Philip, N., Bandara, H. M. H. N., Leishman, S. J., & Walsh, L. J. (2019). Effect of polyphenol-rich cranberry extracts on cariogenic biofilm properties and microbial composition of polymicrobial biofilms. *Archives of Oral Biology*, *102*, 1-6. https://doi.org/10.1016/j.archoralbio.2019.03.026

Title: Effect of polyphenol-rich cranberry extracts on cariogenic biofilm properties and microbial composition of polymicrobial biofilms

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Running title: Ecological effects of cranberry phenols

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Abstract

Objective: To investigate the effect of cranberry extracts on saliva-derived polymicrobial biofilms with regards to biofilm biomass, acidogenicity, exopolysaccharide (EPS)/microbial biovolumes, colony forming unit (CFU) counts, and the relative abundance of specific caries- and health-associated bacteria.

Methods: Saliva-derived polymicrobial biofilms were grown for 96 h in a cariogenic environment and treated for 2 min every 12 h over the entire biofilm growth period with 500 μg/mL cranberry extract or vehicle control. The effect of the cranberry extract on biofilm behaviour was evaluated using different assays and its influence on key cariogenic and health-associated bacterial populations was assessed with a microarray real-time quantitative PCR method.

Results: Cranberry-treated biofilms showed significant drops in biomass (38% reduction, $P < 0.001$), acidogenicity (44% reduction, $P < 0.001$), EPS/microbial biovolume ratios ($P = 0.033$), and CFU counts (51% reduction, $P = 0.001$). Furthermore, the cranberry extracts effected a significantly lower relative abundance of caries-associated *Streptococcus sobrinus* (fold change 0.004, $P = 0.002$) and *Provotella denticola* (0.002, $P < 0.001$), and a significantly higher relative abundance of the health-associated *Streptococcus sanguinis* (fold change 90.715, $P = 0.001$).

Conclusions: The cranberry extract lowered biofilm biomass, acidogenicity, EPS/microbial biovolumes, CFU counts, and modulated a beneficial microbial ecological change in saliva-derived polymicrobial biofilms.

Key Words: Cranberry; Microbial ecology; Dental caries

1. Introduction

Dental caries is a biofilm-mediated disease characterized by a highly dynamic, polymicrobial, and diet-driven pathologic process [Bowen et al., 2018]. Local environmental stresses (e.g. frequent sugar exposures, poor oral hygiene, xerostomia) induce dysbiosis in the resident microbiome resulting in microbial community shifts that ensues dominance of acidogenic/aciduric microorganisms over health-associated commensal species [Marsh, 2018]. If the microbiome dysbiosis is not reversed, the assembled virulent plaque biofilms will cause enamel demineralization, eventually leading to the clinical onset of cavitation [Burne, 2018]. The ecological antagonism between commensal and cariogenic bacteria is thus a major factor shaping the composition and virulence of dental biofilms [Huang et al., 2016]. When in equilibrium, a symbiotic oral microbiome can not only control the growth of cariogenic bacteria, but can also play a positive role in maintaining oral health by delivering important benefits like immunologic priming and down-regulation of excessive pro-inflammatory responses [Marsh et al., 2015].

One consequence of the evolution of caries etiological paradigms is the realization that current caries preventive approaches do not fully address the microbiome dysbiosis that is fundamental to the disease process [Philip et al., 2018a]. Fluoride, which is regarded as the gold standard among anticaries agents, has only limited antimicrobial actions, and is often not able to cope with the massive cariogenic challenge encountered in high-risk individuals [ten Cate and Cummins, 2013]. On the other hand, commonly used biocides are likely to indiscriminately eradicate oral microflora, including those beneficial to health [Philip et al., 2018b]. Once the chemotherapeutic intervention stops, susceptible tooth surfaces are often repopulated with a microbiome similar in composition to one that was eliminated, resulting in the continuation of the disease process [Burne, 2018]. This suggests the need to develop additional measures that can reverse the dental plaque dysbiosis and complement the cariostatic effects of fluoride on the de-/remineralization equilibrium.

