soy Broth (TSB, BactoTM, Becton, Dickinson and Co., Sparks, MD, USA). The plaque bacteria were dislodged from the swab by vortex for 30 s followed by dispersion for 2 min in a sonic cleaner (Fisher Scientific, Waltham, MA, USA). The cell suspension was adjusted to O.D. = 0.2 ± 0.01 at 600 nm and used for further testing. Because no demographic or protected health information was obtained or recorded from the children, this study was exempt from IRB review as nonhuman subject research (protocol #2014-1216, Office for the Protection of Research Subjects, University of Illinois at Chicago).

2.2. Ready-to-Drink (RTD) Beverages Used for S. mutans Testing

The 20 test RTD bottled beverages used were available and marketed in the US. They were purchased from a Jewel-Osco grocery store in Chicago, IL. All RTDs containing Green Tea are noted in the product names and those without notations contain only Black Tea:

- AriZona® Diet Green Tea with Ginseng (AriZona Beverage Co., Cincinnati, OH, USA).
- AriZona® Green Tea with Ginseng and Honey (AriZona Beverage Co., Cincinnati, OH, USA).
- Gold Peak® Green Tea (The Coca Cola Co., Atlanta, GA, USA).
- Gold Peak® Diet Iced Tea (The Coca Cola Co., Atlanta, GA, USA).
- Lipton® 100% Natural Green Tea with Citrus (Unilever, Englewood Cliffs, NJ, USA).
- Lipton® 100% Natural Iced Tea with Lemon (Unilever, Englewood Cliffs, NJ, USA).
- Lipton® 100% Natural Iced Tea with Pomegranate Blueberry (Unilever, Englewood Cliffs, NJ, USA).
- Lipton® Diet Green Tea with Citrus (Unilever, Englewood Cliffs, NJ, USA).
- PureLeaf™ Iced Tea Unsweetened (Unilever, Englewood Cliffs, NJ, USA).
- PureLeaf™ Iced Tea Sweetened (Unilever, Englewood Cliffs, NJ, USA).
- PureLeaf™ Iced Tea Raspberry (Unilever, Englewood Cliffs, NJ, USA).
- Snapple® All Natural Raspberry Iced Tea (Snapple Beverage Corp., Rye Brook, NY, USA).
- Diet Snapple® Cranberry Raspberry (Snapple Beverage Corp., Rye Brook, NY, USA).
- Diet Snapple® Half Lemonade 'n Half Iced Tea (Snapple Beverage Corp., Rye Brook, NY, USA).
- Diet Snapple® Raspberry Iced Tea (Snapple Beverage Corp., Rye Brook, NY, USA).
- Diet Snapple® Raspberry Tea (Snapple Beverage Corp., Rye Brook, NY, USA).
- SoBe™ Honey Green Tea-Lean (PepsiCo., Purchase, NY, USA).
- Ocean Spray® Diet Cranberry (Ocean Spray Cranberries Inc., Lakeville, MA, USA).
- Ocean Spray® Cranberry Juice Cocktail (Ocean Spray Cranberries Inc., Lakeville, MA, USA).
- Simply™ Cranberry Cocktail (Simply Orange Juice Co., Apopka, FL, USA).

2.3. Beverages Used for Children's Supragingival Plaque Testing

All prepackaged tea bags and ready-to-drink (RTD) bottled beverages used for the testing of children's supragingival plaque are available and marketed in the US. These were purchased from a Jewel-Osco grocery store in Chicago, IL:

- Prepackaged tea bags.
    - Lipton® Green Tea (GT; Unilever, Englewood Cliffs, NJ, USA).
    - Bigelow® Black Tea English Teatime (BT-ET; Bigelow Tea, Fairfield, CT, USA).
    - Bigelow® Black Tea Cinnamon Stick (BT-CS; Bigelow Tea, Fairfield, CT, USA).
    - Bigelow® Black Tea Raspberry Royale (BT-RR; Bigelow Tea, Fairfield, CT, USA).
- Ready-to-drink (RTD) bottled beverages.
    - PureLeaf™ Iced Tea Unsweetened (UT; black tea, Unilever, Fairfield, CT, USA).

  - Snapple® All Natural Green Tea (ANGT, Snapple Beverage Corp., Rye Brook, NY, USA).
  - Snapple® All Natural Raspberry iced Tea (ANRT; Black and Green Tea, Snapple Beverage Corp., Rye Brook, NY, USA).
- Cranberry juice cocktails.
  - Ocean Spray® Cranberry Juice Cocktail (CJ-OS, Ocean Spray Cranberries Inc., Lakeville, MA, USA).
  - Simply™ Cranberry Cocktail (CJ-S; Simply Orange Juice Co., Apopka, FL, USA).

