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## **Inhibitory Effects of Fruit Berry Extracts on *Streptococcus mutans* Biofilms**

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### **Abstract**

Dark-coloured fruit berries are a rich source of polyphenols that could provide innovative bioactive molecules as natural weapons against dental caries. High-quality extracts of cranberry, blueberry, strawberry, and a combination of the three berry extracts (Orophenol) were used to treat 24 h old *Streptococcus mutans* (*Strep. mutans*) biofilms. The grown biofilms were treated with the berry extracts at concentrations ranging from 62.5 to 500 µg/ml. Treated biofilms were assessed for metabolic activity, acidogenicity, biovolumes, structural organisation, and bacterial viability. The biofilms treated with the cranberry and Orophenol extracts exhibited the most significant reductions in metabolic activity, acid production, and bacterial/exopolysaccharide (EPS) biovolumes, while their structural architecture appeared less compact than the control treated biofilms. The blueberry extract produced significant reductions in metabolic activity and acidogenicity only at the highest concentration tested, without significantly affecting bacterial/EPS biovolumes or biofilm architecture. Strawberry extracts had no significant effects on *Strep. mutans* biofilms. None of the berry extracts were bactericidal for *Strep. mutans*. The results indicate that the cranberry extract was the most effective in disrupting *Strep. mutans* virulence properties without significantly affecting bacterial viability. This suggests a potential ecological role for cranberry phenols as non-bactericidal agents capable of modulating pathogenicity of cariogenic biofilms.

**Key Words:** Dental caries, Natural Products, Biofilm ecology.

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Dental caries is a polymicrobial disease caused by dysbiosis in the resident microbiome that can lead to progressive demineralization of dental hard tissues (1). The acidogenic/aciduric bacteria responsible for this multifactorial disease are best described as 'pathobionts', as they are present in low numbers even in the dental plaque of caries-free individuals (2). Local environmental stresses, such as frequent sugar-laden dietary exposures or reduced saliva flow, can allow these pathobionts to gain an ecological advantage over commensal bacteria leading to microbiome dysbiosis and subsequent initiation of the caries lesion.

Modern molecular techniques have shown that the microbial consortia associated with the caries process are remarkably diverse, with *Streptococcus mutans* (*Strep. mutans*) accounting for only a small fraction of the bacterial community implicated in disease pathogenesis (2-4). However, *Strep. mutans* is still regarded as a key contributor to the caries process based on its extraordinary ability to produce both soluble and insoluble glucans from dietary sucrose using glucosyltransferases (Gtfs) (5). Among the different extracellular polymeric substances (EPS), the insoluble glucans are considered to be the prime building blocks of cariogenic biofilms, influencing adherence, physical, and biochemical virulence properties of dental plaque (6). Recent reviews have stressed that mutans streptococci (MS) are largely responsible for insoluble glucan synthesis, with relatively few of the other cariogenic bacteria capable of glucan production (7, 8). *Strep. mutans*, even when present in low numbers, appears to play the major role in orchestrating the initial assembly of the cariogenic biofilm matrix. This paves the way for the other relevant resident aciduric bacteria (e.g., bifidobacteria, some lactobacilli, and *Scardovia* spp.) to become dominant as the biofilm matures (7, 9). Hence, *Strep. mutans* still remains a suitable indicator organism to explore cariogenic virulence properties, and how they may be affected by novel antimicrobial agents.

Traditional chemotherapeutic approaches to oral diseases are falling out of favour due to adverse effects often associated with commonly used synthetic oral biocides like chlorhexidine (CHX) and concerns about the emergence of microbial resistance to these agents. Furthermore, the realization of the key health benefits of a symbiotic oral microbiome has resulted in virulence-targeted therapies being preferred over broad-spectrum antimicrobials (10, 11). This is especially vital for biofilm-mediated diseases like dental caries where reversing the microbiome dysbiosis responsible for caries pathogenesis is more important than simply eliminating the dental plaque biofilm (12). Natural products with subtle antimicrobial effects are an attractive alternative to conventional chemotherapeutics, offering the promise of a 'controlled' ecological approach to dental caries prevention (13).

