Inhibitory Effects of Lingonberry Extract on Oral Streptococcal Biofilm Formation and Bioactivity

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Abstract

Phenolic compounds in fruits such as cranberries have been shown to promote a number of biological activities. The purpose of this study was to investigate the effects of polyphenolic compound-containing lingonberry extract on oral streptococci and compare them with the known anti-cariogenic activity of cranberries. Water-soluble and polyphenol-rich fractions (Fractions I and II, respectively) were isolated from cranberries and lingonberries. The effects of those fractions on the biofilm formation ability and bioactivity of Streptococcus mutans MT8148R, Streptococcus sobrinus 6715, and Streptococcus sanguinis ATCC 10556 were then evaluated. Cranberry or lingonberry Fraction II (at 0.5–1 mg/ml) significantly reduced biofilm formation by S. mutans, S. sobrinus, and S. sanguinis. In contrast, cranberry or lingonberry Fraction I (at 0.5–2 mg/ml) increased biofilm formation by S. mutans and S. sobrinus, but not by S. sanguinis. Fractions I and II (at 1–2 mg/ml) also reduced the bioactivity of S. mutans, while Fraction II (at 0.5 mg/ml) enhanced the bioactivity of all tested strains. The results revealed that lingonberries contained a larger amount of polyphenol than cranberries and that they showed almost the same level of activity against the biofilm formation ability and bioactivity of oral streptococci. This indicates that polyphenol-rich lingonberry fraction offers a promising natural food derivative for prevention of dental caries.

Key words: Biofilm — Dental caries — Dental plaque — Mutans streptococci — Polyphenol

Introduction

Dental caries and periodontitis are infectious diseases caused by the bacteria in dental plaque biofilms, and both diseases can ultimately result in loss of teeth28,29). Although more than 700 bacterial taxa have been identified in dental plaque16, streptococci are the most abundant. In the first stage of dental plaque formation, oral streptococci adhere to
the acquired pellicle on the tooth surface. Among the various oral streptococci, *Streptococcus mutans* and *Streptococcus sobrinus* are the most involved in dental caries due to their strong ability to adhere to the tooth surface through protein antigen c and glucans and their acid production. To prevent dental caries, it is important to prevent colonization by these highly cariogenic microorganisms and/or inhibit plaque formation. The conventional method for prevention is mechanical removal of dental plaque by brushing or the use of broad-spectrum bactericidal agents, such as chlorhexidine. Systemic administration of antimicrobials, however, carries the risk of a number of side effects, including selection of drug-resistant microorganisms.

Therefore, natural products, including a broad range of plant metabolites containing phenolic compounds, have been explored for their anti-cariogenic activities.

Berries contain a wide variety of phenolic compounds, such as anthocyanins, flavonols, and phenolic acid. The most commonly harvested berries are of the genus Vaccinium, which includes several popular commercial berry species, such as cranberry (*Vaccinium macrocarpon*) and lingonberry (*Vaccinium vitis-idaea*). These berries are widely consumed by humans in various forms. The phenolic compounds, such as proanthocyanidins, in these fruits have a number of biological effects. Proanthocyanidins are a type of flavanol, and most of the proanthocyanidins in natural products are found as procyanidins. Cranberry consumption has been reported to prevent urinary tract infection; inhibit the adherence of *Helicobacter pylori*; inhibit the adherence or biofilm formation of cariogenic bacteria and the proliferation of periodontopathic bacteria; attenuate acid production by *S. mutans*; and reduce the risk of several cancers. Lingonberries have been reported to reduce ultraviolet A-induced retinal photoreceptor cell damage; radiation-induced damage; and biofilm formation by uropathogenic *Escherichia coli*, and to stimulate antioxidant activity.

Although there have been several studies on the effects of cranberries on oral microorganisms, the only studies on the effects of lingonberry extract on oral microorganisms investigated coaggregation between *S. mutans* and *Fusobacterium nucleatum* or *Actinomyces naeslundii*. Lingonberries have about twice the polyphenol content of cranberries, suggesting that the polyphenolic compounds in lingonberries would have similar effects on cariogenic bacteria.

The purpose of this study was to clarify the anti-cariogenic activity of lingonberries by investigating the effects of their polyphenolic compounds on the biofilm formation ability and bioactivity of oral streptococci and comparing them with those of polyphenolic compounds from cranberries.

**Materials and Methods**

1. Preparation of polyphenol fractions

Concentrated cranberry juice was obtained from Nippon Del Monte Corp. (Tokyo, Japan), and concentrated lingonberry juice from Fushimi Chemical Co., Ltd. (Kyoto, Japan). A total of 450 g concentrated cranberry juice and 200 g concentrated lingonberry juice was fractionated by repeated column chromatography as described below. The column (Amberlite XAD 7HP, 3 cm × 30 cm; Organo Corporation, Tokyo, Japan) was equilibrated with deionized water and washed with 500 ml deionized water. Concentrated cranberry or lingonberry juice (50 g) was applied to the column and eluted with 1,000 ml of 5% ethanol. These water-soluble fractions were pooled to obtain Fraction I. After these fractions were eluted, the polyphenol-rich fraction (Fraction II) was eluted with 1,000 ml of 70% ethanol. Total polyphenol in each fraction was determined by the Folin-Ciocalteu method (gallic acid conversion).

