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Cranberry high molecular weight constituents promote *Streptococcus sobrinus* desorption from artificial biofilm

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Abstract

Dental biofilm harbouring oral bacteria is highly correlated with the progression of dental diseases. Disruption of biofilm formation via anti-adhesion agents is an alternative means to the antibacterial approach. Previous studies have shown that high molecular weight non-dialysable material (NDM) derived from cranberry juice inhibits the adhesion of *Escherichia coli* and the coaggregation of a variety of oral bacteria. In addition, it inhibits the formation of glucans and fructans synthesised by GTF and FTF. In the present study, we examined the anti-adhesion effect of NDM on *S. sobrinus*. NDM promoted desorption of *S. sobrinus* from biofilm in the presence and absence of extracellular glucans and fructans, although the effect was more pronounced in the absence of these polysaccharides. Precoating of the bacteria with NDM reduced their ability to form biofilm. Our results indicate that NDM could be exploited as an anti-biofilm agent.

Keywords: Cranberry juice; NDM; Biofilm; Anti-adhesion; *Streptococcus sobrinus*

1. Introduction

Several methods of interfering with the accumulation of bacteria in biofilm have been tried. The reduction of biofilm mass by the use of anti-adhesion compounds that prevent bacterial accumulation or initiate detachment of bacteria from the oral surfaces, has given promising results [1–3]. This approach has many advantages, as virtually no bacterial resistance is encountered, unlike other antibacterial or antibiotic treatments.

Sobota [4] and Schmidt and Sobota [5] were the first to report that cranberry juice affects the adhesion of *Escherichia coli* to epithelial cells, a finding supported later by Foo et al. [6]. Burger et al. [7] showed that a high molecular weight, non-dialysable constituent of cranberry (NDM), inhibits *Helicobacter pylori* adhesion, while Weiss et al. [8] demonstrated that NDM not only inhibits but reverses already formed coaggregates of many oral bacterial pairs. A pilot clinical study, in which six weeks of daily rinses with NDM-containing mouthwash were used by volunteers, showed a significant reduction in the salivary mutans streptococcus count and total bacterial count, compared with the control group [9].

Mutans streptococci, such as *Streptococcus sobrinus*, are commensal inhabitants of the dental biofilm. These bacteria as well as others (*S. mutans*) play a significant role in the formation of dental caries [10]. Upon exposure to carbohydrate, these bacteria become the primary initiators of tooth decay. Bacteria colonize tooth surfaces by several interacting mechanisms [10,11], including hydrophobic and electrostatic interactions. The sucrose-dependent adhesion is mediated by polysaccharides produced by bacterial extracellular enzymes; glucosyltransferase (GTF), which synthesize glucans and fructosyltransferase (FTF) which synthesize fructans.

Based on previous observations, we tested whether NDM could detach bacteria from biofilms formed in vitro by *S. sobrinus*.

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2. Materials and methods

2.1. Preparation of NDM

Juice from the American cranberry, *Vaccinium macrocarpon*, was obtained from Ocean Spray Inc. After exhaustive dialysis at 4 °C against distilled water in 15,000 MW cut-off dialysis bags, the juice was lyophilised. The high molecular weight, non-dialysable material, designated NDM, exhibits tannin-like properties, is highly soluble in water, is devoid of proteins, carbohydrates and fatty acids and contains 56.6% carbon and 4.14% hydrogen [12].

