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Inhibitory effects of cranberry juice on attachment of oral streptococci and biofilm formation

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Cranberry juice is known to inhibit bacterial adhesion. We examined the inhibitory effect of cranberry juice on the adhesion of oral streptococci strains labeled with [^{3}H]-thymidine to saliva-coated hydroxyapatite beads (s-HA). When the bacterial cells were momentarily exposed to cranberry juice, their adherence to s-HA decreased significantly compared with the control ($P < 0.01$). Their hydrophobicity also decreased dependently with the concentration of cranberry juice. We also evaluated the inhibitory effect of cranberry juice on biofilm formation. By using a microplate system, we found that the high molecular mass constituents of cranberry juice inhibited the biofilm formation of the tested streptococci. The inhibitory activity was related to the reduction of the hydrophobicity. The present findings suggest that cranberry juice component (s) can inhibit colonization by oral streptococci to the tooth surface and can thus slow development of dental plaque.

Key words: adsorption; biofilm; cranberry; oral streptococci

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The existence of microorganisms as the polyspecies consortium known as oral biofilm and called dental plaque has profound implications for the etiology of caries and periodontal diseases (6, 8, 9, 11, 20–22, 25, 26). The adhesion of streptococci to the pellicle on tooth surfaces appears to be the first step in the formation of dental plaque. Mutans streptococci such as *Streptococcus mutans* and *Streptococcus sobrinus* have been strongly implicated as causative organisms of dental caries (8). The adsorption of mutans streptococci to the tooth surface is an essential step in the development of dental caries. It has been noted that the exclusion or reduction of such pathogenic bacteria is beneficial in controlling oral infections such as dental caries (8, 15–17, 24, 31).

The American cranberry is a member of the heath family native to North America. The fruit is widely used in a variety of food products including juices and confectionery. Cranberry juice has been shown to affect the adhesion of uropathogenic microorganisms to uroepithelial cells by interfering with specific receptor-ligand modes of microbial adhesion (13, 23) and to inhibit the sialic acid-specific adhesion of *Helicobacter pylori* to human gastric mucosa and erythrocytes (2, 3). Avorn et al. (1) have demonstrated that drinking cranberry juice decreased the frequency of bacteria with pyuria in elderly women. The high molecular weight nondialyzable material of cranberry juice constituents reversed the coaggregation of the majority of coaggregating bacterial pairs tested (28). In a preliminary clinical trial, nondialyzable material reduced the *S. mutans* count in saliva (29). Inhibition of bacterial colonization is a rational strategy for prevention of chronic oral infectious diseases caused by dental plaque bacteria.

The purpose of this study was to investigate the inhibitory effects of cranberry juice on the adherence of oral streptococci to saliva-coated hydroxyapatite (s-HA) beads and on biofilm formation.

Materials and methods

Microbial strains and culture conditions

The organisms used in this study were *S. sobrinus* 6715 and B13; *S. mutans* MT8148R, JC2, Ingbritt and ATCC 10449; *Streptococcus criceti* E49; *Streptococcus sanguinis* ATCC 10556; *Streptococcus oralis* ATCC 10557; *Streptococcus mitis* ATCC 9811 and *Streptococcus gordonii* Challis. These strains of streptococci were cultured in trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD). The organisms were grown at 37°C for 24–72 h in an anaerobic chamber (N_2 80%, H_2 10%, CO_2 10%). To radiolabel the

bacteria, the organisms were anaerobically grown with [^{3}H]-thymidine at 37°C to the early stationary phase.

Preparation of cranberry juice

Cranberry juice powder (Ocean Spray Cranberries, Inc., Lakeville-Middleboro, MA) was dissolved in water to a 25% concentration of cranberry juice following the manufacturer's instructions. Because a standard commercially available cranberry juice beverage contains around 25–27% juice, we selected 25% juice for this experiment. To avoid any effect of the low pH of cranberry juice on bacterial growth, the juice was dialyzed against distilled water (distilled water) in dialysis bags with a molecular mass cutoff point of 14,000 (Seamless Cellulose Tubing, 20/32, Viskase Sales Corp., Willowbrook, IL) at 4°C for 7 days. The non-dialyzable material was lyophilized. This non-dialyzable material was dissolved in trypticase soy broth and used in the biofilm formation assay as ''high molecular weight constituents of cranberry juice''.