As proanthocyanidins are essentially polymer chains of flavanols, such as catechin, the total amount of flavanols in each fraction was measured as catechin conversion by the vanillin-HCl method.
2. Bacterial strains

Three Streptococcus strains (S. mutans MT8148R\(^{15}\), S. sobrinus 6715\(^{11}\), and Streptococcus sanguinis ATCC 10556\(^{16}\)) were used. These strains were maintained on blood agar plates (Tryptic Soy Agar [Becton Dickinson Microbiology Systems, Cockeysville, MD, USA] supplemented with 5 µg/ml hemin, 0.5 µg/ml menadione, and 10% horse defibrinated blood) at 37°C under anaerobic conditions (80% N\(_2\), 10% H\(_2\), and 10% CO\(_2\)).

3. Evaluation of biofilm formation

Each strain was pre-cultured overnight in Todd Hewitt broth (THB; Becton Dickinson Microbiology System). Then, 5 µl culture was added to each well of a 96-well cell culture plate (Sumitomo Bakelite, Tokyo, Japan) along with 100 µl THB. After the addition of cranberry or lingonberry Fraction I or II to a final concentration of 0, 0.5, 1.0, or 2.0 mg/ml, the plate was incubated at 37°C for 24 hr under anaerobic conditions. Biofilm mass was evaluated as described previously\(^{36}\). Briefly, the broth was removed from the plate and each well washed with phosphate buffered saline (PBS, pH 7.2). The biofilm that remained in the well was stained with 0.1% crystal violet (Wako Pure Chemical Industries, Tokyo, Japan) for 15 min. The plate was then washed with PBS and the remaining dye extracted with 99% ethanol. The absorbance of extracted dye was measured with a microplate reader (Spectra Max M5; Molecular Devices, Sunnyvale, CA, USA) at 595 nm.

4. Evaluation of bactericidal activity in biofilms

Each strain was cultivated for 24 hr in a 96-well cell culture plate containing 0, 0.5, 1.0, or 2.0 mg/ml cranberry or lingonberry Fraction I or II as described above. The viability of the bacteria was then measured by an ATP-bioluminescence assay using BacTiter-Glo (Promega, Madison, WI, USA). After removing the planktonic bacteria by washing with distilled water, 100 µl BacTiter-Glo solution was added to each well and the plate incubated for 10 min at room temperature. The bioluminescence of each well was measured with a microplate reader for 0.5 sec and recorded as relative light units.

5. Statistical analysis

The effect of the cranberry and lingonberry extracts was analyzed with an ANOVA followed by Bonferroni correction using GraphPad Prism v. 5.0f (Graph Pad Software, San Diego, CA, USA). A p value of less than 0.05 was considered statistically significant.

Results

1. Fractionation of extracts

The quantities in the cranberry and lingonberry fractions are listed in Table 1. Dry weights of 217.4 and 139.4 g, respectively, were obtained from the concentrated samples of cranberry juice (450 g) and lingonberry juice (200 g), and these were applied to the column. Fraction I, which was eluted with 5% ethanol, contained 198.0 and 132.3 g, respectively, of the original sample, and Fraction II, which was eluted with 70% ethanol, contained 9.88 and 7.51 g, respectively. In Fraction I, the polyphenol contents from cranberries and lingonberries were 0.22 and 0.19%, respectively, and the flavanol contents were 0.023 and 0.025%, respectively. In Fraction II, the polyphenol contents from cranberries and lingonberries were 57.8 and 58.7%, respectively, and the flavanol contents were 24.5 and 37.5%, respectively. The polyphenol yields in Fraction II from cranberries and lingonberries were 2.6 and 3.2%, respectively.

2. Effects of polyphenol fractions on biofilm formation

The effects of cranberry and lingonberry Fractions I and II on biofilm formation by S. mutans, S. sobrinus, and S. sanguinis cultured in medium are summarized in Figs. 1 and 2. Biofilm formation by S. mutans was significantly enhanced by the addition of 1.0 or 2.0 mg/ml cranberry or lingonberry Fraction I (Fig. 1a), while biofilm formation by S. sobrinus was enhanced by the addition of 2.0 mg/ml cran-
Fraction II, while the bioactivity of *S. sobrinus* was not affected by lingonberry Fraction II. The bioactivity of *S. sanguinis* was significantly increased when incubated with cranberry or lingonberry Fraction II at 0.5 or 1.0 mg/ml (Fig. 4c).