Dark-coloured fruit berries are among the best dietary sources of polyphenols and are used globally as functional food ingredients. A large body of research has firmly established that the dietary

intake of berry fruits has a positive impact on human health (14). With regards to oral health, specific phytochemicals isolated from cranberry have demonstrated inhibitory effects on *Strep. mutans* virulence factors like hydrophobicity, acid production, aciduricity, and glucan synthesis (15-18). Besides cariostatic effects, polyphenol molecules from cranberry and wild blueberry have also shown the potential to prevent periodontal diseases by inhibiting bacterial proteolytic enzymes and the host-inflammatory response (19-21).

The extraction and isolation of specific bioactive phytochemicals from berry fruits is a time-consuming and costly process. An alternative would be to use commercially available berry extracts that have standardized polyphenol concentrations. Such extracts have the advantage of having consistent phytochemical concentrations, overcoming the problem of compositional variability often associated with natural products. Moreover, being a blend of different polyphenol classes of varying sizes and molecular weights, they should ideally be able to exert multi-target biological effects. Until now, there have been no reports examining the influence of standardized extracts of three commonly used fruit berries (cranberry, wild blueberry, and strawberry) on virulence properties of *Strep. mutans* biofilms. Therefore, the null hypothesis of the study was that none of the selected berry extracts had inhibitory effects against *Strep. mutans* biofilms.

## **Materials and Methods**

### *Natural Product Extracts*

High-quality, organic extracts of cranberry, wild blueberry, strawberry, and a combined product of the three berry extracts (Orophenol) were sourced from Diana Food (Champlain, QC, Canada). The total phenolic content of the berry extracts were 40% for cranberry and Orophenol, and 20% for the wild blueberry and strawberry extracts as determined by the Folin-Ciocalteu assay (22). As per the manufacturer's data sheet, 80% of the polyphenols were proanthocyanidins (PACs), with modest amounts of different flavonols (e.g. quercetin, myricetin), anthocyanins, and phenolic acids making up the remainder. Serial dilutions of berry extracts were made in phosphate buffered saline (PBS; Gibco, Thermo Fisher Scientific, Paisley, UK) before each experiment in concentrations ranging from 62.5 to 500 µg/ml. The final pH of the berry extract solutions was in the range of 7.1 to 7.3.

### *Biofilm Model and Substrate*

The well-established Amsterdam Active Attachment (AAA) biofilm model (23) was used to grow the *Strep. mutans* biofilms. Hydroxyapatite (HA) discs (9.5mm x 2mm; Himed, New York, NY, USA) were attached to nylon clamps of the custom-made stainless- steel lid of the AAA model, such that each disc fits into one well of a standard polystyrene 24-well microtiter plate (Costar 3526; Sigma-Aldrich, New York, NY, USA).

### *Saliva Collection and HA Disc Conditioning*

Institutional ethics approval was obtained from the University of Queensland Human Research Ethics Committee (Approval number 2017000519) for the collection of parafilm-stimulated whole saliva from a single donor, who was asked to abstain from food/drink for at least 2 h before saliva collection. The collected saliva was clarified by centrifugation (4000 *g*, 4°C, 20 min), filter-sterilized (0.22 µm polyethersulphone low-protein binding filter; Millipore, Burlington, MA, USA), dispensed into aliquots, and stored at -80°C. Prior to biofilm formation, 2 ml of the thawed saliva was pipetted into each well of a 24-well plate. The HA discs mounted on the AAA model lid were then transferred to the saliva containing plate and incubated for 1 h at 37°C.