Preparation of s-HA

Bacterial attachment to experimental pellicles was studied using s-HA as described by Gibbons et al. (4, 5). Human parotid saliva was collected with collecting devices from a healthy adult donor (16). The salivary flow was stimulated by an acid candy. The saliva was stored at –20°C and was used for the following experiments. A total of 5 mg of spheroidal hydroxyapatite beads (BDH Chemicals Ltd., Poole, England) were equilibrated in 200 μl buffered-KCl (0.05 M KCl, 1 mM potassium phosphate, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH 6.0) at 4°C overnight. These hydroxyapatite beads were treated with 100 μl of saliva by use of a rotator (Model RT50 Taitec Co., Tokyo, Japan) at 5 r.p.m. for 60 min. They were then washed three times with buffered KCl (4, 5). Grown cells labeled with ^{3}[H]-thymidine were harvested by centrifugation. The harvested cells were washed three times in buffered KCl and then suspended in buffered KCl to produce a suspension containing 1.0×10^9 cells per ml.

Binding inhibition assay

A suspension of 100 μl containing a [^{3}H]-labeled bacterial suspension (1×10^9 cells/ml) was incubated with 500 μl of 25% cranberry juice in buffered KCl for 10 s, 10 min or 30 min. Buffered KCl without cranberry juice was used as a control. After washing once with buffered KCl, bacterial cells were suspended in 100 μl of same buffer. The suspension of 100 μl was incubated with s-HA in a rotator at 5 r.p.m. for 60 min. After washing three times with buffered KCl, the number of bacterial cells which had attached to s-HA was determined by direct scintillation counting. The experiments were done independently three times.

Hydrophobicity assay

Hydrophobicity was determined as described by Rosenberg et al. (14). Briefly, bacterial suspensions in PUM buffer, which contains $K_2PO_4 \cdot 3H_2O$ (22.2 g/l), KH_2PO_4 (7.26 g/l), urea (1.8 g/l), and $MgSO_4 \cdot 7H_2O$ (0.2 g/l), were adjusted to an optical density of approximately 0.5 at 400 nm using a 2000U Spectrophotometer (Hitachi, Tokyo, Japan). Duplicate samples of bacterial suspensions (1.2 ml in PUM buffer) were placed in tubes, and 600 μl of hexadecane was added. The tubes were vigorously mixed by vortex stirring for 60 s and left to stand for 15 min. The optical density at 400 nm (OD_{400}) of the aqueous phase was then measured. The percent hydrophobicity was calculated as follows: [(OD_{400} before mixing) – (OD_{400} after mixing)]/(OD_{400} before mixing) × 100. Each isolate was assayed twice, and the values obtained were averaged.

Biofilm formation assay

The inhibitory effect of cranberry juice on the biofilm formation of *S. sobrinus* 6715, *S. mutans* JC2, *S. criceti* E49, *S. sanguinis* ATCC 10556, *S. oralis* ATCC 10557, and *S. mitis* ATCC 9811 on the bottom of cell culture plates (SUMILON Multi Well Plate, Sumitomo Bakelite Co. Ltd, Tokyo, Japan) was examined. Biofilm assays were done using the protocol of Loo et al. (9). Briefly, strains of streptococci were cultured in trypticase soy broth supplemented with 100 μg/ml or 500 μg/ml of high molecular weight constituents of cranberry juice for 1 day under anaerobic conditions. Media and unattached bacterial cells were decanted from the wells, and the remaining planktonic or loosely bound cells were removed by rinsing with distilled water twice. The plates were then blotted on paper towels and air dried, and adherent bacteria were stained with 50 μl of 0.1% crystal violet for 15 min at room temperature. After rinsing twice with 200 μl of distilled water each time, bound dye was extracted from the stained cells by using 200 μl of 99% ethanol. Biofilm formation was then quantified by measuring the absorbance of the solution at 595 nm (OD_{595}) with a microtiter plate reader (Model 3550, Bio-Rad Laboratories, Hercules, CA).