2.4. Preparation of Test Teas/Beverages

Dried tea leaves (1 g) were removed from individual prepackaged tea bags and placed in 10 mL of filtered and electric-kettle-boiled water (100 mg/mL) then allowed to cool to room temperature gradually over a 30-min period. The extract was then filtered through a 0.45 µm filter (Millex®-HV, MilliporeSigma Corp., St. Louis, MO, USA) and used as test solution. All test tea solutions were freshly prepared prior to laboratory testing. All RTD bottled beverages and teas used for testing were serially diluted in sterile double-distilled water. The highest test concentration achievable using our testing protocol was 50% of the test beverages and teas.

2.5. Effects of Selected Teas and RTD Bottled Beverages on In Vitro Growth of Children's Supragingival Plaque Bacteria

Plaque bacteria (10^6 CFU/mL) were grown in a 96-well microtiter plate containing chemically defined medium (CDM) [46] and serially diluted test teas or beverages at 37 °C in an anaerobic chamber (5% H_2, 5% CO_2, 90% N_2; Forma Scientific Anaerobic System, Marietta, OH, USA). Controls contained plaque bacteria and growth medium with water replacing test teas. After 24 h, growth at O.D. 550 nm was determined (Power Wave 200-I, Bio-Tek Instruments®, Winooski, VT, USA). The Minimum Inhibitory Concentration (MIC) was defined as the lowest tealeaf concentration (mg/mL) or % original beverage concentrations where no plaque bacterial growth (O.D. $\leq$ 0.05) was observed. MIC data were expressed as Median (range) obtained from 16 children.

2.6. Effects of Selected Teas and RTD Bottled Beverages on In Vitro Biofilm Formation of Children's Supragingival Plaque Bacteria

Plaque bacteria (10^6 CFU/mL) were grown in CDM supplemented with 1% (*w*/*v*) sucrose and test tea or beverage at 37 °C. Controls contained water in place of test agents. After 48 h of anaerobic incubation, the nonadherent cells in each of the wells were carefully removed and discarded. The wells were washed twice with 200 µL of phosphate-buffered saline (PBS; 0.05 M, pH 6.8), and the adherent biofilm was air-dried and stained with 100 µL of 0.1% Crystal Violet. After 10 min the dye was carefully removed, and the stained biofilm was washed gently with PBS and the dye was extracted with 200 µL of 90% ethanol and the O.D. 550 nm was measured. The Minimum Biofilm Inhibitory Concentration (MBIC) was defined as the lowest tealeaf concentration (mg/mL) or % original beverage concentration where more than 90% of biofilm formation was inhibited [47]. MBIC data were expressed as Median (range) obtained from 16 children.

2.7. Effects of Selected RTD Bottled Beverages on In Vitro Growth and Biofilm Formation of S. mutans

S. mutans UA159 was used in these experiments. The effect of the 20 RTD bottled beverages (Section 2.2) on in vitro growth and biofilm formation of *S. mutans* were determined using methods described above for testing of children's plaque bacteria (Sections 2.5 and 2.6). Water in place of the test beverage was used as control. The MIC and MBIC were obtained and growth and biofilm formation were expressed as % of the nontreated controls.

2.8. Total Polyphenol Content of Test RTD Bottled Beverages and Teas

The total polyphenol content (TPC) of the test beverages and teas were determined using the modified Folin–Ciocalteu's method with gallic acid used as a standard [46,47]. Samples for testing were either obtained from freshly open RTD bottled beverages or freshly prepared tea infusions.

2.9. Statistical Analysis

Statistical analyses were carried out with IBM SPSS version 22. Descriptive statistics including the mean, median, minimum and maximum of variations were calculated. The results were analyzed for significance ($p < 0.05$) with the Friedman's ANOVA test, the Wilcoxon Signed-ranks test, the Kruskal–Wallis ANOVA test and Dunnett's T3 test used for comparisons of combinations of the means.

3. Results

3.1. Effects of RTD Bottled Beverages on S. mutans Growth and Biofilm Formation

In this study, using the protocol described above, the highest achievable test concentration for all beverages was 50% of the original (1:1 dilution). Among the 20 RTD beverages tested, three had no effect on growth, ten inhibited growth between 20% and 60% while seven inhibited >98% of growth (Figure 1). Fourteen out of the twenty test beverages inhibited biofilm between 30% and 90% while six inhibited more than 97% (Figure 2). The seven beverages that inhibited >98% growth were Raspberry Iced Tea (Snapple), Diet Snapple—Half Lemonade 'n Half Iced Tea (Snapple), Diet Raspberry Iced Tea (Snapple), Diet Snapple Raspberry Tea (Snapple), Cranberry Juice Cocktail (Ocean Spray), Diet Cranberry (Ocean Spray) and Cranberry Cocktail (Simply). Except for Diet Snapple Raspberry Tea, six of these seven also strongly inhibited biofilm (>97%). Three of the six were cranberry juice cocktails containing 27% cranberry juice and three were bottled teas with raspberry flavor (Figure 2). The sugar-sweetened Cranberry Juice Cocktail (Ocean Spray) and the sugar-free Diet Cranberry (Ocean Spray) were both effective in reducing more than 90% growth and biofilm, even at 25% (1:4 dilution) beverage concentrations (data not shown). Similar results were noted for the raspberry-flavored iced teas (Snapple) with or without added sugar. The Diet Snapple Half Lemonade 'n Half Iced Tea (Snapple) was the only non-berry-containing test beverage that showed >90% growth and biofilm inhibition at 50% (1:1 dilution) beverage concentration (Figures 1 and 2). Beverages containing citrus fruits were not as effective as those containing cranberry juice. All *S. mutans* biofilms formed in the presence of the test beverages were visibly much less adherent and easily detachable from surfaces.