### *Biofilm Preparation*

Biofilms of *Strep. mutans* (ATCC 25175) were grown for 24 h on the saliva-coated HA (sHA) discs as previously described by EXTERKATE *et al.* (23) with some modifications. Briefly, the inoculation medium was an overnight culture of *Strep. mutans* adjusted spectrophotometrically (Infinite 200 Pro; Tecan, Grödig, Austria) to a standard cell count ( $1 \times 10^7$  colony forming units (CFU)/ml) in brain heart infusion (BHI; Merck, Darmstadt, Germany). Immediately after the saliva conditioning, the sHA discs were transferred to a 24-well plate containing the inoculation medium and incubated for 1.5 h at 37°C in an orbital shaker at 80 r.p.m for bacterial adhesion. After this initial inoculation period, the discs were transferred to a new plate containing fresh BHI supplemented with 0.2% sucrose and incubated for a further 22.5 h under similar conditions.

### *Biofilm Rinsing and Treatment*

At the end of the 24 h growth period, the AAA model lid with the biofilm bearing HA discs was rinsed by moving the lid up and down 5 times into sterile PBS and repeating this rinsing procedure twice with fresh PBS to ensure the removal of all non-adherent cells. The biofilms were then treated by transferring them to a 24-well plate containing 2 ml/well of the berry extracts or the vehicle control and incubated for 1 h at 37°C. For assessing the effect of the berry extracts on biofilm metabolic activity and acid production, four concentrations of each extract (ranging from 62.5 to 500 µg/ml), were compared with their respective control-treated biofilms. The berry extracts that showed inhibitory effects against biofilm metabolic activity/acidogenicity were further selected at their highest 500 µg/ml concentration to examine their influence on bacterial viability, biofilm biovolumes and structural organization. After treatment, and prior to any assays, the HA discs with the treated biofilms were dip-rinsed with sterile PBS to remove any excess of treatment solutions.

### *Biofilm Assays*

The effects of the fruit berry extracts on the treated *Strep. mutans* biofilms were evaluated for biofilm metabolic activity (XTT reduction assay), acid production (using a standard curve colorimetric assay), biovolumes/structural organisation (assessed by confocal microscopic imaging) and bacterial viability (based on CFU counts). Each assay was carried out in triplicate and repeated in at least three independent experiments.

#### *Biofilm metabolic activity: XTT reduction assay*

An indirect quantification of the metabolic activity of biofilm cells was obtained from a colorimetric assay that is based on the bioreduction of a tetrazolium salt 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) to a coloured formazan product, the absorbance of which can be measured spectrophotometrically (24). Briefly, XTT (Sigma, St. Louis, MI, USA) salt solution (1 mg/ml in PBS) was prepared, filter-sterilized (0.22 µm pore-size filter), and stored at -80°C until required. Immediately before each assay, a solution of 0.4 mM menadione (Sigma) was freshly prepared. XTT reaction solution was prepared by mixing PBS, XTT stock, and menadione solution in a 79:20:1 volume ratio. The treated biofilm-coated HA discs were transferred to a new 24-well plate containing the reaction solution and incubated in the dark for 3 h at 37°C. Thereafter, 100 µl of the solution from each well was pipetted to a 96-well plate and optical density (OD) measured with a microplate reader (Infinite 200 Pro, Tecan) at 492 nm absorbance.

#### *Acid production assay*

At the end of the biofilm growth period and subsequent treatment, the AAA model lid with the biofilm bearing HA discs were placed in a new 24-well plate containing 2 ml/well of buffered peptone water (Merck) supplemented with 0.2% sucrose. The model was then incubated anaerobically for 3 h at 37°C to allow acid formation. The amount of lactic acid formed during this period was calculated using a standard curve colorimetric assay by measuring the absorbance at 340 nm (25, 26).