Statistics

The Mann–Whitney *U*-test was used for all experiments in this study to identify statistically significant differences.

Results

Effects of cranberry juice on inhibiting adhesion to s-HA

The inhibitory effects of 25% cranberry juice on the adsorption of seven oral streptococcus strains are summarized in Table 1. The adherence rates of the tested oral streptococci to s-HA beads differed from strain to strain. The adsorption of *S. mutans* ATCC 10449 cells to the s-HA beads was strong, but the adsorption to *S. sobrinus* 6715 was markedly weaker. Momentary exposure to 25% cranberry juice significantly reduced the adherence of all tested oral streptococci except *S. sobrinus* 6715 to s-HA ($P < 0.01$).

Effect of cranberry juice on cell surface hydrophobicity

The effects of the addition of the cranberry juice on the cell surface hydrophobicity of tested streptococci are summarized in Table 2. The hydrophobicity also differed from strain to strain. The hydrophobicity reduction was found to be dependent on the concentration of cranberry juice. *S. mutans* Ingbritt, MT8148R and JC2 and *S. sobrinus* 6715 showed 40–60% hydrophobicity, *S. criceti* had the highest and *S. sobrinus* B13 a low hydrophobicity. The other oral streptococci exhibited more than 80% hydrophobicity. The addition of 12.5% cranberry juice significantly reduced the hydrophobicity of 10 of 11 strains of streptococcus. The addition of 25% cranberry juice significantly reduced the hydrophobicity of all the streptococcus strains tested ($P < 0.05$ to $P < 0.01$).

Effects of the high molecular weight dialyzable materials from cranberry juice on the formation of streptococcal biofilm

The data from the experiments examining the inhibitory effects of the high molecular weight dialyzable materials from cranberry juice on biofilm formation by oral

Table 1. Inhibitory effect of 25% cranberry on adsorption of oral streptococci to 5 mg S-HA beads

Strain	Time of exposure	Bacterial numbers adsorbed to s-HA ($\times 10^6$)	% inhibition of adsorption
S. sobrinus			
6715	Control[†]	1.79 ± 0.24	
	10 s	2.69 ± 0.91	−50.3
	10 min	0.91 ± 0.50	49.2
	30 min	$0.66 \pm 0.21^*$	63.1
B13	Control	3.71 ± 0.37	
	10 s	$1.23 \pm 0.33^*$	66.8
	10 min	$1.12 \pm 0.23^*$	69.8
	30 min	$0.46 \pm 0.24^*$	87.6
S. mutans			
MT8148R	Control	9.19 ± 1.89	
	10 s	$2.45 \pm 1.37^*$	73.3
	10 min	$1.03 \pm 0.22^*$	88.8
	30 min	$1.29 \pm 0.44^*$	86.0
JC2	Control	11.22 ± 2.16	
	10 s	$2.46 \pm 1.42^*$	78.1
	10 min	$1.21 \pm 0.44^*$	89.2
	30 min	$1.03 \pm 0.27^*$	90.8
Ingbritt	Control	4.04 ± 1.06	
	10 s	$1.26 \pm 0.29^*$	68.8
	10 min	$0.58 \pm 0.12^*$	85.6
	30 min	$0.50 \pm 0.04^*$	87.6
ATCC 10449	Control	20.30 ± 9.01	
	10 s	$1.44 \pm 0.64^*$	92.9
	10 min	$1.43 \pm 0.64^*$	93.0
	30 min	$1.30 \pm 0.39^*$	93.6
S. criceti			
E49	Control	12.52 ± 9.33	
	10 s	$0.61 \pm 0.90^*$	95.1
	10 min	$0.51 \pm 0.62^*$	95.9
	30 min	$0.36 \pm 0.33^*$	97.1
S. sanguinis			
ATCC 10556	Control	3.79 ± 0.44	
	10 s	$0.65 \pm 0.08^*$	82.8
	10 min	$0.52 \pm 0.10^*$	86.3
	30 min	$0.58 \pm 0.11^*$	84.7
S. oralis			
ATCC 10557	Control	5.93 ± 2.42	
	10 s	$0.41 \pm 0.18^*$	93.1
	10 min	$0.53 \pm 0.41^*$	91.1
	30 min	$0.38 \pm 0.23^*$	93.6
S. mitis			
ATCC 9811	Control	5.04 ± 3.19	
	10 s	$0.92 \pm 0.58^*$	81.7
	10 min	$1.26 \pm 0.95^*$	75.0
	30 min	$0.90 \pm 0.56^*$	82.1
S. gordonii			
Challis	Control	7.99 ± 5.44	
	10 s	$3.05 \pm 0.15^*$	61.8
	10 min	$0.48 \pm 0.16^*$	94.0
	30 min	$0.37 \pm 0.31^*$	95.4