Plant-derived natural products may be better able to modulate the behaviour of dental biofilms, in line with the concept of targeting virulence rather than broadly affecting microbial growth or viability [Cegelski et al., 2008]. Among the several cariostatic natural products studied, cranberry polyphenols hold particular promise as they can disrupt critical cariogenic virulence factors such as bacterial adhesion, acidogenicity, aciduricity, glucan synthesis, and biofilm structural architecture without affecting microbial viability [Duarte et al., 2006; Feng et al., 2013; Gregoire et al., 2007; Koo et al., 2010; Philip et al., 2019]. Furthermore, microbial resistance is less likely to develop against such natural flavonoids as they are chemically diverse and have multiple modes of antimicrobial action [Koehn and Carter 2005]. This potentially makes it an ideal agent to reduce overall biofilm virulence and ecologically modify the dental plaque microbial community.

Most previous studies of cranberry polyphenols have focussed on its effects on mono-species *Streptococcus mutans* biofilms [Duarte et al., 2006; Feng et al., 2013; Gregoire et al., 2007; Philip et al., 2019]. To date, its influence on polymicrobial biofilms growing in a sucrose-rich environment has not been

examined. There is also a need to ascertain whether cranberry phenols could promote beneficial microbial ecological changes in the biofilm. Thus, the present study was undertaken to explore whether standardized polyphenol-rich cranberry extracts could inhibit the cariogenic virulence properties of saliva-derived polymicrobial biofilms, and the relative abundance of certain health- and caries-associated bacterial species. The null hypothesis of the study was that the cranberry extracts did not did not influence biofilm behaviour or its microbial composition.

2. Materials and Methods

2.1 Cranberry extracts

High-quality organic extracts of cranberry were sourced from Diana Food (Champlain, QC, Canada), and used to treat the polymicrobial biofilms. As per the manufacturer's data sheet, $\geq$ 80% of the polyphenols in these cranberry extracts were A-linked proanthocyanidins (A-PACs), with modest amounts of different flavonols (e.g. quercetin, myricetin), anthocyanins, and phenolic acids making up the remainder. The polyphenol concentrations in these water-soluble, sugar-free cranberry extracts have been standardized by the manufacturer, ensuring uniform therapeutic effects. Based on previous studies of the same extracts against *S. mutans* biofilms, a concentration of 500 µg/mL was chosen [Philip et al., 2019]. The cranberry extract was diluted in 0.15 M phosphate buffered saline (PBS; Gibco, Thermo Fisher Scientific, Paisley, UK) just prior to each treatment. The final pH of the treatment solution was in the range of 7.1-7.2. PBS was used as the vehicle control in the different assays.

2.2 Saliva collection

With approval of the institutional ethics committee (approval number 2017001492), parafilm-stimulated whole saliva was collected from 16 caries-free healthy adult donors who had not used antibiotics/mouthwashes in the previous 3 months. The donors did not brush their teeth the morning before saliva donation and abstained from food for 2 h prior to saliva donation. The saliva was pooled, diluted 2-fold with 60% sterile glycerol, dispensed into aliquots and stored at -80°C as described previously [Exterkate et al., 2010; Huang et al., 2017].

2.3 Polymicrobial biofilm formation and treatments

Saliva-derived polymicrobial dental biofilms were grown for 96 h on hydroxyapatite (HA) discs (9.5mm diameter x 2mm thick; Himed, New York, NY, USA) in the high-throughput Amsterdam Active Attachment (AAA) model [Exterkate et al., 2010]. Briefly, the HA discs were fitted to the custom-made AAA model lid such that each HA disc fitted into one well of a polystyrene 24-well flat-bottomed microtiter plate. Prior to biofilm formation, and in order to partly replicate the salivary pellicle, the HA discs were coated with cell-free clarified human saliva as described previously [Philip et al., 2019]. The saliva-coated HA discs (s-HA) were moved to a 24-well plate containing 2 mL/well of the 500 µg/mL cranberry extract solution as a pre-treatment for 2 min. The pre-treated s-HA discs were shaken gently to remove excess treatment solutions

