

Original Article

Inhibitory Effect of Cranberry Polyphenol on Cariogenic Bacteria

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Abstract

The purpose of this study was to investigate the effect of cranberry polyphenol fraction on mutans streptococci. Hydrophobicity is an important factor in the adherence of bacteria to the tooth surface. We found that cranberry polyphenol fraction significantly decreased the hydrophobicity of *Streptococcus sobrinus* 6715, *Streptococcus mutans* MT8148R and JC2 in a dose-dependent manner ($p < 0.05$). Biofilm formation by *S. sobrinus* 6715 and *S. mutans* MT8148R was inhibited by 100 $\mu\text{g}/\text{ml}$ cranberry polyphenol fraction ($p < 0.01$). When dosage was increased to 500 $\mu\text{g}/\text{ml}$, biofilm formation by *S. mutans* JC2 was significantly inhibited ($p < 0.05$). Addition of 500 $\mu\text{g}/\text{ml}$ cranberry polyphenol fraction to medium inhibited growth of *S. mutans* MT8148R compared with the control ($p < 0.05$).

Key words: Cranberry polyphenol fraction—Cariogenic bacteria—Biofilm—Hydrophobicity

Introduction

Periodontal disease and dental caries are major factors in tooth loss. Oral biofilm-forming bacteria such as *Streptococcus mutans* and *Streptococcus sobrinus*, in particular, are potent pathogens of dental caries⁵. In these pathogens, hydrophobicity is generally an important factor in their ability to attach to the tooth surface^{12,14}. After adhesion, mutans streptococci can colonize and form biofilm by synthesizing glucan and/or adhering to other dental plaque bacteria.

The American cranberry is a member of the heath family native to North America, and it is known in the USA as a healthy drink containing a lot of vitamin C. Cranberry polyphenol fraction has been shown to inhibit adhesion by various bacteria such as infectious pathogens of the urinary tract, *Helicobacter pylori* and pathogens of oral disease^{2–4,8,10,13}.

The purpose of this study was to investigate the inhibitory effect of cranberry polyphenol fraction on the hydrophobicity and growth of oral streptococci and biofilm formation.

Materials and Methods

1. Preparation of cranberry polyphenol fraction

Cranberry juice concentrate (40 g, Brix 35°; Nippon Del Monte Corp., Tokyo, Japan) was applied to a glass column packed with Amberlite XAD 7HP (Rohm and Haas Co., Philadelphia, PA, USA). Water (100 ml) was passed through the column to remove non-phenolic cranberry constituents. To elute cranberry phenolics, aqueous 70% ethanol (100 ml) was passed through the column. The elution was then concentrated under vacuum and lyophilized to give the polyphenol fraction¹⁶⁾ (0.53 g). Total polyphenol was confirmed to be 62% by the Folin & Ciocalteu method¹¹⁾ (Gallic acid conversion) and total flavanol was confirmed to be 34% by the Vanillin-HCl method¹⁾ (Catechin conversion). The total flavanol value represents the sum of monomeric polyphenols, including catechins, and primary components such as proanthocyanidins.

Cranberry polyphenol fraction was dissolved in dimethyl sulfoxide (DMSO) and added to equal amounts of distilled water. The resulting solution was then sterilized through a membrane filter (pore size, 0.45 μm) and stored at 4°C.

2. Bacterial strains and culture conditions

The strains used in this study were *S. sobrinus* 6715 and B13, and *S. mutans* MT8148R and JC2. The organisms were grown in Todd Hewitt broth (THB; Difco Laboratories, Detroit, MI) at 37°C for 12–24 hr in an anaerobic chamber (N_2 : 80%, H_2 : 10%, CO_2 : 10%). Tryptic soy broth (TSB) was used for biofilm assay.

3. Hydrophobicity assay

Hydrophobicity was determined as described by Rosenberg *et al.*⁹⁾. Briefly, bacterial suspensions in PUM buffer, which contains $\text{K}_2\text{PO}_4 \cdot 3\text{H}_2\text{O}$ (22.2 g/l), KH_2PO_4 (7.26 g/l), urea (1.8 g/l) and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g/l) were adjusted to an optical density of approximately 0.5 at 400 nm using a UV-VISIBLE SPECTROPHOTOMETER (Shimadzu, Kyoto,

Japan). Duplicate samples of 600 μl bacterial suspension and an equal volume of two-fold concentration of cranberry polyphenol (500 $\mu\text{g}/\text{ml}$, 1 mg/ml) in PUM buffer were placed in tubes, and 600 μl hexadecane was added. These tubes were vigorously mixed by vortex-stirring for 60 sec and left to stand for 15 min. Optical density at 400 nm (OD_{400}) of the aqueous phase was then measured. Percentage of hydrophobicity was calculated as follows: $\{[(\text{OD}_{400} \text{ before mixing}) - (\text{OD}_{400} \text{ after mixing})] / (\text{OD}_{400} \text{ before mixing})\} \times 100$. Each isolate was assayed 3 times, and the values obtained were averaged.