When total polyphenol contents (TPC) were determined for RTD beverages, the values ranged from 0.24 mg/mL to 0.95 mg/mL with Ocean Spray® Cranberry Juice Cocktail being highest at 0.95 mg/mL. There was no apparent correlation between the total phenolic content vs. their biofilm inhibitory activity. For example, the Raspberry Iced Tea (Snapple) contained 0.335 mg/mL TPC but was able to inhibit >98% of biofilm at 1:1 dilution, while no more than 40% inhibition was noted with PureLeaf™ Iced Tea Sweetened (Unilever) having comparable TPC of 0.346 mg/mL (data not shown).

3.2. Effects of Teas on Growth and Biofilm Formation of Children's Plaque Bacteria

Teas prepared from Green or Black Tea bags with or without flavors strongly inhibited growth and biofilm formation of children's plaque bacteria (Table 1). Among these, Green Tea (GT) demonstrated the lowest minimum inhibitory concentration (MIC) at 0.78 mg/mL, equivalent to 10% concentration of a 250-mL cup of tea brewed from one tea bag (2 g). The nonflavored Black Tea (BT-ET) and cinnamon- (BT-CS) or raspberry- (BT-RR) flavored Black Tea inhibited growth of children's plaque bacteria at median value of MICs ranging from 2.35 to 6.25 mg/mL (Table 1). Overall, Green Tea (GT) had the lowest MIC compared with the three other test teas and was only marginally lower than Black Tea without flavoring (BT-ET) and Black Tea with cinnamon flavoring (BT-CS) ($p > 0.05$).