#### *Biofilm biovolumes and structural organization: Confocal microscopic imaging*

A novel in situ labeling technique (27) that allows simultaneous visualization and quantification of EPS and bacterial cells within intact biofilms was followed with minor modifications. Briefly, 1 µM Alexa Fluor 647-dextran conjugate (10,000 MW; absorbance/fluorescence emission maxima 647/668 nm; Thermo Fisher Scientific, Scoresby, Australia) was added to the 0.2% sucrose supplemented BHI culture medium. This fluorescence-labeled dextran acts as a primer for glucosyltransferases (Gtf) and gets incorporated into newly formed glucans over the course of biofilm development without staining the bacterial cells at these concentrations. The biofilm bacterial components were labeled at the end of biofilm treatment using 1 µM SYTO 9 (480/500 nm; Thermo Fisher Scientific). Imaging of the

bacterial and EPS components was done using a spectral spinning disc confocal microscope (Nikon, Tokyo, Japan) with argon-ion and helium neon lasers tuned to 488 and 637 nm respectively. Each biofilm was scanned at 5 randomly selected positions and the z series generated by optical sectioning (2.5  $\mu\text{m}$  intervals) at each of these positions. Three independent biofilm experiments were performed and 5 image stacks per experiment were collected. The image-processing software IMARIS (Bitplane, Concord, MA, USA) was used to quantify and characterize the 3D structure of the biofilms. Biovolumes (biomass/substratum surface area) of the bacterial and EPS biofilm components were quantified separately and structural architecture assessed qualitatively.

#### *Biofilm bacterial viability: Colony forming units (CFU) determination*

The treated biofilm bearing HA discs were gently detached from the AAA model clamps and placed into sterile tubes containing 1 ml PBS and dispersed by uniform vortexing at maximum speed for 1 min as described previously (28). The resultant suspension containing the detached biofilm cells was serially diluted ( $10^1$ - $10^4$ ) and a 50  $\mu\text{l}$  aliquot from each suspension was plated in duplicate onto BHI agar by means of an automatic spiral plater (Autoplate; Advanced Instruments, Norwood, MA, USA). A 0.12% chlorhexidine (CHX) positive control was included in this assay. The plates were incubated in a 5%  $\text{CO}_2$  incubator at 37°C for 48 h. Subsequently, the number of CFU was totaled with a digital colony counter (Thermo Fisher Scientific) and the CFU values were  $\log_{10}$  transformed prior to analysis.

#### *Statistical analyses*

After normality of data distribution was checked with the Shapiro-Wilk test, either the one-way ANOVA or the Kruskal-Wallis H-test was chosen to look for statistical differences between each berry extract treatment and their respective control treatment. For parametric data, a Dunnett's post hoc test was done for multiple comparisons of the treatment groups to the control group. For non-parametric data, the Dunn-Bonferroni post hoc test for multiple comparisons was used. The level of significance was set at 5%. Statistical software SPSS version 25 (IBM, New York, NY, USA) was used to perform the analyses.

## **Results**

### *Metabolic activity*

The effects of the different berry extracts on metabolic activity of 24 h *Strep. mutans* biofilms are summarized in Table 1. When compared to the control-treated biofilms, the highest reduction in metabolic activity was seen in biofilms treated with the cranberry extract at concentrations of 500  $\mu\text{g}/\text{ml}$  (32% reduction,  $P = 0.000$ ) and 250  $\mu\text{g}/\text{ml}$  (29% reduction,  $P = 0.000$ ), with the 125  $\mu\text{g}/\text{ml}$  cranberry extract effecting a more modest decrease (14% reduction,  $P = 0.005$ ) in metabolic activity



of the *Strep. mutans* biofilms. Wild blueberry extracts significantly suppressed metabolic activity only at the 500 µg/ml concentration (14% reduction,  $P = 0.038$ ), while none of the strawberry extract concentrations appeared to have significant inhibitory effects on biofilm metabolism. However, the combination of the three berry extracts (Orophenol) was also able to significantly reduce metabolic activity at the 500 µg/ml (25% reduction,  $P = 0.000$ ), 250 µg/ml (22% reduction,  $P = 0.000$ ), and 125 µg/ml concentrations (18% reduction,  $P = 0.001$ ).