4. Biofilm formation assay

We investigated the inhibitory effect of cranberry polyphenol fraction on biofilm formation by *S. sobrinus* 6715 and *S. mutans* MT8148R and JC2 on the bottom of cell culture plates (SUMILON Multi Well Plate, Sumitomo Bakelite Co., Ltd., Tokyo). Biofilm assays were done using the protocol of Loo *et al.*⁶⁾. Briefly, strains of streptococci were cultured in TSB supplemented with 100 $\mu\text{g}/\text{ml}$ or 500 $\mu\text{g}/\text{ml}$ cranberry polyphenol fraction for one day under anaerobic conditions. Media and unattached bacterial cells were removed 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 distilled water each time, bound dye was extracted from the stained cells with 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).

5. Evaluation of bacterial growth

Susceptibility of bacterial growth to polyphenol fraction was evaluated by ATP-bioluminescent assay using the Microbial Cell Viability Assay kit (Promega Corporation, Madison, WI). ATP-bioluminescent activity

Table 1 Effect of polyphenol on cell surface hydrophobicity

Strain	Concentration of polyphenol ($\mu\text{g}/\text{ml}$)	% Hydrophobicity	
<i>S. sobrinus</i>	6715	0	88.15 \pm 9.42
		250	27.30 \pm 15.70*
		500	1.40 \pm 7.72*
	B13	0	50.23 \pm 3.74
		250	7.02 \pm 3.76*
		500	13.94 \pm 1.53*
<i>S. mutans</i>	MT8148R	0	77.24 \pm 14.09
		250	43.64 \pm 11.94*
		500	8.90 \pm 4.04*
	JC2	0	88.78 \pm 4.73
		250	48.27 \pm 16.21*
		500	28.87 \pm 8.76*

Data represent means from 3 duplicate experiments with standard deviations.
*: $p < 0.05$ as compared with control for corresponding strain.

was shown as Relative Light units (RLU). Strains of streptococci were cultured in THB supplemented with 100 $\mu\text{g}/\text{ml}$ or 500 $\mu\text{g}/\text{ml}$ cranberry polyphenol for one day under anaerobic conditions. Medium containing DMSO without polyphenol was used as a control. One hundred μl each strain was added to an equal amount of BacTiter-Glo™ reagent for measurement of bioluminescence at 0, 3, 6, 9 and 12 hr.

6. Statistics

The Mann-Whitney *U*-test was used for quantification of biofilm formation and the hydrophobicity test. The bioluminescence assays for growth curve were repeated more than 3 times to confirm proliferation kinetics.

Results

1. Hydrophobicity assay

The effects of addition of cranberry polyphenol fraction on hydrophobicity are summarized in Table 1. Hydrophobicity differed between strains. Reduction in hydrophobicity was found to be dependent on concentration of cranberry polyphenol fraction. We found a 70–90% reduction in hydrophobicity in

S. sobrinus 6715, *S. mutans* MT8148R and JC2. The reduction in *S. sobrinus* B13 was the largest among the tested strains.

2. Biofilm formation assay

The inhibitory effect of cranberry polyphenol fraction on biofilm formation by mutans streptococcus strains is summarized in Table 2. Cranberry polyphenol fraction significantly inhibited biofilm formation. Biofilm formation by *S. sobrinus* 6715 and *S. mutans* MT8148R was significantly inhibited at concentrations of 100 $\mu\text{g}/\text{ml}$ and 500 $\mu\text{g}/\text{ml}$ compared to the control ($p < 0.01$). When the dose was increased to 500 $\mu\text{g}/\text{ml}$, biofilm formation by *S. mutans* JC2 was significantly inhibited ($p < 0.05$).

3. Evaluation of bacterial growth

To evaluate the effects of cranberry polyphenol fraction on bacterial growth, we used an ATP-bioluminescent assay. The relationship between viable cell count (CFU) and ATP-bioluminescent activity is shown Fig. 1. This result confirmed that ATP-bioluminescence reflected viable cell counts.

The inhibitory effect of cranberry polyphenol fraction on growth of *S. mutans* MT8148R is shown in Fig. 2. The ATP-bioluminescence

Table 2 Inhibitory effect of cranberry polyphenol on biofilm formation by mutans streptococci

Strain	Concentration of polyphenol ($\mu\text{g}/\text{ml}$)	Biofilm formation (OD_{595})
<i>S. sobrinus</i> 6715	0 (control)	1.158 ± 0.685
	100	$0.317 \pm 0.133^*$
	500	$0.169 \pm 0.010^*$
<i>S. mutans</i> MT8148R	0 (control)	1.561 ± 0.387
	100	$0.657 \pm 0.090^*$
	500	$0.193 \pm 0.026^*$
<i>S. mutans</i> JC2	0 (control)	0.808 ± 0.557
	100	0.787 ± 0.400
	500	$0.208 \pm 0.056^{**}$

Cranberry polyphenol was dissolved in TSB. Data represent means from two fourfold experiments with standard deviations. *: $p < 0.01$ as compared with control for respective bacteria. **: $p < 0.05$ as compared with control for respective bacteria.