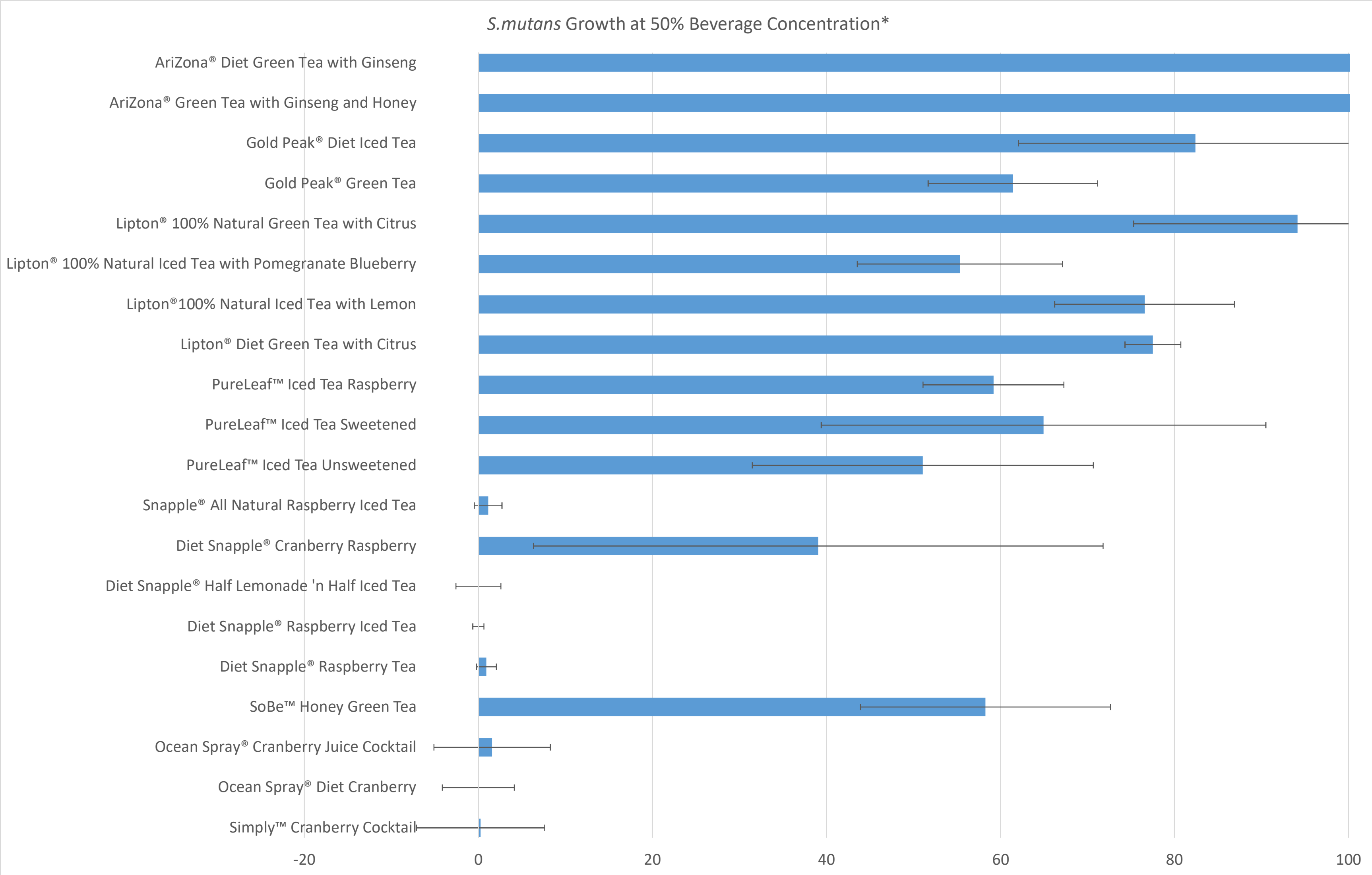

Figure 1. Growth of *S. mutans* in the presence of RTD bottled teas and beverages. * Growth expressed as % Growth = OD $_{treated}$/OD $_{control}$ × 100%.