#### *Acid production*

Significant reductions in lactic acid production were seen when the *Strep. mutans* biofilms were treated with the 500 µg/ml of cranberry (46% reduction,  $P = 0.001$ ) and Orophenol extracts (40% reduction,  $P = 0.000$ ) when compared to their respective control-treated biofilms (Table 1). Significant reductions in lactic acid production were also observed for biofilms treated with 125-250 µg/ml concentrations of the cranberry and Orophenol extracts, while only the 500 µg/ml concentration of the wild blueberry extract was able to effect a significant decrease in lactic acid synthesis (24% reduction,  $P = 0.000$ ). The strawberry extract did not elicit any significant effect on acid production. The decrease in acid production largely corresponded with the reduced metabolic activity shown in the XTT assays for all the test agents.

#### *Biovolumes and structural organization*

Analysis of the confocal imaging data revealed that the cranberry and Orophenol extracts produced both quantitative (Table 2) and qualitative alterations (Fig. 1) in the *Strep. mutans* biofilms. The cranberry treated biofilms exhibited significant reductions in biovolumes of both bacterial ( $P = 0.03$ ) and EPS ( $P = 0.007$ ) components compared to the control-treated biofilms, while the biofilms treated with Orophenol also showed significantly lower biovolumes of bacteria ( $P = 0.04$ ) and EPS ( $P = 0.015$ ). The wild blueberry extract did not exert significant effects on EPS or bacterial biovolumes. Qualitatively, treatments with cranberry and Orophenol resulted in biofilms that were less compact, with the biofilm structure interspersed with areas of porosity (Fig. 1).

#### *Bacterial counts*

The test agents did not significantly impact bacterial viability of the treated *Strep. mutans* biofilms as indicated by CFU counts (Fig. 2). The population of bacterial colonies recovered from the biofilms treated with the berry extracts showed slightly lower numbers of recoverable viable cells compared with the vehicle control (<0.5 log decrease in CFU/ml), however none of them appeared to be bactericidal for *Strep. mutans* in the biofilms. In contrast, CHX severely affected the viability of *Strep. mutans* (>3 log decrease in CFU/ml).

## Discussion

The results of this study indicate that certain dark-coloured berry extracts may provide useful biofilm effects by inhibiting virulence behavior of *Strep. mutans* without significantly affecting bacterial viability. The organic berry extracts used in this study were standardized in terms of their polyphenol content, overcoming problems of compositional variation often associated with phytochemical extraction and geographic or seasonal influences. The high content of PACs in these extracts can be particularly advantageous, as PACs are known to be the most effective flavonoids against *Strep. mutans* virulence factors. Furthermore, the natural origin of these extracts suggest that they may find easier public acceptance for long-term control over biofilm-mediated diseases like dental caries. These water soluble sugar-free molecular extracts are thus ideal for a potential incorporation into oral care products for daily use.

The AAA biofilm model used in this study has been recommended for the initial screening of new anticaries agents (29). Important advantages of this model are that the biofilm formation is highly reproducible, there is active attachment of bacteria to the substrate, it allows high-throughput screening of multiple compounds and concentrations in a single experiment, and the treatment and rinsing steps can be consistently controlled (23, 30). Although the monospecies biofilm used in the present study does not reflect the complex nature of dental plaque, it is useful for investigating specific effects of the test agents on *Strep. mutans* metabolism, growth, and biofilm architecture, as a prelude to later studies using polymicrobial biofilms.