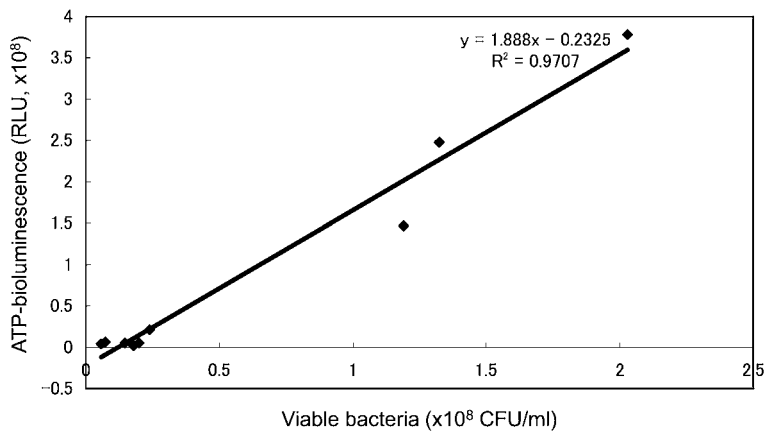


Fig. 1 Relationship between viable cell count (CFU) and ATP-bioluminescence

in *S. mutans* MT8148R treated with $500 \mu\text{g}/\text{ml}$ cranberry was lower than that in the control, suggesting that cranberry polyphenol fraction inhibited growth of *S. mutans* MT8148R in a dose-dependent manner. Cranberry polyphenol fraction, however, showed no inhibitory effect on the growth of the other strains used in this study.

Discussion

Cranberries and cranberry constituents have been shown to possess cariostatic properties.

Cell surface hydrophobicity is an important factor in oral bacterial adherence to the tooth surface¹²⁾. The hydrophobicity of *S. mutans* is believed to be mainly associated with its cell surface proteins⁷⁾. The present study revealed that the cell surface hydrophobicity of mutans streptococci was reduced by the addition of cranberry polyphenol fraction. An earlier study found that cranberry juice decreased hydrophobicity in oral streptococci¹⁵⁾. The results of the present study support that finding, showing a reduction in hydrophobicity in mutans streptococci by addition of cranberry polyphenol fraction. It is probable that polyphenol

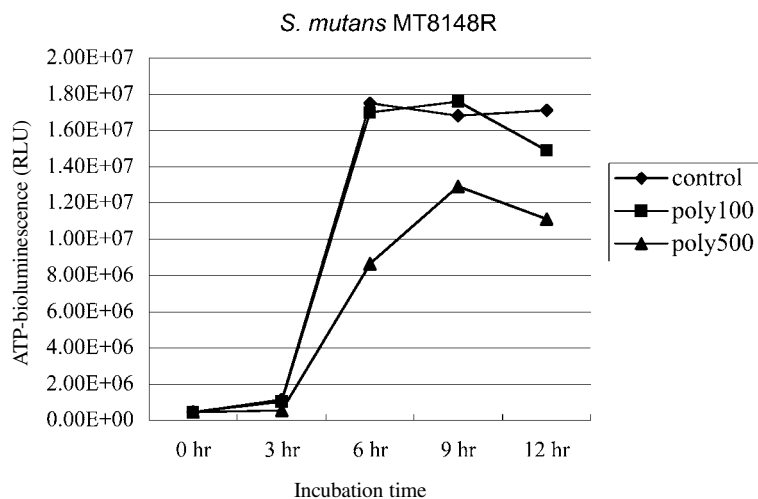


Fig. 2 Inhibitory effect of cranberry polyphenol on growth of *S. mutans* MT8148R. Poly 100, 500 mean cranberry polyphenol, 100 or 500 μ g/ml, respectively.

components, especially proanthocyanidins, bind to and/or mask hydrophobic proteins on the cell surface of oral streptococci.

The ATP-bioluminescence assay revealed that addition of cranberry polyphenol fraction inhibited growth of *S. mutans* MT8148R at 500 μ g/ml, but had no effect on the viability of the other 3 strains tested. This suggests that susceptibility of mutans streptococci to cranberry polyphenol differs among strains. In an earlier study, cranberry juice showed no influence on growth of *S. mutans* MT8148R¹⁵. The cranberry polyphenol fraction used in this study may have contained higher doses of active components than that of cranberry juice.