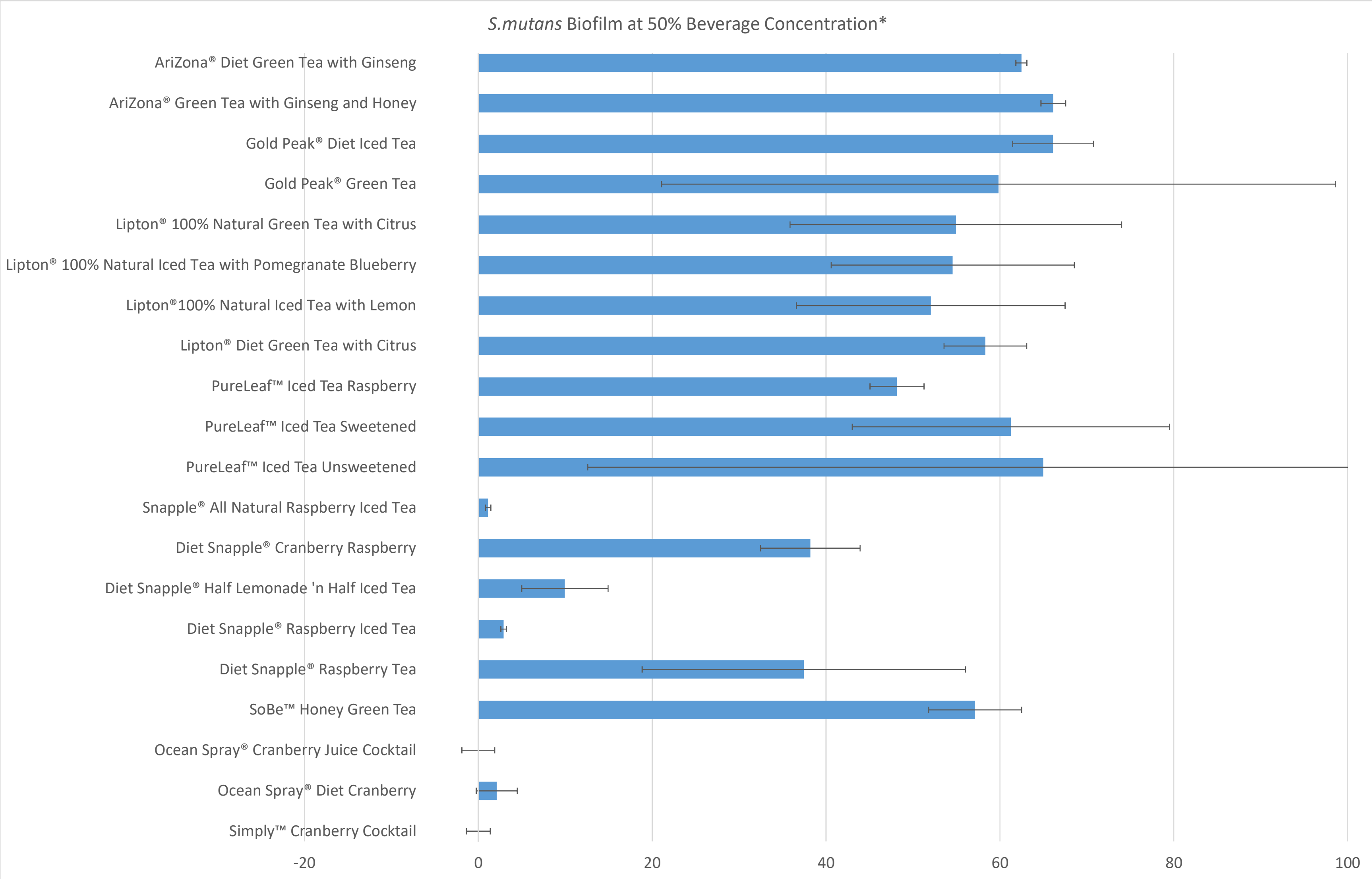

Figure 2. Biofilm formation of *S. mutans* in the presence of RTD bottled teas and beverages. * Biofilm expressed as % Biofilm formation = $OD_{treated}/OD_{control} \times 100\%$.

Table 1. Inhibition of growth and biofilm formation of children's supragingival plaque bacteria by teas ($n = 16$).

Test Teas	Growth (MIC, mg/mL) *	Biofilm (MBIC, mg/mL) *
	Median (Range)	Median (Range)
Green Tea (GT)	0.78 (0.39–3.13)	1.56 (0.39–25)
Black Tea-English Teatime (BT-ET)	3.13 (0.39–6.25)	0.78 (0.39–6.25)
Black Tea-Cinnamon Stick (BT-CS)	2.35 (0.39–6.25)	1.56 (0.78–3.13)
Black Tea-Raspberry Royale (BT-RR)	6.25 (0.78–25)	1.56 (0.39–25)

* MIC, Minimum Inhibitory Concentration; MBIC, Minimum Biofilm Inhibitory Concentration. Values represent median (range) of tealeaf concentrations (mg/mL) obtained from 16 children. MIC values differed significantly among test teas (Friedman ANOVA, $p < 0.001$).

All four test teas inhibited children's plaque biofilm formation, with minimum biofilm inhibitory concentration (MBIC, median values) ranging from 0.78 to 1.56 mg/mL with no statistically significant difference (Friedman Test, $p > 0.05$). This concentration equaled from 10% to 20% of a cup of brewed tea (Table 1). Compared with the nontreated control, all plaque biofilm formed in the presence of teas was loosely adherent and was easily dislodged from surfaces. These physical properties of the treated biofilm were similar to the *S. mutans* biofilms in the presence of the RTD bottled beverages.

The total polyphenol contents of the four test teas were 8.8 mg/mL for GT; 5.287 mg/mL for BT-ET; 4.602 mg/mL for BT-CS and 4.636 mg/mL for BT-RR. The packaged Green Tea contained the highest amount of TPC. Again, no positive correlation was noted between TPC and bioactivity of the test teas.

3.3. Effects of Ready-to-Drink (RTD) Bottled Teas and Beverages on Growth and Biofilm Formation of Children's Plaque Bacteria

Three RTD tea beverages and two cranberry juices that were inhibitory to *S. mutans* were selected for further testing against children's plaque bacteria. As noted in the Methods (Section 2.4), the highest achievable testing concentration using our test protocol was 50% of the test beverages. The MIC values for the five RTD bottled teas and beverages differed significantly (Friedman ANOVA, $p < 0.05$). The two RTD bottled cranberry cocktails (CJ-OS and CJ-S) completely inhibited plaque bacterial growth, with MIC at 50% of the beverage concentration (1:1 beverage dilution, Table 2). Although the three RTD bottled teas (All Natural Green Tea, ANGT; Unsweetened Tea, UT; and All Natural Raspberry Iced Tea, ANRT) did not completely inhibit growth at 50% beverage concentration, significant inhibition was noted at 70.21% for ANRT, 62.39% for ANGT and 52.37% for UT compared with the water control (one-way ANOVA, $p < 0.05$, Table 3).

Table 2. Inhibition of growth and biofilm formation of children's supragingival plaque bacteria by ready-to-drink (RTD) bottled beverages ($n = 16$).

Test Beverages	Growth (MIC *, % Beverage)	Biofilm (MBIC *, % Beverage)
	Median (Range)	Median (Range)
All Natural Raspberry Iced Tea (ANRT)	>50	9.38 (1.56–25)
All Natural Green Tea (ANGT)	>50 (50–>50)	>50
Unsweetened Tea (UT)	>50	4.68 (1.56–12.5)
Cranberry Juice Cocktail (CJ-OS)	50 (3.13–50)	>50 (1.56–>50)
Cranberry Cocktail (CJ-S)	50 (3.13–50)	50 (1.56–>50)

* MIC, Minimum Inhibitory Concentration; MBIC, Minimum Biofilm Inhibitory Concentration. Values represent % original beverage concentration expressed as Median (range) obtained from 16 children.