and transferred to a new 24-well plate containing the 2 mL/well of the biofilm inoculum. The inoculation medium for the polymicrobial biofilms was 50-fold dilution of the pooled saliva-glycerol mixture into the mucin-rich McBain medium [McBain et al., 2005] supplemented with 1% (w/v) sucrose. The AAA model was then incubated anaerobically at 37°C in an orbital shaker at 80 rpm. The sucrose-containing growth medium was replenished every 8 h, while subsequent biofilm treatments took place every 12 h, until the end of the 96 h growth period. All treatment exposures were for 2 min, with the biofilm-laden HA discs dip-rinsed 5 times in PBS before each treatment to remove non-adherent cells. The biofilms were also rinsed post-treatment with PBS to remove residual treatment solutions.

2.4 Biofilm analyses

After the final treatment, the influence of the 500 µg/mL cranberry extract on the 96 h-old polymicrobial biofilms was assessed using different assays to estimate biofilm biomass, acidogenicity, exopolysaccharide (EPS)/microbial biovolumes, structural organization, and colony forming unit (CFU) counts. All the biofilm assays were performed in triplicate and repeated in 3 independent experiments. The relative abundance of specific caries- and health-associated bacteria was estimated from duplicate biofilms obtained from 3 separate experiments using a real-time quantitative polymerase chain reaction (qPCR) assay. The assay procedures are briefly described below.

2.4.1 Biofilm biomass: Crystal violet (CV) assay

The CV assay was used to quantify biofilm biomass at 96 h. The biofilms were carefully rinsed in PBS three times and stained with 0.1% CV solution for 15 min at 25°C without shaking. After staining, the cells were again washed in PBS to remove excess stain and air-dried at room temperature. The discs with the stained biofilms were then transferred to a 24-well plate containing 2 mL/well of 10% (v/v) acetic acid and incubated for 30 min at 25°C. At the end of the incubation period, 100 µL of the acetic acid solution was transferred to a 96-well plate and its absorbance measured at wavelength of 570 nm [Bandara et al., 2016].

2.4.2 Acidogenicity: Acid production assay

The biofilm bearing HA discs were transferred to a 24-well plate containing 2 mL/well of buffered peptone water (BPW, Merck, Darmstadt, Germany) supplemented with 0.2% sucrose and incubated anaerobically at 37°C for 3 h under anaerobic conditions. Lactic acid concentrations formed by the biofilms during this period was then determined using the lactate dehydrogenase assay [Exterkate et al., 2010].

2.4.3 EPS/Microbial biovolumes and structural organization: Confocal microscopy

The effect of the cranberry extract on structure of the polymicrobial biofilms was evaluated by confocal microscopic imaging as described previously [Klein et al., 2011]. Briefly, 1 µM Alexa Fluor 647-labeled dextran conjugate (647/668 nm; Thermo Fisher Scientific, Scoresby, Australia) was added to the culture medium to label the EPS component of the biofilm during the biofilm growth phase. Biofilm microbial

components were labeled at the end of the growth period using 1 µM SYTO 9 (480/500 nm; Thermo Fisher Scientific). The images were obtained using a spectral spinning disc confocal microscope (Nikon, Tokyo, Japan) at 5 randomly selected positions for each biofilm. Three dimensional (3D) renderings of biofilms and the quantification of EPS/microbial biovolumes was assessed with IMARIS 8.0 (Bitplane, Concord, MA, USA). The biovolume was defined as the volume of the biomass (μm^3) divided by substratum surface area (μm^2). The effect of the treatment and control solutions on biofilm structural architecture was assessed qualitatively from the confocal 3D images.