**Discussion**

In the present study, the yield of polyphenol-rich Fraction II from lingonberries was nearly identical to that from cranberries. Lingonberries have been reported to have about twice the polyphenol content of cranberries\(^\text{10}\). In the present study, the proportions of total polyphenols in the cranberry and lingonberry extracts fractionated from 450 g of concentrated cranberry juice and 200 g of concentrated lingonberry juice were similar, which is in agreement with that earlier report. The polyphenol-rich Fraction II from lingonberries significantly attenuated biofilm formation by oral streptococci, and the magnitude of the reduction in biofilm formation by lingonberry Fraction II was similar to that of cranberry Fraction II.

Lingonberry Fraction II reduced the biofilm formation ability of *S. mutans*, *S. sobrinus*, and *S. sanguinis* to the same extent as cranberry Fraction II. Extract or polyphenol fraction of cranberries was reported to inhibit the adherence of oral streptococci\(^\text{11}\) and the formation of *S. mutans* biofilm\(^\text{4.40}\), and the
activity of cranberry Fraction II observed in the present study agrees with these previous findings. This activity has been attributed to the presence of proanthocyanidins, as proanthocyanidins with degrees of polymerization from 4 to 12 showed strong inhibition activity against glucosyltransferase B, which is involved in the production of extracellular polysaccharide by *S. mutans*. Kylli *et al.* reported that lingonberries and cranberries are rich in proanthocyanidins, with them comprising 63–71% (257.0 mg/100 g and 299.3 mg/100 g dry weight, respectively) of the total phenolic content. The percentages of flavonols/total polyphenolic content in cranberry and lingonberry Fraction II in the present study, at 42.4 and 63.9%, respectively, were similar. Riihinen *et al.* reported that a fraction containing procyanidin polymers from lingonberry inhibited *S. mutans* biofilm formation. Therefore, it is possible that lingonberry Fraction II also contains procyanidin.
At 2 mg/ml, cranberry or lingonberry Fraction II attenuated the bioactivity of S. mutans biofilm cells. Cranberry extract has been reported to inhibit acid production and the metabolic activity of oral streptococci\(^2\),\(^18\), and the present results also suggest that cranberry or lingonberry Fraction II attenuates the metabolic activity of S. mutans. In contrast, Fraction II at 0.5 mg/ml significantly enhanced the bioactivity of all three strains.

It is possible that this was caused by a discrepancy between the production and consumption of ATP due to the effect of Fraction II. This phenomenon has also been observed in microorganisms treated with antibiotics at levels below the minimum inhibitory concentration\(^36\). It is possible that at low concentrations, the extracts caused stress, leading to an increase in bioactivity. In S. sobrinus, the bioac-
tivity of cells treated with 0.5 mg/ml cranberry Fraction II showed an increase, while the bioactivity of cells treated with 0.5 mg/ml lingonberry Fraction II showed no change. This suggests that susceptibility to polyphenols varies among the different species of oral streptococci.

Biofilm formation by *S. mutans* or *S. sobrinus* showed an increase with addition of cranberry or lingonberry Fraction I, whereas that by *S. sanguinis* was unaffected. One explanation for this species-dependent discrepancy is differences in glucan production. *Streptococcus mutans* and *S. sobrinus* produce both water-soluble and water-insoluble glucans, whereas *S. sanguinis* produces only water-soluble glucans. It is possible that Fraction I contains components that enhance the production of water-insoluble glucans. Further analysis is required to test this hypothesis, however. The activity of Fraction I towards *S. mutans* and *S. sobrinus* suggests that it has the potential to promote dental caries. A previous report indicated that cranberry juice inhibits biofilm formation by oral streptococci, however. The results of the present study indicate that the reduction in biofilm formation induced by Fraction II is stronger than the enhancement of biofilm formation induced by Fraction I, and that the polyphenol-rich fraction is more active than the unfractionated material. In contrast to biofilm formation, the bioactivity of oral streptococci treated with Fraction I showed a decrease. This suggests that Fraction I also contains components that attenuate metabolic activity. Further analysis is required to clarify the relationship between this reduction in metabolic activity and increase in biofilm formation.

**Conclusions**

Lingonberries contain a larger amount of polyphenol than cranberries and exhibit almost the same level of activity against biofilm formation by oral streptococci. This indicates that polyphenol-rich fraction of lingonberries offers a promising natural food derivative for prevention of caries.

**Conflict of Interest**

This study was funded in part by Kikkoman Corporation (Noda, Japan). The sponsor of the study had no role in the study design, conduct of the study, data collection, data interpretation, or preparation of the report.

Emiko Kinoshita is an employee of Kikkoman Corporation and was involved in isolating the samples. She had no role in the study design, conduct of the study, data collection, data interpretation, or preparation of the report.

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**Authorship**

All authors except Emiko Kinoshita made substantial contributions to all of the following: (1) the conception and design of the study, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be submitted.

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