When the effects of RTD bottled beverages on children's plaque bacteria biofilm were examined, both of the cranberry juice cocktails (CJ-S and CJ-OS) showed comparable inhibition at 50% beverage concentration (71.76% and 57.48% respectively; $p > 0.05$, Wilcoxon Signed-ranks test; Table 3). Among the three bottled teas tested, All Natural Raspberry Tea

(ANRT) and Unsweetened Tea (UT) inhibited plaque biofilm, with MBIC as low as 9.38% and 4.68% beverage concentrations (Table 2). The All Natural Green Tea did not inhibit biofilm formation at 50% beverage concentration (Tables 2 and 3). Similar to what was observed in *S. mutans*, the plaque biofilms formed in the presence of RTD bottled beverages were also observed to be loosely attached to surfaces and were as easily rinsed off.

Table 3. The inhibition of ready-to-drink (RTD) bottled beverages at 50% concentration (1:1 dilution) on growth and biofilm formation of children's supragingival plaque bacteria (*n* = 16).

Beverages	Growth		Biofilm Formation	
	% Control *	% Inhibition †	% Control *	% Inhibition †
All Natural Raspberry Iced Tea (ANRT)	29.79 ± 20.23	70.21	1.16 ± 2.49	98.84
All Natural Green Tea (ANGT)	37.61 ± 34.32	62.39	118.94 ± 50.58	NI ‡
Unsweetened Tea (UT)	47.63 ± 37.30	52.37	2.16 ± 1.49	97.84
Cranberry Juice Cocktail (CJ-OS)	4.41 ± 6.99	95.59	42.52 ± 51.99	57.48
Cranberry Cocktail (CJ-S)	5.42 ± 14.77	94.58	28.24 ± 63.65	71.76

Values represent Mean ± SD. * % Control = $OD_{treated}/OD_{control} \times 100\%$. † % inhibition = 100% − % control. ‡ NI: No inhibition.

4. Discussion

It is well known that diet, especially sugars, plays an important role in influencing the caries experience. Most strategies used to prevent caries focus on reduction of sugar intake and frequency of snacking. In recent years, attempts have been made to investigate the protective effects of certain foods and food components, other than free sugar, against oral diseases thus benefit oral health [9,11,23]. Many plant-based foods that are part of the human diet contain polyphenols that could interfere with oral bacterial adhesion and co-aggregation. These polyphenols have been widely used in the food industry and are commonly consumed in beverage form. In recent years, the consumer market has witnessed an influx of beverage products with a variety of health-related functional claims, i.e., functional beverages.

In this study, *S. mutans*, the prominent cariogenic bacteria of the dental plaque was used as the indicator species for initial testing of beverage products. A chemically defined medium was used in all assays instead of a high protein complex medium (e.g., BHI) was based on our previous findings showing inconsistencies in MIC/MBIC values due to protein-polyphenols interaction [48]. The choice of the 20 marketed RTD bottled beverages for testing against *S. mutans* was based on our previous research on teas and cranberries. Except for the two RTD Green Tea with ginseng and the one with citrus, the remaining 17 test beverages inhibited *S. mutans* growth by 20% to 100%. All 20 beverages inhibited biofilm from 30% to 100%. Based on our testing, the RTD beverages contained polyphenols with TPC ranging from 0.24 to 0.95 mg/mL. However, there was no correlation between TPC values and bioactivity of the beverages. For example, the Ocean Spray® Cranberry Juice Cocktail with the highest total phenolic content (TPC) at 0.95 mg/mL completely inhibited *S. mutans* biofilm, while Snapple® All Natural Raspberry Iced Tea with similar inhibition (>98%) only tested 0.335 mg/mL for TPC. Since the type and content of teas and other ingredients in the beverages were not fully disclosed, it is likely that the various beverage ingredients (e.g., Vitamin C or organic acids) could have interfered with TPC detection or there are active ingredients in addition to polyphenols that contributed to the activity. Nevertheless, our data showed that various marketed tea beverages and cranberry juice cocktails inhibited growth and biofilm formation of *S. mutans*.

Among the 20 RTD beverages, the six that demonstrated >97% inhibition of *S. mutans* growth and biofilm formation included teas with raspberry flavors and the cranberry juice cocktails frequently consumed by children (Figures 1 and 2). These results provided the rationale for the selection of teas and beverages for testing against the complex biofilm community of supragingival plaque. Because the microbial compositions of plaque biofilm can vary from one individual to another, we have observed variation in the MIC and MBIC values obtained from plaque bacteria of the 16 participating children. Therefore, these values were expressed as Median and Range instead of Mean ± SD (Tables 1 and 2). Future studies with larger numbers of participants should help minimize the variations.