2.4.4 Microbial counts

The treated biofilms were dispersed in 1 mL sterile PBS by uniform vortexing at maximum speed for 1 min and serially diluted as previously described [Philip et al., 2019]. Briefly, 50 µL aliquots of the microbial suspensions were plated on tryptic soy agar blood plates and incubated anaerobically at 37°C for 48 h. Microbial colonies were counted from the dilution that allowed visualization of distinct isolated colonies. The CFU/mL values were calculated after correcting for the relevant dilution factor.

2.4.5 Ecological effects: DNA extraction and real-time quantitative PCR (qPCR)

The treated polymicrobial biofilms were dispersed as described previously for the CFU assay. Bacterial DNA was then extracted from the microbial suspension using the MO BIO Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) following manufacturer's instructions. RNA was removed using RNAse A (Thermo Fischer Scientific) and the amount of isolated DNA was quantified spectrophotometrically.

The bacterial load of each of the 14 bacterial species of interest were determined using a custom-made qPCR array (16 x 24 format; Qiagen, Hilden, Germany). The bacteria selected included 8 caries-associated bacterial species (*Actinomyces gerensceriae*, *Lactobacillus gasseri*, *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus parasanguinis*, *Scardovia wiggsiae*, *Provotella denticola* and *Veillonella parvula*) and 6 health-associated commensal bacterial species (*Streptococcus sanguinis*, *Streptococcus mitis/oralis*, *Steptococcus salivarius/thermophilus*, *Corynebacterium durum*, *Rothia aeria/dentocariosa* and *Neisseria flavescens*). The target was the 16S rRNA gene of the relevant bacterium, with probes designed using the GreenGene database for 16S sequences. The DNA sample was mixed with a proprietary master mix and robotically dispensed into a 384-well plate (10µL/well, 7 ng DNA/well) containing freeze-dried primers and fluorogenic probes for each of the bacterial 16S rRNA genes tested. Arrays also contained a positive PCR control to test for inhibitors in the sample, and a non-template control to account for assay background. Reactions were performed with the 384-well plate QuantStudio™ 6 Flex Real-Time PCR sequence detection system (Thermo Fisher Scientific) with the following cycling conditions: enzymatic activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 2 min. Data was analysed using the sequence detection system software (QuantStudio v1.3; Thermo Fisher Scientific).

2.5 Statistical analysis

Depending on the normality of data distribution, either an independent samples t-test or the Mann-Whitney *U*-test was chosen to compare the cranberry- and control-treated biofilms for the biomass, acid production, EPS/microbial biovolumes, and CFU counts. Data was considered statistically significant if the 2-tailed *P*-value was < 0.05. For the qPCR assay, the fold change of each bacterial amplicon in cranberry- versus control-treated biofilms was calculated using the comparative cycle threshold method (ΔΔCt). Briefly, for each sample, the Ct value of the individual bacterial species was normalized to the mean Ct value of all bacterial species ($\Delta Ct^{\text{bacteria species}} = Ct^{\text{bacteria species}} - Ct^{\text{mean of all bacterial species}}$). The ΔΔCt for cranberry- versus control-treated biofilms was then calculated for each bacterial species as follows: $\Delta\Delta Ct = \Delta Ct^{\text{bacterial species (cranberry)}} - \Delta Ct^{\text{bacterial species (control)}}$. Fold increase, or decrease, in abundance was calculated based on the formula $2^{-\Delta\Delta Ct}$. For each bacterial species, independent t-tests were performed to test for differences between the cranberry- and control-treated biofilms using the ΔCt values [Yuan et al., 2006]. *P*-values were considered significant only if they were less than the Simes critical *P*-value [Simes,1986]. Statistical software SPSS version 25 (IBM, New York, NY, USA) was used to perform the analyses.

3. Results

3.1 Cranberry extract reduces biomass of polymicrobial biofilms

The cranberry extract significantly inhibited *(P* < 0.001) the biomass of the 96 h polymicrobial biofilms, with the cranberry-treated biofilms showing 38% mean reduction in biomass compared to the control-treated biofilms (Table 1).