2.2. Effect of NDM on bacterial detachment from biofilms

A *Streptococcus sobrinus* 6715 biofilm was constructed on hydroxyapatite (HA) as described by Schilling and Bowen [13]. In brief, 40 mg of HA beads (80 μm in diameter) (Bio-Rad Laboratories, Hercules, CA) were equilibrated with phosphate-buffered-saline (PBS) and incubated for 1 h at 37 °C with clarified whole saliva. After three washes with PBS, the saliva-coated HA (sHA) were incubated for 1 h at 37 °C with cell-free GTF or FTF, prepared as described by Rozen et al. [14]. The saliva and enzyme-coated HA beads were washed three times with PBS and further incubated at 37 °C with radioactively labelled *S. sobrinus* (^{3}H radiolabelled bacteria were prepared by supplementing the bacterial growth medium with 5 mci/ml of ^{3}H-thymidine. After overnight incubation, the bacteria were washed thoroughly and the suspension was adjusted to OD_{540}, 1.0). Following the formation of biofilm for 1 h, the beads were washed three times to remove loosely bound bacteria. NDM at concentrations between 0 and 1.33 mg/ml was added to the coated HA beads, which were further incubated for 1 h at 37 °C. Bacteria-coated HA beads were washed and the number of bacteria remaining on the beads was calculated based on their specific radioactivity (Beta-matic, Kontron, Switzerland). The results are presented as percent adherence.

2.3. Effect of NDM on bacterial desorption from biofilm

The effect of NDM on an existing biofilm of *S. sobrinus* was tested in a constant-depth film fermenter (CDFF). Biofilms were grown by a method similar to that described by Pratten et al. [15]. The CDFF consisted of a rotating turntable with adjusted polytetrafluoroethylene (PTFE) cylinders on which the biofilms were grown as follows: *S. sobrinus* were incubated overnight at 37 °C in 700 ml of brain heart infusion (BHI). Next, the inoculum was pumped into the CDFF for 7 h at 37 °C via a peristaltic pump (ISMATEC SA, Labortechnik-Analytik, Zurich, Switzerland) at a rate of 100 ml/h. BHI supplemented with 1% sucrose was delivered into the CDFF at a rate of 100 ml/h. After 5 days at 37 °C, the PTFE cylinders were removed from the CDFF, washed gently with sterile PBS, and incubated at 37 °C for an additional 16 h with 0. 2 or 4 mg/ml of NDM. Bacteria in the biofilms were then stained with propidium iodide (PI) as follows: plugs were immersed in ethanolamine (1:10) for 10 min. After three washes, the samples were stained with PI (1:100) for 30 min and excess PI was removed by washing. Fluorescence emission was measured using a Zeiss LSM 510 (Carl Zeiss Microscopy, Jena, Germany) confocal laser scanning microscope (CLSM). In each experiment, exciting laser intensity, background level, contrast and electronic zoom size were maintained at the same level. At least two random fields were analysed in each experiment. Z-series optical cross section images were acquired at 5-μm depth intervals from the surface through the vertical axis of the specimen, using a computer-controlled motor drive. Fluorescence intensity (average grey values or pixel intensity) was quantitated and expressed in arbitrary fluorescence units per square micron area, using a computerised image analysis program (Zeiss LSMDummy. LNK Jena Germany).

2.4. Effect of pre-exposed bacteria to NDM on biofilm thickness

The effect of NDM on desorption of biofilm bacteria, which were pre-exposed to NDM, was examined in the CDFF. *S. sobrinus* were incubated with 2 mg/ml NDM in 700 ml of BHI overnight at 37 °C. The inoculum was then pumped into the CDFF, while BHI supplemented with 1% sucrose was pumped into the CDFF through another port. After 48 h of incubation at 37 °C, the PTFE cylinders coated with biofilms were washed, stained with PI and the biofilms were analysed by CLSM as described above.

2.5. Statistical analysis

Data analysis and determination of significance were performed using the Bonferroni test (the data are presented as the mean ± standard deviation). $P \leq 0.05$ was considered statistically significant compared to control group.

3. Results

S. sobrinus detachment from biofilms composed of conditioning films formed from immobilised cell-free GTF or FTF in the presence or absence of sucrose, is shown in Figs. 1 and 2, respectively. In the presence of NDM, extensive desorption of bacteria from GTF biofilms in the absence of in situ formation of glucans (no sucrose) was observed (Fig. 1). The desorption effect was less pronounced in biofilms formed in the presence of sucrose. A similar trend was recorded in biofilms formed from FTF conditioning film. In the absence of in situ formation of fructans, significant bacterial desorption was observed, whereas NDM was less effective in the presence of extracellular fructans.

CLSM analysis of the effect of NDM on bacterial desorption from an established biofilm is shown in Fig. 3;