Analysis of our data showed that infusions prepared from the four test teas (GT, BT-ET, BT-CS and BT-RR) demonstrated comparable antigrowth and antibiofilm activity against children's plaque (Table 1). At the concentrations tested, Green Tea (GT) exhibited the strongest growth inhibition, whereas Black Tea (BT-ET) was best at inhibiting biofilm formation. This is supported by our previous studies demonstrating that tea polyphenols, especially the catechin EGCG, suppressed gene expressions of *S. mutans* enzymes involved in acid production and the GTF enzymes responsible for adherent glucan matrix synthesis in the biofilm [31]. Since the extracellular polymeric substances (EPS) are essential for the development of biofilm architecture, we believe that the tea polyphenols affected the structure and stability of the plaque biofilm which were loosely adherent and easily disrupted from surfaces.

Many natural products or plant extracts, including that from cinnamon, are frequently used as flavoring agents in foods and beverages. Flavoring agents have been reported to possess antimicrobial activity [49,50]. We have previously found that cinnamon aldehyde suppressed growth of *S. mutans* and other oral bacteria [51]. Therefore, we anticipated that Black Tea with added cinnamon flavor may enhance inhibition against plaque bacteria. However, analysis of our data showed that the effect on growth of plaque bacteria by Black tea with cinnamon (BT-CS) was comparable with that of Black Tea without flavor (BT-ET). This suggested that the addition of cinnamon flavor to Black Tea did not appear to enhance or negatively affect the antibacterial properties of BT-ET (Table 1). Although Black Tea with Raspberry flavor (BT-RR) demonstrated less growth inhibition compared with the others, all four test teas showed comparable inhibition of children's plaque biofilm at 0.78–1.56 mg/mL ($p > 0.05$, Table 1), concentrations equaled from 10% to 20% of a cup of brewed tea. While we are aware that the total weight of tea leaves per flavored tea bag may be less than that of the nonflavored Black Tea (BT-ET), they still demonstrated comparable inhibition of plaque biofilm.

As for whether the inhibitory activity was a reflection of the TPCs contents of the test teas, our data showed that there was no positive correlation between TPCs and bioactivity. For example, Black Teas with or without flavors (TPCs 4.6 to 5.2 mg/mL) demonstrated comparable inhibitory activity to that of Green Tea with the highest TPC of 8.8 mg/mL. Although both Black and Green Teas originate from the same plant, *Camellia sinensis*, the oxidation level of the plant leaves and the plant types have been shown to influence their polyphenol profiles [46]. Green Tea is minimally oxidized and contains high levels of catechins (flavanols and flavanol gallates) while Black Tea is fully oxidized and through enzymatic process, catechin content declines and complex flavanols such as theaflavins and thearubins are formed. Our previous work has shown that both the purified Green Tea catechins and Black Tea theaflavins inhibited *S. mutans* biofilm. The Folin–Ciocalteu's method provides a general polyphenolic content of the beverages and may not be optimal for the differentiation of the different classes of tea polyphenols. Moreover, the commercially prepackaged teas and tea beverages often contain other nondisclosed additives and ingredients which may interfere with the TPC assay. However, based on our data, we can conclude that the presence polyphenols in these products, regardless of their levels, contributed to the inhibition observed.

Among the three RTD tea beverages evaluated against children's plaque bacteria, both All Natural Raspberry Tea (ANRT) and Unsweetened Tea (UT) inhibited plaque formation at less than 10% of beverage concentration (Table 2). At 50% beverage concentration, both of these bottled teas inhibited >98% plaque biofilm in the children being tested (Table 3). The ingredient labels revealed that the ANRT contained both Black and Green Teas, while UT contained only Black Tea. Since the exact contents of each ingredient were not disclosed, we suspect that the addition of Raspberry flavor did not antagonize the inhibitory action of the tea component. It was surprising to note that the All Natural Green Tea (ANGT) had no biofilm inhibition at 50% beverage concentration, since it should also contain the antimicrobial polyphenols. This may have been due to the variations in tea contents of different RTD beverages. However, it is undeniable that Black Tea, either in the form of prepared tea bags or as an RTD, possessed antibiofilm activity against both *S. mutans* and children's plaque bacteria. Although one should not compare antimicrobial activity between the infusions prepared from tea bags and the RTD beverages, due to differences in the beverages' ingredients and compositions, analysis of the current study data has shown consistently that Black or Green Tea with or without flavors was able to suppress children's plaque bacteria and the adherent properties of dental plaque biofilm. This could explain some of the results from clinical studies showing dental plaque reduction, i.e., plaque index reduction, in children after rinsing with tea or cranberry juice extract [50].