The XTT assay results indicate that the cranberry and Orophenol extracts effected the most significant reductions in biofilm metabolic activity. The influence of the different berry extracts on bacterial metabolism largely corresponded with their effects on acid production and EPS biovolumes. The difference in the inhibitory effects between the test agents can be attributed to the higher phenolic content in the cranberry and Orophenol extracts compared to the blueberry and strawberry extracts (40% vs. 20%), and, more importantly, to the different types of phytochemicals present in these extracts. For instance, the double interflavan A-linked proanthocyanidins (A-PACs) are uniquely found only in cranberry, with the PAC subunits in other berries and fruits usually linked via a single B-type bond (31). The A-PACs have been shown to have the most potent effects against *Strep. mutans* glycolytic enzymes and Gtfs when compared with other berry flavonoids (15-17). As the inhibitory effects of the combined Orophenol extract was not significantly better than the cranberry extract alone, its biological activity could predominantly also be attributed to the cranberry phenols, rather than potential synergism between the three extracts present in it.

Considering that the production of lactic acid is an important characteristic of cariogenic biofilms, it is noteworthy that the biofilms treated with cranberry and Orophenol extracts showed

significantly reduced acidogenicity. This is consistent with previous reports showing cranberry flavonoids inhibiting *Strep. mutans* glycolytic activity. These studies suggested that the polyphenols responsible for inhibitory activity against bacterial acid production were mainly the quercetin flavonols and a low molecular weight PAC dimer called procyanidin A<sub>2</sub> (16, 17). It is unlikely that the larger PAC oligomers can directly affect intracellular bacterial glycolytic enzymes, though they may still have deleterious effects on membrane components of the glycolytic pathway (32). Quercetin is also known to be a non-competitive inhibitor of the proton-translocating F-ATPase activity that is critical to the survival of acidogenic bacteria in the low pH biofilm environment they create (33). Suppressing dental plaque acidogenicity and aciduricity can potentially create a more favourable environment for commensal bacteria to grow and dominate the biofilm microbial community.

Post-treatment confocal images (Fig. 1) indicate that bioactive phytochemicals present in the cranberry and Orophenol extracts disrupted biofilm integrity and structural architecture. This qualitative assessment was consistent with quantitative data showing biovolumes of both the EPS and bacterial components were significantly reduced (Table 2). Like acidogenicity, EPS are critical to *Strep. mutans* virulence as they provide structural integrity and bulk to dental biofilms, enhance bacterial adherence and co-aggregation, and can act as a substrate source for sustained acidogenesis (6). The polyphenols responsible for the reduced EPS are believed to be the flavonols and A-linked PAC oligomers. Flavonol-mediated Gtf inhibition has been related to its unsaturated double bond between C2 and C3, as anthocyanins that lack the C2-C3 double bond exhibited only modest inhibitory activity against glucan synthesis (16). For the PACs, those with degrees of polymerization between 4 and 12 were considered optimal for interaction and inhibition of Gtfs, as any greater polymerization actually diminished inhibitory effects (32). It is reasonable to infer that the polyphenol-mediated inhibition of glucan synthesis (particularly of the insoluble glucans) would result in a debilitated biofilm structure and disturbed dynamics. This could potentially result in cariogenic biofilms becoming more susceptible to inimical influences of remineralizing agents and antimicrobials.

The anti-Gtf effects of berry phenols could also indirectly be responsible for the decrease in bacterial biovolumes observed in the study. A drop in the EPS content not only reduces overall biofilm bulk and volume, but also affects bacterial biomass (especially in the hydrated stage) by limiting binding sites for bacterial adhesion and co-aggregation. Besides disrupting Gtf-mediated bacterial adhesion, a high molecular-weight non-dialyzable fraction of cranberry has been shown to inhibit bacterial cell surface hydrophobicity, further affecting bacterial adhesion to tooth surfaces (18, 34). The inhibitory effects of cranberry polyphenols on bacterial adhesion could largely be responsible for the reduced bacterial biomass seen in the confocal 3D images, rather than any bactericidal effects. Further investigation is required to corroborate the anti-adhesion effects of cranberry phenols.