Data of bacterial numbers adsorbed to s-HA are the means from three quintuple experiments with standard deviations.
$^*P < 0.01$ as compared with control for respective bacteria.
[†]Buffered KCl was used for control (without cranberry).

Table 2. Effect of cranberry on cell surface hydrophobicity

Strain	Time of exposure	% of hydrophobicity
S. sobrinus		
6715	0	42.19 ± 11.90
	12.5	23.04 ± 3.76
	25	$16.24 \pm 10.04^{**}$
B13	0	24.07 ± 7.75
	12.5	$-2.10 \pm 4.50^*$
	25	$-0.30 \pm 5.71^*$
S. mutans		
MT8148R	0	58.52 ± 15.00
	12.5	$28.90 \pm 14.00^{**}$
	25	$6.62 \pm 16.45^*$
JC2	0	57.85 ± 13.01
	12.5	$25.95 \pm 1.14^*$
	25	$12.43 \pm 12.86^*$
Ingbritt	0	43.30 ± 7.37
	12.5	$-1.28 \pm 2.00^*$
	25	$-4.44 \pm 5.90^*$
ATCC 10449	0	88.79 ± 10.45
	12.5	$17.50 \pm 7.86^*$
	25	$8.37 \pm 6.10^*$
S. criceti		
E49	0	97.26 ± 1.32
	12.5	$-3.93 \pm 8.41^*$
	25	$-6.10 \pm 3.37^*$
S. sanguinis		
ATCC 10556	0	88.03 ± 5.44
	12.5	$23.10 \pm 3.30^*$
	25	$8.02 \pm 2.15^*$
S. oralis		
ATCC 10557	0	94.74 ± 2.52
	12.5	$21.64 \pm 6.50^*$
	25	$2.83 \pm 4.16^*$
S. mitis		
ATCC 9811	0	88.37 ± 4.61
	12.5	$34.29 \pm 10.05^*$
	25	$-1.38 \pm 2.14^*$
S. gordonii		
Challis	0	82.90 ± 8.86
	12.5	$23.27 \pm 32.41^*$
	25	$-1.71 \pm 6.10^*$

Data are the means from three duplicate experiments with standard deviation.
$^*P < 0.01$ and $^{**}P < 0.05$ as compared with 0 % of cranberry for respective bacteria respectively.

streptococcus strains are summarized in Table 3. The prepared high molecular weight constituents of cranberry juice clearly inhibited the biofilm formation of the streptococci. Biofilm formations by *S. mutans*, *S. criceti*, *S. oralis* and *S. mitis* were significantly inhibited by the cranberry constituents at both 100 μg/ml and 500 μg/ml concentration compared to control ($P < 0.01$). When the dose of the cranberry constituents was increased up to 500 μg/ml, the biofilm formations by *S. sobrinus* 6715 and *S. sanguinis* ATCC 10556 were significantly inhibited ($P < 0.05$).