In the current study, we reported that the two cranberry juice cocktails consistently suppressed growth and biofilm formation of both *S. mutans* and children's supragingival plaque bacteria. These antiadhesive properties are not reported in apple, grape or orange juices [52]. The Simply™ Cranberry Cocktail seemed to perform better when compared with Ocean Spray® Cranberry Juice, but the difference was not statistically significant ($p > 0.5$, Tables 2 and 3). Both of these juice drinks contained 27% of cranberry juice, with Ocean Spray® Cranberry Juice Cocktail prepared from juice concentrate while the Simply™ Cranberry Cocktail was not. Since the identities and quantities of other ingredients in these beverages were not clearly listed, it is difficult to compare the two juices. The plaque biofilms grown in the presence of these cranberry juice drinks were loosely attached and easily dislodged from surfaces. We believe that the antimicrobial polyphenolic compound proanthocyanidins (PACs) present in the RTD cranberry juice drinks contributed to their inhibition. PACs have been reported to suppress glucosyltransferase (GTF) enzymes that catalyze the synthesis of the sticky glucan matrix, thus disrupting the exopolysaccharide matrix of plaque biofilm [53]. We have previously showed that the acidity of cranberry juice did not contribute to its biofilm inhibition [37]. Based on our data, we believe that cranberry juice represents a much healthier alternative over the other sugar-added test beverages used in this study. Recent studies at Wu's laboratory have shown that exposure of dental plaque bacteria from both children and adults to cranberry juice in vivo suppressed further regrowth, acid production and biofilm formation [54,55].

The raspberry fruit *Rubis idaeus* is known for its richness in polyphenols, especially tannins, which are responsible for its inhibition against microorganisms including *Candida albicans* biofilm [56,57]. The antibiofilm effects observed in all the raspberry-containing beverages or the raspberry-flavored teas tested in this study might have been contributed by the polyphenols from both teas and raspberries. However, the nature and the source of the raspberry component in these beverages were not disclosed. It has been observed that sugar is commonly added to enhance the palatability of teas or cranberry juices. Analysis of our data indicated that the addition of sugar did not negatively affect the antibiofilm properties of cranberry juice or raspberry-flavored beverages, since comparable levels of inhibition were noted between sugar-added and sugar-free (diet) products (Figures 1 and 2). This suggested that the berries' polyphenols may counteract the deleterious effect of dietary sugars. Therefore, cranberry or raspberry juice drinks with added sugars are still more desirable than the popular high sugar soft drinks which offer no protective effect but to promote acid production and biofilm formation by dental plaque bacteria.

The RTD bottled cranberry juice drinks and cranberry cocktails have been popular among children and consumers, but many are concerned about the high acidity of these beverages [58]. While cranberry juice is acidic, it contains more fluoride (1.3 to 6 times higher) and tannins and much lower amounts of total carbohydrate (60% of dry weight) compared with other fruit juices. Although the acidic nature of popular fruit drinks has been linked to increased risk of dental erosion, this concern has been based primarily on many in vitro studies with controversial data that did not support the association between acidic drinks and dental erosion [59]. It was concluded that many other factors can affect the erosive process, including intake frequency and length of exposure. To demonstrate a positive link between acidic beverages and dental erosion, additional well-controlled in vivo human dietary studies are warranted.

Frequent consumption of sugar-sweetened beverages by children is one of the risk factors for dental caries. However, an understanding of the anticariogenic potential of natural foods or commercially available beverages containing dietary plant-derived polyphenols can help consumers, especially children, make better dietary decisions to promote oral health. The awareness of healthy beverages should not only emphasize general health but also promote oral health, which is intricately associated with overall well-being. With the discoveries made in this study, dietary plant-derived polyphenols present in commercially available teas and beverages may be preferred over sugar-sweetened popular soft drinks. Further in vivo evaluations of the effects of these beverages on human dental plaque are in progress. The concept of oral disease prevention using natural foods or beverages in the diet may be a novel, practical and acceptable approach toward reducing caries incidences in children.

5. Conclusions

Commercially marketed, ready-to-drink bottled tea beverages and cranberry juice drinks containing plant-derived antimicrobial polyphenols were found to inhibit the growth and biofilm formation of *Streptococcus mutans*. Infusions prepared from prepackaged teas and cranberry juice drinks also suppressed growth, biofilm formation and matrix mechanical properties of children's supragingival plaque bacteria. These beverages contain plant-derived polyphenols that are natural alternatives to traditional antimicrobial chemicals in targeting cariogenic virulence factors. If sequenced properly between meals and performance of oral hygiene, these beverages exhibit potential oral health benefits and are preferable to commonly consumed sugar-sweetened beverages. The concept of caries management using natural foods or beverages in the diet is a novel, practical and innovative approach to promote oral health in children.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of University of Illinois at Chicago (UIC). Because no demographic or protected health information was obtained or recorded from the children, this study was exempt from IRB review as non-human subject research (protocol #2014-1216).

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