Dental plaque is a biofilm composed of polyspecies of bacteria, and the adhesive ability of these microorganisms seems to be an important pathogenic factor. Biofilm is a mass of bacteria capable of evading host defense mechanisms and resisting antibiotics. We demonstrated that polyphenol fraction inhibited biofilm formation, and in former studies, we reported that high molecular mass constituents of cranberry juice such as non-dialyzable material (NDM) inhibited the biofilm formation of oral streptococci¹⁵.

Labrecque *et al.*⁴) reported that NDM inhibited biofilm formation by *P. gingivalis*. NDM fraction contains 0.35% anthocyanins and 65.1% proanthocyanidins^{4,13}. Our polyphenol fraction sample contained 34% flavonol, including catechins and primary components such as proanthocyanidins. Gregoire *et al.*³) identified the bioactive constituents in cranberry against *S. mutans* using highly purified isolated compounds, and showed the combined effects of specific flavonol and proanthocyanidins on glucan synthesis and acidogenicity. They suggested that the biological activity of cranberry extracts resulted from the complex mixture of flavonoids rather than a single active compound. These active constituents may have been involved in the polyphenol fraction used in this study.

We examined the effects of cranberry polyphenol fraction on the hydrophobicity, biofilm formation and bacterial growth of mutans streptococcus strains. The results of this study, taken together with those of earlier studies, suggest that daily use of mouthwashes, toothpaste or chewing gum containing cranberry polyphenol fraction might prevent the development of dental plaque.

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References

- 1) Broadhurst RB, Jones WT (1978) Analysis of condensed tannin using acidified vanillin. *J Sci Food Agric* 29:788–794.
- 2) Duarte S, Gregoire S, Singh AP, Vorsa N, Schaich K, Bowen WH, Koo H (2006) Inhibitory effects of cranberry polyphenols on formation and acidogenicity of *Streptococcus mutans* biofilms. *FEMS Microbiol Lett* 257: 50–56.
- 3) Gregoire S, Singh AP, Vorsa N, Koo H (2007) Influence of cranberry phenolics on glucan synthesis by glucosyltransferases and *Streptococcus mutans* acidogenicity. *J Appl Microbiol* 103: 1960–1968.
- 4) Labrecque J, Bodet C, Chandad F, Grenier D (2006) Effects of a high-molecular-weight cranberry fraction on growth, biofilm formation and adherence of *Porphyromonas gingivalis*. *J Antimicrob Chemother* 58:439–443.
- 5) Loesche WJ (1986) Role of *Streptococcus mutans* in human dental decay. *Microbiol Rev* 50: 353–380.
- 6) Loo CY, Coliss DA, Ganeshkumar N (2000) *Streptococcus gordonii* biofilm formation. Identification of genes that code for biofilm phenotypes. *J Bacteriol* 183:1374–1382.
- 7) McBride BC, Song M, Krasse B, Olsson J (1984) Biochemical and immunological differences between hydrophobic and hydrophilic strains of *Streptococcus mutans*. *Infect Immun* 44:68–75.
- 8) Raz R, Chazan B, Dan M (2004) Cranberry juice and urinary tract infection. *Clin Infect Dis* 38:1413–1419.
- 9) Rosenberg M, Gutnik D, Rosenberg E (1980) Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol Lett* 9:29–33.
- 10) Sharon N, Ofek I (2002) Fighting infectious diseases with inhibitors of microbial adhesion to host tissues. *Crit Rev Food Sci Nutr* 42:267–272.
- 11) Singleton VL, Orthofer R, Lamuela-Raventos RM (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol* 299:152–178.
- 12) Weiss E, Rosenberg M, Judes H, Rosenberg E (1982) Cell-surface hydrophobicity of adherent oral bacteria. *Curr Microbiol* 7:125–128.
- 13) Weiss EI, Kozlovsky A, Steinberg D, Lev-Dor R, Bar Ness Greenstein R, Feldman M, Sharon N, Ofek I (2004) A high molecular mass cranberry constituent reduces mutans streptococci level in saliva and inhibits *in vitro* adhesion to hydroxyapatite. *FEMS Microbiol Lett* 232: 89–92.
- 14) Westergren G, Olsson J (1983) Hydrophobicity and adherence of oral streptococci after repeated subculture *in vitro*. *Infect Immun* 40: 432–435.
- 15) Yamanaka A, Kimizuka R, Kato T, Okuda K (2004) Inhibitory effects of cranberry juice on attachment of oral streptococci and biofilm formation. *Oral Microbiol Immunol* 19:150–154.
- 16) Yamanaka A, Kouchi T, Kasai K, Kato T, Ishihara K, Okuda K (2007) Inhibitory effect of cranberry polyphenol on biofilm formation and cysteine proteases of *Porphyromonas gingivalis*. *J Periodontal Res* 42:589–592.

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