Discussion

Cranberry juice has been demonstrated to inhibit the adherence of some bacteria (13, 32). Human daily consumption of cranberry juice can reduce urinary tract infections caused by *Escherichia coli* (1). High molecular mass constituents isolated from cranberry juice have been shown to inhibit the adhesion of *E. coli* and *H. pylori* (2, 13, 29). Howell et al. (7) found that high molecule mass proanthocyanidin (condensed tannin) from cranberry juice prevented the expression of the p-fimbriae of *E. coli* and inhibited its adherence activity. In addition, Weiss et al. (29) reported that a high molecular weight constituent of cranberry inhibited the adhesion and coaggregation activities of oral bacteria. These are known to be stable phenolic compounds that exhibit antiviral, antibacterial, antiadhesive, and/or antioxidant properties (18, 19). In an unpublished study, we found that our extracted high molecular weight

Table 3. Inhibitory effect of a high molecular weight constituent of cranberry on the biofilm formation of oral streptococci

Strain	Concentration of cranberry constituent (μg/ml)	Biofilm formation (OD_{595})
S. sobrinus		
6715	0 (Control)	0.311 ± 0.037
	100	0.290 ± 0.058
	500	$0.278 \pm 0.029^{**}$
S. mutans		
JC2	0 (Control)	0.337 ± 0.098
	100	$0.221 \pm 0.048^{*}$
	500	$0.192 \pm 0.026^{*}$
S. criceti		
E49	0 (Control)	1.863 ± 0.252
	100	$1.363 \pm 0.309^{*}$
	500	$1.229 \pm 0.536^{*}$
S. sanguinis		
ATCC 10556	0 (Control)	0.209 ± 0.033
	100	0.192 ± 0.033
	500	$0.180 \pm 0.026^{**}$
S. oralis		
ATCC 10557	0 (Control)	0.920 ± 0.120
	100	$0.531 \pm 0.089^{*}$
	500	$0.551 \pm 0.188^{*}$
S. mitis		
ATCC 9811	0 (Control)	0.755 ± 0.204
	100	$0.504 \pm 0.101^{*}$
	500	$0.522 \pm 0.067^{*}$

Lyophilized high molecular weight constituent of cranberry was dissolved in trypticase soy broth. Data are means from three quintuple triplicate experiments with standard deviations.
$^{*}P < 0.01$ and $^{**}P < 0.05$ as compared with control for respective bacteria respectively.

constituents of cranberry juice did not kill *S. mutans* JC2 but that exposing oral streptococci to 25% cranberry juice for 10 s resulted in a significant reduction of their adsorption to s-HA beads. It is necessary to identify which major ingredients of this high molecular weight extract possess this inhibitory activity in a future study. Our results suggest that cranberry juice inhibits the colonization of streptococci in the initial phase of biofilm formation.

Cell surface hydrophobicity is one of the important factors involved in oral bacterial adherence to the tooth surface (27). Westergren et al. (30) showed that surface hydrophobicity-lacking mutant strains of *S. mutans* and *S. sanguinis* could not adhere to s-HA beads. The hydrophobicity of *S. mutans* is believed to be mainly associated with its cell surface proteins (12). Matsumoto et al. (10) have reported that oolong tea extract polyphenols may inhibit bacterial adherence to the tooth surface by reducing the cell surface hydrophobicity of mutans streptococci. The present study revealed that the cell surface hydrophobicity of some oral streptococci was reduced by the addition of the cranberry juice and that the reduction was dependent on the concentration of the juice. It is probable that the cranberry juice components bond to and/or mask the hydrophobic protein (s) on the cell surface of oral streptococci.

Dental plaque is a biofilm composed of polyspecies of bacteria, and the adhesive ability of these microorganisms seems to be an important pathogenic factor. Therefore, we investigated the inhibitory effect of cranberry juice on the biofilm formation of oral streptococci. The high molecular weight constituents of cranberry juice inhibited the biofilm formation of oral streptococci, including cariogenic strains, suggesting that the daily use of cranberry juice could inhibit dental plaque development.

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