3.2 Cranberry extract inhibited polymicrobial biofilm lactic acid production

The cranberry extract caused a 44% reduction in lactic acid production (Table 1). Lactic acid concentrations of 6.2 ± 1.9 mM/L were produced from the cranberry-treated biofilms compared to the 11.2 ± 3.8 mM/L lactic acid generated from the control-treated biofilms (*P* < 0.001).

3.3 Cranberry extract reduced EPS/microbial biovolumes and disrupted biofilm structure

The cranberry-treated biofilms showed significantly lower biovolumes for both the EPS and microbial biofilm components (Table 2), with the EPS/microbial biovolume ratios significantly lower than that of the control-treated biofilms (*P* = 0.033).

The architecture of the cranberry-treated biofilms also appeared less compact, with distinct areas of porosity seen within the biofilm structure (Fig. 1). The 3D confocal images indicated that the cranberry extract altered the biofilm structural organization, with microcolonies (depicted in green) appearing more closely interspersed within the EPS matrix (depicted in red) in the control-treated biofilms than in the cranberry-treated biofilms.

3.4 Cranberry extract lowered CFUs recovered from polymicrobial biofilms

Significantly fewer microbial cells were recovered from the cranberry-treated biofilms compared to the control-treated biofilms (51% reduction, $P = 0.001$). The CFU/mL counts (mean ± S.D) were $4.9 \times 10^7 \pm 1.2 \times 10^7$ and $1.0 \times 10^8 \pm 3.2 \times 10^7$ for the cranberry- and control-treated biofilms respectively (Fig 2).

3.5 Cranberry extract beneficially modulated microbial ecology of polymicrobial biofilms

The qPCR analysis demonstrated that compared to the control-treated biofilms, the cranberry-treated biofilms had significantly lower abundance of the caries-associated *S. sobrinus* (fold change 0.004, $P = 0.002$) and *P. denticola* (fold change 0.002, $P < 0.001$), and significantly greater abundance of the health-associated *S. sanguinis* (fold change 90.715, $P = 0.001$). Two bacterial species (*L. gasseri* and *S. parasanguinis*) were not detected in any of the treated biofilms and hence were not included in the comparisons. For the other bacterial species, there was a consistent trend towards a reduction in caries-associated bacterial species (except for *A. gerensceriae*) and a fold increase for all the health-associated bacteria (Fig. 3), however these changes were not statistically significant.

4. Discussion

The results of the study indicate that the A-PAC-rich cranberry extract inhibited cariogenic virulence behaviour of polymicrobial biofilms and promoted a beneficial change in the microbial ecological balance of the biofilm. Such extracts could have value for inclusion into daily use oral care products to complement the remineralizing effects of fluoride, as part of a long-term caries management strategy. In the present study, saliva-derived polymicrobial biofilms were grown on HA discs in sugar-rich conditions to partly mimic the complexity of dental plaque developing under cariogenic environments. Pooled saliva from multiple donors ensured microbial diversity of the *in vitro* polymicrobial biofilms. To maintain a cariogenic environment, the growth medium was replenished regularly with the sucrose supplemented growth medium.

The CV assay results demonstrated that the cranberry extract reduced the biomass of the saliva-derived polymicrobial biofilms, consistent with the significant decreases observed in the biovolumes of EPS and microbial components. These changes likely reflect the inhibitory effects cranberry flavonols and A-PACs have on the *S. mutans*-derived glucosyltransferase (Gtf) exoenzymes. While the presence of sucrose in the biofilm growth environment enable Gtfs to rapidly assemble a glucan-rich EPS matrix, cranberry phenols can disrupt this process and thereby affect biofilm bulk and structural integrity. 3D confocal images confirmed that the cranberry extract altered the biofilm structural architecture. The cranberry polyphenol-mediated disruption to biofilm structural organization may render the biofilm less capable of maintaining acidic pH conditions at the tooth-biofilm interface.