The CFU data confirms that none of the berry extracts had a significant effect on the population of viable cells in contrast to the CHX control (Fig. 2). The ability of berry phenols to target cariogenic virulence properties without significantly affecting microbial viability is an important advantage over the broad bactericidal effects of traditional oral biocides. The use of agents like CHX for caries prevention in high-risk patients often results in susceptible tooth surfaces being repopulated with the same disease-causing microbiome that was eliminated, once the chemotherapeutic intervention stops (9). On the other hand, natural products that can disrupt biofilm acidogenicity, glucan synthesis, and structural architecture are more likely to exert an ecological pressure that favours health-associated microbial plaque communities. Animal model studies have confirmed that natural products with virulence-targeting properties can reduce caries incidence and severity despite lacking bactericidal activity (15, 35).

It is possible that berry phenols will have both short-term and long-term effects on bacterial biofilms. Cranberry A-type PACs are known to irreversibly bind to proteins, forming protein-polyphenol complexes, that can impair the activity of glycolytic and Gtf enzymes over a period of time (16, 17). Furthermore, cranberry PACs binding to salivary proteins may confer improved substantivity beyond their brief period of exposure in the mouth (36). PACs in other fruit berries lack the rigidity conferring A-type linkage partly explaining their lower biological effects observed in this study.

Prevention of dental caries has traditionally depended on limiting dietary sugar exposures and meticulous oral hygiene using fluoride dentifrices. With dental caries now recognized as a disease of stress-induced microbiome dysbiosis, there is an increased focus on ecological preventive measures that can shift the dental plaque biofilm from a state of dysbiosis to a state of symbiosis (37). While fluoride will remain the cornerstone of caries prevention, its cariostatic effects are primarily physiochemical, exhibiting only short-term reversible biological effects against biofilm metabolism even at high concentrations (38). Provision of non-fluoride biofilm-modifying interventions, that are designed to repopulate teeth with health-associated microbiomes, can deliver small but relevant benefits over a prolonged period, and these could be particularly useful for caries control in high-risk populations (1, 9). Cariogenic virulence-targeting natural products are one of the several ecological caries preventive approaches currently under investigation (12). Among the potential cariostatic natural products, dark-coloured berry fruits hold particular promise because of their high phytochemical content, wide consumption, and proven effects on systemic health and disease. There is a growing body of evidence that suggests biological activities of berry phenols extend beyond their anti-oxidant effects to include regulating metabolic enzymes, gene expression, and subcellular signaling pathways (14), all actions that can potentially play a role in reducing virulence of *Strep. mutans*.

Prudency is required when interpreting the presented data to avoid over evaluation of the cariostatic effects. Our in vitro monospecies biofilms clearly does not reflect the complex polymicrobial, ecological, and environmental interactions taking place in the oral cavity. However, we have demonstrated that the cranberry extract used in this study is particularly effective in inhibiting *Strep. mutans* pathogenicity without necessarily killing the target bacteria, and are thus promising candidates for development as ecological caries preventive agents. They could possibly act by modulating dental plaque virulence properties, while retaining the benefits of the symbiotic resident oral microbiome. Further studies using polymicrobial biofilm models and pilot clinical trials with the cranberry extract incorporated into oral care products are currently underway to better elucidate whether the shown biological effects can actually result in a favourable microbial ecological change in dental plaque.

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### Conflicts of interests

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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### Figure Legends

*Fig. 1.* 3D confocal image stacks: representative reconstruction of the structural organization of 24 h *Strep. mutans* biofilms following treatment with (A) vehicle control, (B) cranberry, (C) Orophanol, and (D) wild blueberry. The structures depicted in green (SYTO 9) are metabolically active bacterial cells, while the structures in red (Dextran, Alexa Fluor) represent the extracellular polymeric substances. The biofilms treated with cranberry and Orophanol appear less compact than the control biofilms with areas of porosity.

*Fig. 2.* Average number of CFUs recovered from 24 h old *Strep. mutans* biofilms after treatment with the different test agents. No significant differences were found between the berry extracts and the control-treated biofilms, in contrast to the CHX-treated biofilm which significantly affected viability of *Strep. mutans*.