S. mutans, as the main producer of insoluble glucans among oral bacteria, plays the key role in orchestrating the EPS biofilm matrix. The glucan-rich EPS matrix resets the biofilm microenvironment for other “heavyweight” acidogenic-aciduric bacteria (e.g. certain lactobacilli, bifidobacteria, and some *Scardovia* spp.) to thrive and become established [Bowen et al., 2018; Burne, 2018; Hajishengallis et al., 2017]. Furthermore, the glucan-rich EPS scaffold also makes it difficult to mechanically remove plaque

biofilms from tooth surfaces, while its physiochemical properties protect the embedded bacteria by reducing drug access and triggering antimicrobial tolerance [Bowen et al., 2018]. Previous studies have shown that cranberry A-PACs inhibited Gtf-mediated glucan synthesis in *S. mutans* mono-species biofilms [Duarte et al., 2006; Feng et al., 2013; Gregoire et al., 2007; Philip et al., 2019]. The current study indicates that polyphenol-rich cranberry extract lowered EPS biovolumes in polymicrobial biofilms too, and this could potentially result in a less pathogenic biofilm environment.

Acidogenicity is another important characteristic of cariogenic biofilms. Sustained biofilm acid production not only favours enamel demineralization, but also results in microbial dysbiosis in the dental plaque biofilm. The present study demonstrated a significant decrease in lactic acid concentrations from the polymicrobial biofilms treated with the cranberry extract. Specific cranberry flavonoids like the low molecular weight A-PAC dimer (called procyanidin A_2) have been shown to affect intracellular bacterial glycolytic enzymes, while larger A-PAC oligomers can have deleterious effects on membrane components of the glycolytic pathway [Duarte et al., 2006; Gregoire et al., 2007]. These mechanisms could account for the reduced lactic acid concentrations produced from the cranberry-treated biofilms. Curbing bacterial acid production would result in a more homeostatic environment for the optimal functioning of non-aciduric commensal organisms and preventing the emergence of highly aciduric organisms in the biofilm microbial community.

The reduced microbial counts observed in the CFU assay and confirmed in the lower microbial biovolumes from the confocal biofilm images are intriguing observations. Several studies have demonstrated that cranberry polyphenols did not have inhibitory effects on microbial growth or viability [Duarte et al., 2006; Girardot et al., 2014; Koo et al., 2010; Philip et al., 2019; Weiss et al., 2004]. A likely reason for the present findings are the anti-adhesion effects of flavonoids present in the cranberry extract, rather than any biocidal effects. Initial bacterial adhesion to the saliva-coated HA surfaces may have been impeded since cranberry polyphenols are known to irreversibly bind to salivary proteins, thereby affecting pellicle-mediated adhesion [Bennick, 2002; Girardot et al., 2014]. In addition, high-molecular weight cranberry fractions can also interact with bacterial cell surface proteins, reducing their hydrophobicity, and thus hindering their adhesion to tooth surfaces [Weiss et al., 2004; Yamanaka et al., 2004]. The A-PAC-mediated inhibition of glucan synthesis can also significantly affect adhesion, as the α1,6-linked glucans are known to provide ample sites for microbial adhesion and co-aggregation [Koo et al., 2010]. Together, these mechanisms could have impaired bacterial adhesion to saliva-coated HA surfaces and to the developing biofilm, explaining the lower CFUs recovered from the cranberry-treated biofilms.

Within the limitations of the experimental conditions used, it is notable that the cranberry extract was able to influence the microbial ecology of the polymicrobial biofilms. The selection of the bacterial profile for the qPCR analysis was based on previous studies that identified bacterial species commonly associated with dental caries or with health [Aas et al., 2008; Becker et al., 2002; Gross et al., 2012; Tanner et al., 2016]. The polymicrobial biofilms treated with the cranberry extract showed significantly lower relative abundance

of two caries associated bacteria (*P. denticola* and *S. sobrinus*) compared to the control-treated biofilms. *P. denticola* showed the highest fold decrease among all the caries-associated bacteria assessed in the qPCR assay. This *Provotella* species has been associated both with initial white spot lesions as well as with advanced dentinal lesions [Chhour et al., 2005; Tanner et al., 2016; Torlakovic et al., 2012]. *S. sobrinus* is often found as a co-colonizer with *S. mutans*, and these species are collectively referred to as mutans streptococci [Gross et al., 2012]. The pathogenic potential of *S. sobrinus* has been established in several studies and its co-colonization with *S. mutans* has been consistently associated with greater caries risk [Choi et al., 2009; Okada et al., 2005]. *S. sobrinus* has also been shown to have higher acidogenic potential than *S. mutans* [de Soet et al., 1989], and this could explain why caries increment in children with both *S. mutans* and *S. sobrinus* was found to be four times higher than those with *S. mutans* alone [Okada et al., 2005]. Furthermore, unlike *S. mutans*, *S. sobrinus* is not detected in plaque of caries-free subjects, suggesting it may be a more specific predictor of the disease than *S. mutans* [Gross et al., 2012]. The qPCR results did indicate a fold decrease for *S. mutans* in the cranberry-treated biofilm, although its relative abundance in the cranberry- vs. control-treated biofilms was not significant (fold change 0.238, $P = 0.055$). The lack of significance could possibly be due to the fact that *S. mutans* is usually present, albeit in lower numbers, even in health-associated saliva or plaque.

Among the bacteria associated with health, levels of *S. sanguinis* was found to be significantly higher in the cranberry-treated biofilm than in the control-treated biofilm. *S. sanguinis* is known to have a well-established arginine deaminase (ADS) activity that can elevate biofilm pH through ammonia and CO_2 production from salivary and dietary substrates [Nascimento, 2018]. The elevated pH not only neutralizes acids produced from carbohydrate metabolism, but also creates an environment that favours growth of health-associated, acid-sensitive bacteria over cariogenic aciduric bacteria [Burne, 2018]. Thus, the observed increase in *S. sanguinis* numbers in the cranberry-treated biofilms should be beneficial for the microbiome. All the other health-associated bacteria also showed fold increases in the cranberry-treated biofilm, although these changes were not statistically significant.

A common problem with using natural products as therapeutic agents is their compositional variability due to their geographical location, seasonal influences, and time of harvest. In this regard, cranberry is a particularly feasible and sustainable source of standardized bioactive compounds, as it is a chemically and genetically well-characterized fruit, with highly standardized methods for extracting the biologically active components (Koo et al, 2010).

In summary, the inhibitory effects of the cranberry extract on biofilm acidogenicity, EPS biovolumes, microbial adhesion, and structure architecture is likely to have also effected a beneficial modulation of the microbial populations of polymicrobial biofilms. There is a need for well-designed clinical trials to evaluate whether the proposed cariostatic effects of cranberry phenols can translate into reversing the microbiome dysbiosis and preventing dental caries in high-risk patients.

Figure Legends

Fig. 1. Representative 3D rendered images depicting structural organization of polymicrobial biofilms following treatment with A) 500 µg/mL of the cranberry extract and B) PBS control. Microbial cells are depicted in green (SYTO 9) and EPS in red (Dextran, Alexa Fluor), with 20x magnification.

Fig. 2. Box-plots of total CFUs recovered from the polymicrobial biofilms after treatment with the cranberry extract or vehicle control (n = 9). * indicates significantly different CFU values ($P < 0.05$) for the cranberry-treated biofilm compared to control-treated biofilms.

Fig. 3. Fold change of A) cariogenic and B) commensal bacterial species in cranberry- vs. control-treated biofilms. A ratio below 1 indicates lower relative abundance; a ratio above 1 indicates higher relative abundance. * indicates statistically significant results after the Simes adjustment for multiple comparisons. *P*-values in brackets are from independent t-tests.

Conflict of interest

None

Acknowledgements

The authors would like to thank Diana Food (Champlain, QC, Canada) for providing the cranberry extracts used in the study.

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